The Modulation of the Cystic Fibrosis Transmembrane Conductance Regulator Protein by Sphingosine-1-Phosphate in the Cerebral Microvascular System

by

Firhan Atir Malik

A thesis submitted in conformity with the requirements for the degree of Doctorate of Philosophy
Department of Physiology
University of Toronto

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Doctorate of Philosophy
Department of Physiology
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Abstract

In the vascular system, peripheral resistance is regulated by the myogenic response (MR), which is the innate ability of a resistance artery to adapt its diameter to changes in transmural pressure. Sphingosine-1-phosphate (S1P) is an endogenous regulator of the MR, and alterations in S1P’s production or degradation affect the responses. The Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) protein uptakes S1P, and contributes to S1P degradation. Since pharmacologic or genomic inhibition of CFTR function upregulates S1P-signaling, and cerebral myogenic reactivity is enhanced, modulation of CFTR expression/function may affect S1P’s bioavailability. This thesis assesses whether S1P directly modulates CFTR’s function, the mechanism(s) involved, and the implications in cerebral microvascular function.

The three studies comprise biochemical and physiological evidence for the S1P and CFTR interplay. First, the iodide efflux assays in stably transfected baby hamster kidney cells (BHK) demonstrate that S1P inhibits CFTR channel function. The effect is reliant upon S1P₁R-AMPK-dependent phosphorylation of CFTR’s serine-737 residue. Second, the show that inhibition of the S1P₁R-AMPK-signaling pathway stimulates iodide efflux in BHK-ΔF508-CFTR cells. In mice, the ΔF508-CFTR mutation enhances the microvascular reactivity of
isolated proximal posterior cerebral arteries (PCAs), and S1P1R antagonism attenuates the responses. This demonstrates that S1P1R-AMPK aggravates the disease-causing mutant’s phenotype. Finally, CFTR is established as a therapeutic target in heart failure (HF), by using a cell model of HF, and show treatment with the CFTR-specific corrector compound (C18) enhances CFTR protein expression and normalizes CFTR function. Furthermore, in HF mice C18 treatment normalizes the myogenic reactivity of isolated PCAs, and *in-vivo* treatment normalizes the cerebral blood flow.

The thesis establishes the S1P-signaling pathway as a novel modulator for CFTR function, and implicates it in murine cerebral microvascular behavior. Also, CFTR is a novel therapeutic target for the cerebral effects of HF, and corrector compounds may be repurposed in the clinical setting.
Acknowledgments

Isaac Asimov once wrote, “The most exciting phrase to hear in science, the one that heralds new discoveries, is not ‘Eureka!’ but ‘That’s funny.’” This single quote defines my entire journey. All researchers strive for those big moments that have the potential to change the entire field, but it takes courage (and a little naivety) to look at the intricacies of a result. Often times an unexpected result is caused by factors other than human error, and the investigation of those strange things can alter not only your view of science, but also self-perception.

There are several people I would like to thank for their help throughout this degree. First and foremost is my supervisor, Dr. Steffen-Sebastian Bolz, who I want to thank for accepting a biochemistry student into his thriving microvascular physiology laboratory. Dr. Bolz’s approach to assessing scientific problems, and coming up with novel solutions is rare. Additionally, your ability to communicate complex information in a straightforward manner is inspirational. I also sincerely thank all members of my supervisory committee: Dr. Christine Bear, Dr. Patricia Brubaker, and Dr. Felix Ratjen. They are three top biochemical and physiological researchers, and they consistently challenged the way I thought about, interpreted, and explained data. The meetings were never easy, but their encouragement to look at a bigger picture, and apply a simple cell culture finding into a much bigger physiological model, cannot be acknowledged enough. A special thank you to Dr. Gregory Hare for his involvement as a departmental internal examiner during the thesis editing process. The two hours I spent talking to you, in person on a cloudy Tuesday afternoon, changed how I viewed my entire thesis, and clarified the context of it all. Thank you to members of the examination committee, Dr. John Hanrahan (External Appraiser), Dr. Scott Heximer, Dr. Robert Jankov, Dr. Jeffrey Medin, and Dr. Herman Yeger. Thank you for taking the time to critically read through my thesis and your insight.
Thank you to all past and present members of the Bolz laboratory. Individual acknowledgements and contributions to experiments are given within the thesis. However, I would like to say a special personal thank you to: Dr. Jeffrey Kroetsch for his vast knowledge as the first Bolz PhD graduate, encouragement, sensibility, and for keeping his desk almost as dirty as mine; Ms. Meghan Sauvé for the funny stories, the Carl-quotes, the Spanx, the random texts, and overall being an incredible lab buddy; Ms. Sonya Hui for the rapid-fire Whatsapp messages, skinny iced café mochas, and random emails just wondering how things are going; Dr. Andrew Levy for the shoulder rubs, high fives, boys’ breakfasts, and for providing quotes that I still smile at; Mr. Danny Dinh and Ms. Jessica Fares, for being incredibly ambitious and hardworking MSc. students. Your questions often stumped me, but I knew if I could answer them to your liking then I would be able to survive in research.

Thank you to all members, past and present, of the Bear laboratory. There were difficult times throughout this process, and I always felt welcomed by every single one of you. Even if I could only stop by 20 minutes to pick up an iodide voltage probe, the brief interactions we had brought just enough sunshine to gloomy days. Thank you also to all members, past and present, of the Heximer laboratory. Our labs have been neighbours for over five years, and there could never have been a better set than your lab.

A very special thank you to my family and friends. You all have heard every conceivable lab story, and at the end of the day still do not know what my degree is in. Those things will always keep me humble.

Finally, last but definitely not least, thank you to my wonderful wife and fellow Department of Physiology PhD student, Sabiha Gardezi. I am not sure what else I could write that I have not already said to you in person, other than you are the: smartest, most resilient, and
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- FM
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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>[Ca^{2+}]</td>
<td>Concentration of calcium ions</td>
</tr>
<tr>
<td>[\Gamma]</td>
<td>Concentration of iodide ions</td>
</tr>
<tr>
<td>(\alpha)-tubulin</td>
<td>Alpha tubulin</td>
</tr>
<tr>
<td>(\Delta F508)-CFTR</td>
<td>Deletion of phenylalanine residue 508 in the CFTR protein</td>
</tr>
<tr>
<td>ABC-transporter</td>
<td>ATP-binding cassette transporter</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMPK-activated protein kinase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BHK</td>
<td>Baby hamster kidney cell line</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>C18</td>
<td>1-(benzo[d][1,3]dioxol-5-yl)-N-(5-((2-chlorophenyl)(3-hydroxypyrrolidin-1-yl)methyl)thiazol-2-yl)cyclopropanecarboxamide</td>
</tr>
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<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<tr>
<td>CBF</td>
<td>Cerebral blood flow</td>
</tr>
<tr>
<td>CdC</td>
<td>Compound C, AMPK inhibitor</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complimentary DNA</td>
</tr>
<tr>
<td>CF</td>
<td>Cystic fibrosis</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic Fibrosis Transmembrane Conductance Regulator</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary cell line</td>
</tr>
<tr>
<td>CO</td>
<td>Cardiac output</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<td>Lipid phosphate phosphatase</td>
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<td>MAP</td>
<td>Mean arterial pressure</td>
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<td>Acronym</td>
<td>Full Form</td>
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</tr>
<tr>
<td>MLC</td>
<td>Myosin light chain</td>
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<td>Myosin light chain phosphatase</td>
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<td>Rho kinase</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>S1P</td>
<td>Sphingosine-1-phosphate</td>
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<tr>
<td>S1P-FITC</td>
<td>Sphingosine-1-phosphate fluorescein isothiocyanate</td>
</tr>
<tr>
<td>S1PR</td>
<td>Sphingosine-1-phosphate receptor</td>
</tr>
<tr>
<td>SAC</td>
<td>Stretch-activated channel</td>
</tr>
<tr>
<td>Sec.</td>
<td>Second</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SER</td>
<td>Smooth endoplasmic reticulum</td>
</tr>
<tr>
<td>SEW</td>
<td>SEW 2871 S1P&lt;sub&gt;1&lt;/sub&gt;R agonist drug</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
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<tr>
<td>SphK</td>
<td>Sphingosine kinase</td>
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<tr>
<td>SPPI</td>
<td>Sphingosine-1-phosphate phosphohydrolase</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TPR</td>
<td>Total peripheral resistance</td>
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<tr>
<td>VOCC</td>
<td>Voltage-operated Ca&lt;sup&gt;2+&lt;/sup&gt; channel</td>
</tr>
<tr>
<td>VPC</td>
<td>VPC 23019 S1P&lt;sub&gt;1,3&lt;/sub&gt;R antagonist drug</td>
</tr>
<tr>
<td>VSMC</td>
<td>Vascular smooth muscle cell</td>
</tr>
<tr>
<td>VSM</td>
<td>Vascular smooth muscle</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
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Chapter 1

1 INTRODUCTION
1.1 Ohm’s law and the cerebral microvascular system

The cardiovascular system is composed of the heart, the vessels (e.g., arteries, veins), and blood, and this system’s role is to provide blood to the major organs within the body. The microvascular system is a component of the cardiovascular system, and is composed of the smallest vessels, like the arterioles, the venules, and the capillaries of the vascular tissue beds. The microvascular system is responsible for tissue perfusion, which is a requirement for cellular processes because cells within the tissues receive oxygen (O$_2$) and nutrients via blood and the vascular capillary beds. These vascular systems promote the appropriate distribution of blood to various organs and tissues that is necessary for homeostasis. In the brain, blood perfusion via the cerebral tissue vascular capillary beds is important because of the high metabolic demand of the neurons and other cells (e.g., astrocytes) within the brain tissue (Cipolla 2009, van Beek et al. 2008).

Ohm’s law is an important law in physics, and this law states that current is equal to the difference in voltage, divided by the resistance. Ohm’s law can be applied to the flow of blood: blood flow is equal to the perfusion pressure divided by the vascular resistance, which is the force that opposes the flow (e.g., Newton’s Third Law of Motion). The application of Ohm’s law to blood flow forms the central characteristic of the vascular systems, and explains the role of the microvascular system in regulating local tissue perfusion. In the cerebral microvascular system, the law would predict that cerebral blood flow (CBF) is equal to the cerebral perfusion pressure (CPP) divided by the cerebral vascular resistance (CVR). CPP is the difference
between the average systemic arterial pressure, or mean arterial pressure (MAP), and the intracranial pressure (i.e., pressure within the cranium) (Mchedlishvili 1980, Heilbrun et al. 1972). Alternatively, CPP is defined as the pressure gradient that is required for supplying blood to the brain, from the heart. Since CPP is directly proportional to CBF, an increase in perfusion pressure will increase CBF. However, an increase in CBF can affect tissue perfusion and homeostasis unless it is regulated. Since CVR is inversely proportional to CBF, CVR is a regulator of blood flow in response to changes in perfusion pressure. CVR is inversely proportional to the radius of a cerebral artery to the fourth power (i.e., \( r^4 \)), small changes in the radius of the small arteries (e.g., resistance arteries, see Section 1.2.1), and arterioles, which provide blood to the cerebral tissue vascular capillary beds, can have large effects on the CVR. Thus, blood flow to the capillaries is regulated by a decrease in these vessels’ radii (i.e., vasoconstriction), or an increase in these vessels’ radii (i.e., vasodilation). It is important to note that Ohm’s law is not fully applicable to the cerebral microvascular system because CBF is constant over a wide range of perfusion pressure values, and this leads to the phenomenon of autoregulation, discussed shortly. The rapid constrictory and dilatory responses within the brain’s individual vascular beds acutely control local blood flow (Hall and Guyton 2011), and ensure the constancy of CBF in response to changes in perfusion pressure, described in greater detail in Section 1.1.2. Regulation in response to changes in perfusion pressure is the most important adaptation process. Therefore, changes in CVR affect CBF, and it is important to understand the mechanisms that regulate CVR in response to changes in perfusion pressure.

### 1.1.1 Cerebral vascular resistance (CVR) and cerebral blood flow (CBF)

Hypoxia and hypercapnia are two physiologically relevant examples of CVR regulators, and demonstrate how changes in CVR can regulate CBF. Hypoxia is defined as the reduction in
tissue or blood O2 content, and acute hypoxia promotes vasodilation in the cerebral microcirculation via enhanced production of metabolites, including potassium (K+) and prostaglandins (reviewed in Pearce 1995), or by stimulating the release of excitatory amino acids from neurons (Pearce 1995, also see Iadecola and Nedergaard 2007, Attwell et al. 2010).

Hypoxia can also promote vasodilation via nitric oxide (NO) production in the endothelial cells (Van Mill et al. 2002), or through hyperpolarization of vascular smooth muscle cells (VSMCs) (reviewed in Pearce 1995) possibly by activating K+ channels (Taguchi et al. 1994). The hypoxia-dependent vasodilation enhances CBF (Steiner et al. 2003), by reducing the CVR.

Hypercapnia refers to the increase in the concentration of carbon dioxide (CO2). Inhalation of 5 to 7% CO2 by healthy subjects has been shown to promote vasodilation of cerebral arteries, as demonstrated by a reduction in CVR (Kety and Schmidt 1948). CBF was shown to increase in subjects who inhaled 5 to 7% CO2. Hypercapnia is thought to promote contraction of vascular smooth muscle (VSM) by elevating the extracellular concentration of hydrogen ions (Cipolla 2009, Kontos et al. 1977). For example, Kontos and colleagues demonstrated that treating cat pial cerebral arteries with acidic cerebrospinal fluid promotes vasodilation of the arteries. Implanting a transparent cranial window into the skull allowed for assessment of changes in the arteries’ diameter. Vasodilation was visualized with a Vickers image-splitting device, and a closed-circuit television camera and monitor (Kontos and Raper 1977).

1.1.2 Cerebral autoregulation

In the 1930’s, two studies by Fog and colleagues assessed the vasoconstrictory responses of pial cerebral arteries in the brain of cats, through a cranial window, in response to changes in pressure (Fog 1938, Fog et al. 1934, also see Agnoli et al. 1968). The studies demonstrated that an increase or a decrease in blood pressure, by changing the blood volume, promoted
vasoconstriction or vasodilation respectively of the cerebral arteries (Fog 1938). In a 1959 review article, Dr. Niels Lassen described the constancy of CBF, in response to steady-state changes in MAP, called the autoregulatory range (see Section 1.2.2). The finding first established cerebral autoregulation (Lassen 1959, also see Tzeng and Ainslie 2014, Strandgaard and Paulson 1984). Harper detected similar observations, through the use of a krypton radioisotope ($^{85}$Kr) clearance in the carotid artery of dogs (Harper 1966). Agnoli and colleagues measured regional cerebral blood flow in normotensive human subjects, via clearance of $^{85}$Kr, and they observed no change in CBF measurements after intravenous infusion with angiotensin amide, a compound that was shown to increase the CPP in the normotensive subjects (Agnoli et al. 1968). The microvascular responses that ensure the constancy of CBF, after changes in perfusion pressure, encompass a mechanism called cerebral autoregulation. Lassen’s work established the static nature of cerebral autoregulation, but CBF can also be changed by dynamic or rapid changes in MAP (e.g., by a blood pressure cuff around the thigh) (Tzeng and Ainslie 2014, Tiecks et al. 1995). Others have described the static and dynamic nature of cerebral autoregulation (Tzeng and Ainslie 2014, Liu et al. 2013, Aries et al. 2010, Panerai 2009, Panerai 2008, van Beek et al. 2008). In an effort to keep this overview straightforward, cerebral autoregulation will be discussed in a general sense. Cerebral autoregulation is important in the brain because of requirements for blood supply and water hemostasis (Cipolla 2009), and contributes to the $O_2$ and nutrient delivery that safeguards cognitive function and integrity of the brain (Walsh and Cole 2013, Aoi et al. 2012, Cipolla 2009). Impairments in cerebral autoregulation have been observed in various conditions and diseases, such as subarachnoid hemorrhages (SAH) in rats (Rasmussen et al. 1992), the vasospasms associated with SAH in human patients (Otite et al. 2014), the ischemia associated with acute stroke in humans (Olsen et al. 1983), the reduced CBF associated with heart failure in humans and mice (see Section 1.5.4).
and in animal models of Alzheimer’s disease, like mice that overexpress mutated forms of the human amyloid precursor protein genes (Claassen and Zhang 2011). In normal or healthy humans, the importance of cerebral autoregulation is observed in the simple task of standing up from sitting on a chair, also known as orthostasis. In an upright standing posture, venous return and cardiac output are reduced (Harms et al. 2003, Matzen et al. 1991, Blomqvist and Stone 1984, Goetz 1950). However, despite the drop in CPP, CBF is maintained due to a decrease in CVR via vasodilation of small cerebral arteries, like the middle cerebral artery (Savin et al. 1995). The orthostasis example demonstrates that perturbations in CPP are buffered by an autoregulatory mechanism. This mechanism is governed by changes in the CVR, and CBF is maintained within a tight range at rest.

1.1.3 The role of CVR in cerebral autoregulation

There are several components or mechanisms that have been shown to or are thought to regulate CVR and CBF. These factors may contribute to cerebral autoregulation and examples include, the cerebrovascular endothelium and its associated molecules (e.g., NO) (Hlatky et al. 2003), the astrocytes of the brain via uptake of extracellular K⁺ (Peterson et al. 2011, Newman et al. 1984), microvascular communication between nearby endothelial cells via connexins (de Wit et al. 2006, Li and Simard 2002, also see Peterson et al. 2011), and catecholamines like norepinephrine (Tuor et al. 1986). Cellular metabolism refers to the changes in cellular metabolism associated with increased or decreased cell activity (Novak 2012), and can also affect CBF. In the brain, increased activity of neurons promotes the increased utilization of O₂, and glucose, and produces higher concentrations of CO₂ and waste metabolites (e.g., lactate) (Hall and Guyton 2011, Peterson et al. 2011, Cipolla 2009). These products reduce the pH within the local tissue, and promote vasodilation. Other metabolites like K⁺ and hydrogen ions
have also been shown to promote vasodilation and affect CBF in rats (Nielson et al. 2001), and there is tight coupling between neuronal metabolism and CBF (Buxton and Frank 1997, also see Paulson et al. 1987). Zoremba and colleagues demonstrated that reductions in CPP, via elevations in intracranial pressure, significantly increased the lactate concentration to pyruvate concentration ratio, the concentration of glutamate, and decreased the concentration of glucose in the cortex of pigs (Zoremba et al. 2007). However, the effects on CVR and CBF were not assessed, and autoregulation of CBF by cerebral metabolism is unclear in humans (Ainslie and Brassard 2014). Furthermore, Peterson and colleagues state that metabolism is separate from the effects of CPP on CBF (Peterson et al. 2011). There may be a neurogenic component in cerebral autoregulation that is controlled by nerve fibers connected to the small arteries of the brain, and autonomic signaling (Novak 2012). For example, cutting the carotid sinus nerves has been shown to abolish cerebral autoregulation in dogs (Sagwa and Guyton 1961), but studies in cats and humans suggest the contribution of the neurogenic component is controversial (reviewed in Ainslie and Brassard 2014). There is another component that regulates CVR and maintains CBF in a tight range of perfusion pressures, and this is the myogenic component described next.

An increase or decrease in CPP changes the transmural pressure, which is the pressure within the lumen of an artery, and affects the tension in the artery’s wall (Walsh and Cole 2013). The change in tension promotes contraction or relaxation of the vascular smooth muscle cells (VSMCs), and vasoconstriction or vasodilation occurs in response (see Sections 1.2 and 1.3). Therefore, this intrinsic mechanism is driven by the VSM, and is called the myogenic response (MR) (see Section 1.2). The MR comprises the myogenic component of cerebral autoregulation. The myogenic component is independent of tissue metabolism (Peterson et al. 2011), and the endothelium and neurogenic factors (Tzeng and Ainslie 2014), but the MR can be modulated by these extrinsic factors (Walsh and Cole 2013, see Section 1.2.3). Pressure-dependent
vasoconstriction has been observed in isolated and cannulated cerebral arteries from several species like human pial arteries (Thorin-Trescases et al. 1997, Wallis et al. 1996), rat middle cerebral arteries (Johnson et al. 2011, El-Yazbi et al. 2010), and distal posterior cerebral arteries (PCAs) from gerbils (Yang et al. 2012). If the MR is considered as a major component to ensure appropriate CVR, this may explain why CBF is maintained in a tight window almost at all times, like in orthostasis. The importance of the MR and its role in cerebral autoregulation is further observed in the heart failure (HF) disease model, where there are no orthostatic challenges. For example in mice with HF, CBF measured with magnetic resonance imaging (MRI) techniques is significantly less in all regions of the brain, relative to control mice (Yang et al. 2012). The reduction in CBF cannot be completely explained by changes in the MAP, since in HF mice MAP decreases less dramatically than CBF. The result could be attributed to the enhancement in CVR, as demonstrated by increased myogenic reactivity of isolated and cannulated small cerebral arteries (see Section 1.5.4.). Therefore, abnormalities in the cerebral MR contribute to the reduction in CBF in HF mice, and this points to the importance of the MR in regulating CVR, and thus cerebral autoregulation. Additionally, abnormalities in the MR of small cerebral arteries (e.g., resistance arteries) have been shown to or are thought to contribute to abnormal cerebral autoregulation in hypertensive rats (Tullos et al. 2013), and ischemic and reperfusion injury in rats (Coucha et al. 2013). An evolving field is the investigation into novel regulators of the cerebral MR, because studying novel mechanisms or signaling pathways that regulate the response may provide insight into the impaired cerebral autoregulation observed in diseases. My thesis assesses the biochemical link between two novel regulators: a vasoactive lipid mediator and a transporter protein. First, an overview on the MR and the classical mechanisms that regulate it are presented in Sections 1.2 and 1.3.
1.2 The myogenic response

In the microvascular system, constant blood flow is maintained by autoregulation when there are changes in the transmural pressure (Stanfield 2011, Johnson 1986). The MR, also known as the Bayliss effect, is the primary mechanism underlying autoregulation. The MR is defined as a decrease of an artery’s diameter after an increase in transmural pressure, or an increase of an artery’s diameter after a decrease in transmural pressure (Lidington et al. 2013, Hill et al. 2009, Schubert and Mulvany 1999, Meininger and Davis 1992, Bayliss 1902). By adjusting an artery’s diameter, the MR provides protection to the capillary beds against systemic pressure fluctuations. The MR also contributes to the regulation of tissue perfusion (Schubert et al. 2008), and the regulation of systemic blood pressure (Metting et al. 1989) by establishing peripheral resistance. Thus, the primary function of the MR is to match the vascular resistance to the prevalent pressure (Walsh and Cole 2013). The MR of cerebral resistance arteries contributes to the autoregulation of CBF, and ensures CBF remains constant during systemic pressure variations (Walsh and Cole 2013, Orsol et al. 2002, Davis and Hill 1999, Olsen et al. 1981).

1.2.1 Types of arteries

There are three types of arteries in the cardiovascular system: elastic, muscular, and resistance arteries (Moore and Dalley 1999). Elastic arteries, like the aorta, are large diameter arteries that contain an internal elastic lamina consisting of smooth muscle and an elastic matrix. In contrast, muscular arteries are medium-sized and are smaller than elastic arteries, but larger than resistance arteries. Muscular arteries, like the radial artery, contain more smooth muscle content and less elastic tissue than elastic arteries. Both elastic and muscular arteries provide low resistance because of their large lumen. The lumen to wall ratio is large because of the thin,
fiber rich and low-compliant artery walls (Boron and Boulpaep 2011), and elastic and muscular arteries have functional characteristics of conduit arteries (i.e., unimpeded blood flow).

Resistance arteries, such as the middle cerebral arteries, do not contain a significant amount of elastic tissue relative to elastic or muscular arteries, and measure less than 200-300 \( \mu \text{m} \) in diameter, which make them the smallest of the arteries localized to the terminal parts of the arterial tree (Walsh and Cole 2013, Khavandi et al. 2009, Christensen and Mulvany 2001). The resistance arteries contain more smooth muscle content than elastic or muscular arteries, and this allows them to contribute to resting resistance, and respond actively to changes in pressure, independently of neurological or hormonal influences. There is a great deal of heterogeneity between vascular beds, but all with the exception of one contain resistance arteries that are myogenically active, such as cerebral tissue (Yang et al. 2012, Salomone et al. 2008), mesentery tissue (Meissner et al. 2012, Hoefer et al. 2010), and skeletal muscle (Hoefer et al. 2010, also see review by Hill and Meininger 1993).

1.2.2 The MR is an inherent feature of resistance arteries

Resistance arteries have unique pressure ranges at which they are myogenically active, and this is called the autoregulatory range (Walsh and Cole 2013, Schubert and Mulvany 1999, Knot et al. 1998). In humans, the systemic arterial pressure’s (i.e., mean arterial pressure) autoregulatory range is between 60 to 150 mmHg (Lidington et al. 2013), and within this range the blood flow remains constant as the pressure increases. The autoregulatory range for CPP is between 60 to 160 mmHg (Cipolla 2009, Phillips and Whisnant 1992). If the pressure is below the lower limit, the change in artery wall tension does not meet the threshold because there is too little blood flow, and the relationship between pressure and blood flow is linear (Osol et al. 2002). The same linear relationship is observed at pressures above the upper limit, because the
artery cannot generate enough force to decrease the diameter in response to the elevated pressure, and therefore there is too much blood flow (Walsh and Cole 2013).

The amplitude of the MR increases from proximal to distal parts of the resistance artery terminal bed. This can be seen in the proximal and distal portions of the posterior cerebral arteries (PCAs) of the brain that comprise the brain’s posterior circulation, along with the basilar and vertebral arteries, and supply blood to the posterior cortex, midbrain and the brainstem. Relative to the circle of Willis, the proximal portion of PCAs, which is greater than 150 µm in diameter, has low myogenic responsiveness, and exhibits functional characteristics similar to conduit arteries of the systemic circulation (Yang et al. 2012). This portion of the PCA brings blood to the circle of Willis, where it is directed to deeper regions of the brain tissue. In contrast, the distal portion of PCAs is less than 150 µm in diameter, and is more myogenically active (Yang et al. 2012, Brekke et al. 2002). Recently, our laboratory demonstrated that the CBF in mice is reduced when the myogenic responsiveness of proximal PCAs is augmented (Yang et al. 2012).

1.2.3 The MR originates in the vascular smooth muscle

The vascular smooth muscle cell (VSMC) layer of resistance arteries is underneath the endothelium, and directly controls the MR (Narayana et al. 2013, Litteri et al. 2012, Carlson and Beard 2011, Carlson and Secomb 2005, Schubert and Brayden 2005, MacPherson et al. 1993). Since these cells have no apparent structural sarcomeric components (Abrahamsen and Lorens 2013), it is difficult to define their contractile apparatus (Cipolla et al. 2002). However, in response to mechanical tension stimuli VSMCs contract through rearrangement of the actin cytoskeleton (Smith et al. 2000, Petroll et al. 1993), and actin filament gliding through actin and myosin interactions (Warshaw et al. 1990). In resistance arteries, an increase in pressure
activates signaling pathways that promote actin-myosin interactions, and thus contraction of VSMCs, and subsequent constriction of the artery occur.

The endothelial cells can modulate the MR through a nitric oxide (NO)-dependent mechanism (Bolz et al. 2003, de Wit et al. 1998). The sympathetic nervous system can also modulate the MR by affecting the vasomotor function and the baroreceptors (Wieling and Groothuis, Chapter 39 in Robertson et al. 2012). For example, electrical field stimulation of free nerve endings in isolated resistance arteries from the tails of rats was shown to enhance the MR at a low-pressure range (e.g., 10 to 40 mmHg) (Anschütz and Schubert 2005). Thus, the MR is driven by the VSM and there are modulatory roles by the endothelium and sympathetic nervous system.

1.3 Mechanisms of the MR

An elevation in pressure causes the development of tension in the artery wall (Johnson et al. 1989, also see Schubert et al. 2008, Schubert and Brayden 2005). Mechanosensors transduce the tension, and downstream targets are activated that cause constriction of the artery (see reviews by Lidington et al. 2013, Walsh and Cole 2013, Hill and Meininger 2012). The mechanosensor is currently unknown, but possible candidates include mechanosensitive ion channels that respond to the artery’s stretch and the wall tension directly (e.g., transient receptor potential channels) (Sharif-Naeini et al. 2008, Pederson and Nillius 2007, also see Schubert and Brayden 2005, Schubert and Mulvany 1999, Meininger and Davis 1992), and G-protein coupled receptors (GPCRs) (e.g., angiotensin II receptor) (Kauffenstein et al. 2012, Schnitzler et al. 2011, Schnitzler et al. 2008). The primary mechanosensors convert the initial stretch stimulus into vasoconstriction (Silver 2006), through mechanisms that regulate the actin-myosin cross-bridge interaction. Several mechanisms have been identified that lead to pressure-dependent
vasoconstriction (see reviews by Lidington et al. 2013, Walsh and Cole 2013, Schubert et al. 2008), and the most established mechanisms, as described below, regulate the interaction of actin-myosin by directly affecting intracellular VSMC Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]), or are reliant upon increased sensitivity to Ca\(^{2+}\) (summarized in Figure 1-1).

1.3.1 Mechanism No. 1: Elevation of intracellular Ca\(^{2+}\) concentration leads to activation of the myosin light chain kinase (MLCK)

An increase in pressure leads to the influx of Ca\(^{2+}\) ions from the extracellular environment, then intracellular [Ca\(^{2+}\)] is increased, and downstream targets are activated that cause vasoconstriction. The activation of voltage-operated Ca\(^{2+}\) channels (VOCCs), present in the membrane of VSMCs, is primarily responsible for the influx of Ca\(^{2+}\) ions (see reviews by Walsh and Cole 2013, Ratz et al. 2005), and pharmacologic inhibition of VOCCs decreases the pressure-induced vasoconstriction (Wesselman et al. 1996), demonstrating their importance in regulating the MR. Although there is some evidence that VOCCs are directly activated by the alteration in wall tension (McCarron et al. 1997), most evidence links their activation to membrane depolarization. Mechanosensitive ion channels can depolarize the membrane in response to increased pressure (Welsh et al. 2002) and the change in artery wall tension. The best-known mechanosensitive channels are the stretch-activated channels (e.g., epithelial sodium channels, transient receptor potential channels) (Kim et al. 2012, Narayanan et al. 2013, Guan et al. 2009, Welsh et al. 2002). Other candidates for mechanosensitive channels include Ca\(^{2+}\)-gated potassium channels, voltage-gated potassium channels, and chloride channels (see reviews by Schubert et al. 2008, Schubert and Mulvany 1999).

The elevated intracellular [Ca\(^{2+}\)] saturates the four Ca\(^{2+}\)-binding sites of calmodulin, a calcium-binding messenger protein, and then the Ca\(^{2+}\)-calmodulin complex binds to the 125 kDa isoform of the myosin light-chain kinase (MLCK) (Raina et al. 2009, Schimizu et al. 2006,
Welsh et al. 2002, Delaey and Voorde 2000, Gallagher et al. 1997). The binding displaces MLCK’s catalytic autoinhibitory pseudosubstrate domain from smooth muscle myosin II’s head, and MLCK is activated (Cole and Welsh 2011, Ogut and Brozovich 2003). MLCK then phosphorylates the serine (Ser)-19 residue of myosin II’s 20 kDa regulatory light chain (MLC20) (Kamm and Stull 2001, Sakurada et al. 1998), and the myosin II heads bind to the actin filaments. Contraction of the VSM follows, and results in the MR (Walsh and Cole 2013).

When extracellular Ca\(^{2+}\) is removed, the pressure-dependent elevation in intracellular [Ca\(^{2+}\)], the phosphorylation of MLC\(_{20}\) (Zou et al. 2000, Hui et al. 1995), and ultimately the MR is abolished (Schubert et al. 2008). This reinforces that the increase in intracellular [Ca\(^{2+}\)] is required for the MR of resistance arteries, and suggests a direct relationship between [Ca\(^{2+}\)] and the magnitude of pressure-dependent vasoconstriction. However, it has been reported that after the initial pressure-dependent elevation of intracellular [Ca\(^{2+}\)], the MR gains strength even though the concentration remains constant (VanBavel et al. 1998, also see Schubert et al. 2008). Thus, the MR is directly mediated by an initial increase in intracellular [Ca\(^{2+}\)], and this leads to MLCK-signaling. In addition, there is a simultaneous mechanism that allows for the MR without further enhancement of the intracellular [Ca\(^{2+}\)].

1.3.2 Mechanism No. 2: Calcium sensitization is mediated by the inhibition of myosin light chain phosphatase (MLCP)

Ca\(^{2+}\)-sensitization refers to the pressure-dependent vasoconstriction that occurs when the intracellular [Ca\(^{2+}\)] remains constant (Baek and Kim 2011, Somlyo and Somlyo 2003). The presence of the MR even when the intracellular [Ca\(^{2+}\)] was clamped at an elevated level by [K\(^{+}\)]-dependent membrane depolarization (i.e., activates VOCCs) (Lagaud et al. 2002), provided evidence for sensitization, and suggested a simultaneous, Ca\(^{2+}\)-calmodulin independent mechanism for the pressure-dependent vasoconstriction. This simultaneous mechanism is
controlled by the smooth muscle myosin light-chain phosphatase (MLCP). The activation of MLCP causes its PP1c-δ phosphatase or its myosin phosphatase target subunit 1 (MYPT1) regulatory subunit to bind to myosin II, and then MLC$_{20}$ is de-phosphorylated, and the cross-bridge cycle is inhibited. However, phosphorylation of MYPT1 (Cole and Welsh 2011, Khromov et al. 2009, Somlyo and Somlyo 2000), at residues threonine (Thr)-697 and Thr-855 (Muranyi et al. 2005, Feng et al. 1999), decreases the catalytic activity of MLCP and the de-phosphorylation of MCL$_{20}$ is inhibited (Cole and Welsh 2011). Therefore, myogenic vasoconstriction through the Ca$^{2+}$-sensitization mechanism relies on the inhibition of MLCP catalytic activity.

Our laboratory demonstrated that the transfection of dominant-negative mutants of the RhoA guanosine triphosphate (GTP)-ase or Rho associated protein kinase (ROK), and pharmacological inhibition of ROK with the Y27632 drug, nearly abolished the MR of isolated hamster gracilis muscle resistance arteries (Bolz et al. 2003). There was no effect on the initial pressure-dependent elevation of intracellular [Ca$^{2+}$], with both strategies, and this implicates the RhoA/ROK-signaling pathway in the Ca$^{2+}$-sensitization mechanism of the MR. More recent work by the laboratories Drs. Cole and Welsh, using rodent resistance arteries, demonstrated that pharmacologic inhibition of ROK with the H1152 drug decreases pressure-dependent phosphorylation of MYPT1, and the phosphorylation of MLC$_{20}$ and myogenic vasoconstriction were also reduced (Moreno-Dominguez et al. 2013, Johnson et al. 2009). This supports that RhoA/ROK-signaling is a regulator of the Ca$^{2+}$-sensitization mechanism in an MLCP-dependent manner.

Vasoactive mediators in the VSMCs are stimulated by an increase in pressure, and are released in an autocrine manner to the extracellular environment. These mediators bind to cell-surface GPCRs, and can regulate the Ca$^{2+}$-sensitization mechanism of the MR through
RhoA/ROK-signaling. There are several agonists that regulate microvascular tone and promote vasoconstriction of resistance arteries in a RhoA/ROK-dependent manner (see Kauffenstein et al. 2012 for a comprehensive review), including arachidonic acid and its metabolites (Randriamboavanjy et al. 2003), extracellular nucleotides (e.g., ATP, UDP) (Kauffenstein et al. 2010, Koltsova et al. 2009), and sphingosine-1-phosphate (S1P) (Keller et al. 2006, Bolz et al. 2003).

1.3.3 Summary of the MLCK/MLCP-dependent mechanisms of the MR

The MR is regulated by several mechanisms, and the two best-characterized mechanisms are dependent upon the opposing functions of MLCK and MLCP. The Ca\(^{2+}\)/calmodulin dependent activation of MLCK and MLC\(_{20}\)’s phosphorylation relies on increased intracellular [Ca\(^{2+}\)], while the prevention of MLC\(_{20}\) de-phosphorylation requires inhibition of MCLP. The RhoA/ROK signaling-pathway is an inhibitor of MLCP. Emerging evidence advocates simultaneous action and inter-connectivity of these two mechanisms (Lidington et al. 2013, Kauffenstein et al. 2012, Lagaud et al. 2002, Bolz et al. 2000). There is some evidence that high [K\(^{+}\)]-mediated depolarization can activate components of the RhoA/ROK-signaling pathway (Salomone et al. 2010, Sakaruda et al. 2003). Although pressure-dependent depolarization of the membrane may indirectly activate components of the pathway, a direct stimulation of RhoA or ROK has not been observed. For this reason, it has been problematic to identify the regulatory interface, or divergence point, for the simultaneous activation of the two mechanisms. The ideal candidate would possess key characteristics such as the ability to regulate both intracellular [Ca\(^{2+}\)] and MLCK, and also regulate MLCP through RhoA/ROK-signaling. Our laboratory’s published work demonstrates the S1P phospholipid is a regulator of the MR, because S1P production and signaling via GPCRs has effects on intracellular [Ca\(^{2+}\)] and Ca\(^{2+}\)-sensitization,
discussed in Section 1.4. Therefore, S1P is an intriguing candidate for the regulatory interface between two well-established mechanisms of the MR.
Figure 1. The myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP) dependent pathways of the myogenic response (MR).

The MR is regulated by the simultaneous activation of a MLCK in a Ca\textsuperscript{2+}-dependent manner, and the inactivation of MLCP in a Ca\textsuperscript{2+}-independent manner.
1.4 The regulation of the MR by S1P

An elevation in transmural pressure promotes the production of S1P (Bolz et al. 2003), a ubiquitous bioactive lipid that is a critical vasoactive mediator of VSMCs. S1P is an intriguing candidate for the regulatory divergence point, because its pressure-dependent production has two effects: (i) intracellular S1P increases the \([\text{Ca}^{2+}]\) and contributes to MLCK-mediated constriction, and (ii) extracellular S1P binds to cell-surface S1P-receptors (S1PRs) (Kim et al. 2012, Peter et al. 2008), which are G-protein coupled, to activate the RhoA/ROK signaling pathway that contributes to inhibition of MLCP. The balance between S1P’s production and degradation is tightly regulated and determines the bioavailability of S1P (Maceyka et al. 2009, Takabe et al. 2008, Spiegel and Milstien 2003, Graeler et al. 2002). Therefore to understand the role of S1P in the MR, it is pivotal to study the mechanisms that ultimately regulate its bioavailability. This section describes what is known about the mechanisms, and then will provide information on a novel transporter-dependent mechanism that regulates S1P bioavailability. The studies in the thesis focus on that transporter protein, its relationship to S1P, and its role in the regulation of the MR.

1.4.1 Production of S1P by sphingosine kinase 1 (SphK1)

S1P is produced from the sphingosine phospholipid. Sphingosine is converted from its ceramide precursor in a reaction catalyzed by ceramidases, localized in the plasma membrane (Romiti et al. 2001), that act to remove the amide-linked fatty acid attached to the sphingoid base (Maceyka et al. 2009). Then sphingosine undergoes a further catalytic reaction where it is phosphorylated by sphingosine kinases (SphKs), to form S1P. In mammals, there are two well-characterized isozymes of SphK, SphK1 and SphK 2, (Pitson 2011, Spiegel and Milstien 2003).
Bolz and colleagues characterized a possible role for the SphK1 isoform as a physiological regulator of MR by overexpressing the enzyme in smooth muscle cells of hamster gracilis muscle resistance arteries. This manipulation augmented MR relative to arteries expressing endogenous SphK1 only. In contrast, transfection of a dominant-negative SphK1 mutant (hSK-G82D) attenuated the MR (Bolz et al. 2003). Since the hSK-G82D mutant is functionally inactive and lacks the ability to produce S1P (Bolz et al. 2000), the results strongly suggest SphK1-dependent production of S1P regulates the MR of resistance arteries, under physiological conditions.

There are various stimuli that activate SphK1, such as cytokines (Pitson 2011, Stein et al. 2003, Xia et al. 2002), hormones (Nava et al. 2002), and elevation of transmural pressure in resistance arteries (Lim et al. 2012, Salomone et al. 2010, Lidington et al. 2009, Keller et al. 2006). These stimuli may activate the Ras GTPase signaling pathways (Peyssonnaux and Eychène 2001), and promote the phosphorylation and activation of extracellular signal-regulated kinase 1/2 (ERK 1/2). ERK 1/2 then directly phosphorylates the serine-225 (Ser-225, S225) residue of SphK1, and SphK1 is activated (Pitson et al. 2003). The Pitson laboratory provided in-vitro evidence, using a fibroblast cell-line, that phosphorylation of the S225 residue also contributes to the translocation of SphK1 from the cytoplasm to the plasma membrane (Pitson 2011, Jarman et al. 2010, Pitson et al. 2005). Furthermore, in hamster gracilis muscle resistance arteries pressure elevation was shown to have no effect on the translocation of the S225A-SphK1 mutant, and the transfection of the mutant also attenuated the MR (Lidington et al. 2009). Recently, Kim and colleagues used immunofluorescence and microscopy to demonstrate that pressure elevation promotes the translocation of SphK1 in isolated rabbit proximal PCAs (Kim et al. 2012) and this confirms work by the Bolz laboratory. Although, the non-translocated SphK1 possesses constitutive catalytic activity in-vitro, its translocation to the plasma membrane
enhances the activity (Pitson et al. 2005). The translocation brings activated SphK1 into close proximity to sphingosine, which is in the inner outlet of the phospholipid bilayer (Takabe et al. 2008), and then S1P is produced. S1P is released to both intracellular and extracellular compartments (Pitson et al. 2003), through presently unknown mechanisms, and there are distinct compartmentalized effects that regulate the MR.

1.4.2 Intracellular effects of S1P

Bolz and colleagues reported that overexpression of SphK1 enhances the resting tone of isolated resistance arteries by elevating the intracellular [Ca\(^{2+}\)] (Bolz et al. 2003), and therefore intracellular S1P promotes pressure-dependent vasoconstriction of resistance arteries in a Ca\(^{2+}\)-dependent manner (Lidington et al. 2009, Peter et al. 2008, Bolz et al. 2003). Although, the exact mechanism is not known, an in-vitro study in a kidney cell line provided some insight. Meyer zu Heringdorf and colleagues incubated cells with a Ca\(^{2+}\)-sensitive dye and a photolyzable derivative of S1P, caged S1P. The group found that photolysis of caged S1P, with short pulses of light, increased the intracellular [Ca\(^{2+}\)] by mobilizing the ions from thapsigargin-sensitive intracellular stores, like the sarcoplasmic reticulum, independent of ryanodine receptors (Meyer zu Heringdorf et al. 2003). Additional mechanisms for the S1P-dependent change in intracellular [Ca\(^{2+}\)] have been proposed, and these include: (i) activation of the PLC/inositol triphosphate (IP\(_3\)) pathway (Berridge et al. 2000, Ghosh et al. 1990), possibly through allosteric action of S1P on IP\(_3\)-gated Ca\(^{2+}\) channels (Heringdorf et al. 2003), (ii) stimulation of Ca\(^{2+}\) influx, shown in neutrophils (Itagaki and Hauser 2003), and (iii) activation of SCaMPER, an endoplasmic reticulum Ca\(^{2+}\) channel (Cavalli et al. 2003, Mao et al. 1996, also see Chen et al. 2010), but this is controversial (Meyer et al. 2003, Schnurbus et al. 2002). Thus, the best-characterized effect of intracellular S1P is to increase intracellular [Ca\(^{2+}\)], but the exact mechanism remains unclear. This S1P-dependent increase occurs simultaneously with the
depolarization-dependent influx of Ca\(^{2+}\) (Bolz \textit{et al.} 2003), and contributes to the activation of MLCK (see Section 1.3.1.).

1.4.3 Extracellular effects of S1P

A 2009 study by Lidington and colleagues provided vascular evidence that an extracellular pool of S1P is involved in regulating the MR. Hamster gracilis muscle resistance arteries were transfected with an S225A-SphK1 construct that contained a membrane anchor (mp-S225A-SphK1), to overcome the translocation deficiency and force localization of the enzyme to the plasma membrane (Lidington \textit{et al.} 2009). The intracellular effects associated with S1P were restored, such as intracellular resting and stimulated Ca\(^{2+}\) signals relative to mp-WT-SphK1, but the myogenic tone and sensitivity to Ca\(^{2+}\) were not affected. The latter two parameters rely on extracellular S1P, and were weaker in the arteries transfected with mp-S225A-SphK1 relative to those transfected with mp-WT-SphK1. The “incorrect subcellular positioning” of SphK1 (Lidington \textit{et al.} 2009) in the plasma membrane of VSMCs, and therefore the reduced supply of extracellular S1P, as theorized by the authors, may account for the effects on myogenic tone and Ca\(^{2+}\)-sensitivity. The export of S1P from the intracellular to extracellular environments is thought to occur in an autocrine manner (Kim \textit{et al.} 2009), where the transported S1P from one cell binds to cell-surface receptors present in the same cell. Then S1P in the extracellular environment regulates myogenic vasoconstriction by binding to G-protein coupled S1PRs.

1.4.3.1 The S1P-Receptors (S1PRs)

In the extracellular compartment, many of the cellular and physiological effects attributed to S1P are mediated by its binding to cell-surface GPCRs, the S1PRs, of which there are five known (S1P\(_{1-5}\)) (Spiegel and Milstien 2003), although more could exist because of alternative splicing (Neidernberg \textit{et al.} 2003). The S1PRs share common structural features including seven
transmembrane domains, an extracellularly located N-terminus, and an intracellularly located C-terminus (O’Sullivan and Dev 2013, Rosen et al. 2013). These receptors are expressed ubiquitously throughout the physiological systems, and couple to a variety of G-proteins (Spiegel and Milstien 2003), described below. The diverse coupling of the G-proteins (also known as guanosine nucleotide-binding proteins) supports S1P as being a significant player in many cellular and physiological processes. In the vascular system, S1P₁R, S1P₂R, and S1P₃R have been shown to regulate the MR of resistance arteries (Lim et al. 2012, Yang et al. 2012, Hoefer et al. 2010, Salomone et al. 2010, Peter et al. 2008, Salomone et al. 2008, Hu et al. 2006, Rosenfeldt et al. 2003). The roles of these three S1PRs in the MR are discussed next (summarized in Figure 1-2).

1.4.3.1.1 S1P₁R

The S1P₁R is expressed in both VSMCs and endothelial cells (Hu et al. 2006, Allende et al. 2003), and its expression is reflective of its role in extracellular S1P-triggered vasoconstriction and also vasodilation, of isolated resistance arteries. Salomone and colleagues used rodent basilar arteries to show that S1P₁R inhibition, with the S1P₁/3R antagonist drug VPC 23019, enhances vasoconstriction in response to exogenous S1P. This effect was abolished when the endothelium was denuded (Salomone et al. 2008). The dilatory effect mediated by S1P₁R could be associated with NO production, because S1P-S1P₁R stimulates NO production in endothelial cells (Igarashi et al. 2003, Shu et al. 2002, Igarashi and Michel 2000, also see Levkau 2008). However, Salomone et al. also found direct stimulation of the S1P₁R, with the specific receptor agonist drug SEW 2871, did not cause dilation of the basilar arteries even when the endothelium was functionally intact (Salomone et al. 2008). Therefore the role of S1P₁R in the endothelium is unclear, but it could have a vasodilatory effect in response to exogenous S1P though an NO-dependent mechanism.
Lim and colleagues demonstrated that inhibiting the S1P₁R, with the specific receptor antagonist drug W146, decreased the effect of exogenous S1P on vasoconstriction in rabbit proximal PCAs (Lim et al. 2012). The authors also assessed the effects on the myogenic responsiveness of PCAs by pre-treating the arteries with S1P, a treatment that enhances the MR of the proximal portion of PCAs (Yang et al. 2012). Lim et al. found antagonism of the S1P₁R attenuated the effect of S1P on the MR (Lim et al. 2012). Therefore, S1P₁R expression in VSMCs contributes to regulating S1P-signaling in the myogenic reactivity of proximal cerebral arteries.

The S1P₁R couples primarily to the Gᵢ₄ protein-signaling pathway (Windh et al. 1999). The Gᵢ proteins are inhibitors of the adenlyate cyclase enzyme, and therefore can decrease the concentration of cAMP in cells (Quilliam and Brown in Naccache 1989). The inhibition is primarily linked to the GTP-hydrolysis dependent dissociation of the β and γ subunits. These subunits bind to the Gₐ₄ protein and deactivate adenylate cyclase (Quilliam and Brown in Naccache 1989). In the context of the MR, inhibition of adenylate cyclase activity would reduce the cAMP-dependent activation of PKA, and PKA-dependent phosphorylation of MLCP is thought to be involved in reducing MLCP activity (see Somlyo and Somlyo 2003). Therefore, S1P-signaling via S1P₁R- Gᵢ₄ could augment myogenic responsiveness or promote S1P-dependent vasoconstriction. However, a role for the Gᵢ₄ subunit as an inhibitor of adenylate cyclase activity has not been documented in resistance arteries, and thus far has only been documented in the cardiovascular system (e.g., cardiac myocytes) (reviewed in El-Armouche et al. 2003). Furthermore, the S1P₁R-adenylate cyclase link has thus far only been documented in lymphocytes (Xu et al. 2013). There is also some in-vitro evidence that ERK1/2 is activated by the S1P₁R in cardiomyocytes (Tao et al. 2009), but this pathway may not be applicable to the VSMCs. The activation of PLC-β by S1P-S1P₁R and Gᵢ₄, leading to the release of Ca²⁺ from the
sarcoplasmic reticulum in VSMCs (Okamoto et al. 1998) has also been proposed as a vasoconstriction mechanism, but studies from Murthy’s group argue against this (Hu et al. 2006, Zhou and Murthy 2004). The inconsistencies in the above studies could be due to the different cell types used, and hence different cell-specific signaling pathways. Finally, the S1P1R is not involved in RhoA/ROK signaling pathways (Murakami et al. 2010, Hu et al. 2006). Therefore the S1P1R is involved in regulating the microvascular function of PCAs, but the contradictory results from the mechanistic studies make it difficult to decipher which pathway is involved.

1.4.3.1.2 S1P2R

The S1P2R is a pro-constrictor because genetic inhibition of the receptor in isolated resistance arteries, by transfection of antisense nucleotides, attenuates the vasoconstriction in response to exogenous S1P (Peter et al. 2008). There is a similar effect of pharmacologic inhibiton of S1P2R by pre-treating isolated resistance arteries with the antagonist drug JTE-013 (Lim et al. 2012, Peter et al. 2008). Antagonism with JTE-013 has also been shown to attenuate the myogenic responsiveness of isolated hamster and murine resistance arteries (Hoefer et al. 2010, Lidington et al. 2009), and decrease the S1P-dependent enhancement of myogenic reactivity in rabbit proximal PCAs (Lim et al. 2012). Since antagonism of the S1P2R did not affect resting tone, resting [Ca\textsuperscript{2+}], or pressure-mediated elevation of intracellular [Ca\textsuperscript{2+}] (Lidington et al. 2009), S1P-signaling through the S1P2R appears to regulate the MR in a Ca\textsuperscript{2+}-independent manner. Although JTE-013 treatment has been shown to attenuate the myogenic tone (Yang et al. 2012), and the vasoconstriction to exogenous S1P (Salomone et al. 2008) in resistance arteries from S1P2R knock out (KO) mice, the effects of JTE-013 appear to be specific to S1P-signaling because pre-treatment does not affect norepinephrine-dependent vasoconstriction (Scherer et al. 2010).
The S1P$_2$R couples to the G$_{q/11}$ and G$_{12/13}$ proteins (Skoura and Hla 2009, Hu et al. 2006, Windh et al. 1999), and this coupling contribute the regulation of microvascular function (see reviews by Levkau 2013, Levkau 2008). There is some in-vitro evidence the S1P$_2$R mediates Ca$^{2+}$-dependent signaling via G$_{q11}$ and PLC$\beta$ activation (Hu et al. 2006), but our laboratory’s work with isolated resistance arteries (Lidington et al. 2009) suggests Ca$^{2+}$-signaling is not the dominant pathway. Instead, the activation of the RhoA/ROK-signaling pathway appears to regulate vasoconstriction in response to exogenous S1P-S1P$_2$R binding (Lim et al. 2012, Bolz and Pohl 2003, also see reviews by Kroetsch and Bolz 2013, Kauffenstein et al. 2012).

1.4.3.1.3 S1P$_3$R

The role of the S1P$_3$R is less characterized than S1P$_1$R and S1P$_2$R, but it shares functional characteristics with the other two receptors. S1P treatment can stimulate NO production in endothelial cells through the S1P$_3$R (Nofer et al. 2004), possibly through the Rac1 GTP-ase dependent activation of endothelial nitric oxide synthase (Igararshi and Michel 2009). The production of NO may contribute to the vasodilation of resistance arteries, such as the rat coronary arteries, in response to exogenous S1P (Mair et al. 2010). However, in rodent basilar arteries genetic inhibition of S1P$_3$R by knock out (KO) or transfection with antisense nucleotides, attenuates exogenous S1P-mediated vasoconstriction (Salomone et al. 2008, Salomone et al. 2003). Coupled with in-vitro studies that demonstrate S1P-S1P$_3$R stimulates RhoA signaling, and increases the intracellular [Ca$^{2+}$] (Murakami et al. 2010), it appears the S1P$_3$R is also a pro-constrictor, like S1P$_2$R. Indeed, a recent study with proximal PCAs demonstrated that antagonism of the S1P$_3$R, with the specific receptor antagonist drug CAY10444, inhibited S1P’s ability to enhance pressure-dependent vasoconstriction (Lim et al. 2012).
The S1P$_3$R couples to $G_{\alpha q/11}$ and $G_{12/13}$ proteins (Murakami et al. 2010, Waeber et al. 2004, also see reviews by Kauffenstein et al. 2012, Igarashi and Michel 2009), like the S1P$_2$R. The S1P$_3$R does not appear to couple to $G_{i1}$ (Salomone et al. 2003), unlike the S1P$_1$R. Thus, the compiled data suggests S1P$_3$R is a pro-constrictor of resistance arteries, and can regulate S1P-signaling in the myogenic responsiveness of proximal PCAs.
Figure 1-2. Summary of the role of sphingosine-1-phosphate (S1P) in the myogenic response (MR) and vasoconstriction of resistance arteries.

The increase in wall tension of a resistance artery, after the pressure is increased, leads to the activation of ERK1/2-signaling. This phosphorylates SphK1, and causes the enzyme to activate and translocate to the plasma membrane. Here, SphK1 stimulates the production of S1P into an intracellular and extracellular compartment. In the intracellular compartment, S1P is responsible for increasing the \([\text{Ca}^{2+}]\) by mobilizing it from the sarcoplasmic reticulum. S1P is also released in an autocrine manner to the extracellular environment, where it can bind to its GPCRs, the S1P-receptors (S1PRs). There are five known S1PRs found on the surface of cells. Of these, three S1PRs can contribute to the effect of S1P on the myogenic response of resistance arteries. (1) The S1P1R may enhance the \([\text{Ca}^{2+}]\) by intracellular signaling mechanisms, such as IP3 or PLC-β. (2) The S1P2R is well characterized to inhibit MLCP through the RhoA/ROK-signaling pathway. (3) The S1P3R can also stimulate the RhoA/ROK-signaling pathway.
1.4.3.2 Summary

S1P in the extracellular compartment controls the Ca\(^{2+}\)-sensitization mechanism by binding to G-protein coupled S1PRs on the surface of VSMCs. The exact receptor-mediated effects can differ, depending upon the vascular bed. However, a recent study demonstrated that S1P\(_{1-3}\)R all promote S1P-dependent myogenic vasoconstriction in proximal PCAs (Lim et al. 2012). Presumably, cooperative action among the receptors exists, at least in the cerebral microvasculature.

1.4.4 Degradation of S1P

The tight control of S1P bioavailability (i.e., the steady state between S1P’s production and degradation) is critical for an effective regulation of the MR. In prokaryotes, lyases have been demonstrated to irreversibly degrade S1P (Huwiler et al. 2011), but in mammalian cells the degradation of S1P is independent of S1P-lyase (Peest et al. 2008). Furthermore, S1P-lyases produce vasoactive metabolites other than sphingosine (Ikeda et al. 2004). Plasma membrane lipid phosphate phosphatases (LPPs) have also been shown to degrade extracellular S1P (Brindley 2004, also see reviews by Brindley and Pilquil 2009, Sigal et al. 2005), but LPPs do not act specifically on S1P (Jasinka et al. 1999). Intriguing candidates that specifically convert S1P into sphingosine are the S1P-phosphohydrolases, such as SPP1 and SPP2. Our laboratory characterized the effect of degradation on endogenous S1P-signaling, by assessing SPP1 involvement in the MR of isolated hamster gracilis muscle resistance arteries. We found that overexpression of SPP1 reduced the myogenic responsiveness, whereas SPP1 inhibition with antisense oligonucleotides enhanced the myogenic responsiveness (Peter et al. 2008). In HEK 293 cells that overexpress the murine SPP1 isoform, the enzyme is predominantly localized to
intracellular membranes (e.g., membrane of the ER), but there is residual expression in the plasma membrane (Le Stunff et al. 2002). Our laboratory was unable to detect plasma membrane localization of SPP1 in isolated hamster gracilis muscle resistance arteries, yet we demonstrated that SPP1 overexpression reduces exogenous S1P-dependent vasoconstriction, and inhibition of SPP1 enhances the vasoconstriction in response to exogenous S1P (Peter et al. 2008). The vascular data supports the idea that S1P degradation or dephosphorylation by intracellular S1P phosphatases is critical to its bioavailability. Although LPPs were not assessed, the finding that the overexpression of SPP1 attenuates S1P-mediated vasoconstriction, and the inhibition of SPP1 with antisense nucleotides augments vasoconstriction, suggests SPP1 contributes to the S1P degradation mechanism in resistance arteries.

1.4.5 Summary of the S1P-dependent regulation of the MR

An elevation in transmural pressure stimulates the production of the vasoactive mediator S1P through ERK 1/2 signaling and SphK1. This occurs simultaneously with the pressure-dependent elevation in [Ca^{2+}]. Then S1P has compartmentalized effects (i.e., intracellular and extracellular). Intracellular S1P promotes vasoconstriction by increasing the [Ca^{2+}], which contributes to the initial elevation in [Ca^{2+}]. This pathway constitutes a Ca^{2+}-dependent mechanism for regulating the MR. In contrast, extracellular S1P binding to the S1P_{2}R and the S1P_{3}R stimulates RhoA/ROK-signaling, through activation of G-proteins, and MLCP activity is inhibited. Then sensitivity of the VSMC contractile apparatus to Ca^{2+} is increased, by the prevention of MLC_{20} de-phosphorylation. S1P binding to S1P_{1}R and S1P_{2}R can also stimulate alternate Ca^{2+}-dependent pathways that may also contribute to myogenic vasoconstriction (Figure 1-2).

The degradation of S1P, and thereby its extracellular effects, are regulated by intracellular SPP1. However, S1P is a hydrophobic zwitterion (Garcia-Pacios et al. 2009), and
this eliminates the possibility for it to spontaneously flip-flop across the plasma membrane (see reviews by Rosen et al. 2013 and Hannun and Obeid 2008). Therefore, intracellular SPP1 access to S1P requires uptake of the phospholipid. Our laboratory found the uptake of S1P to be regulated by the ABCC7 transporter, also known as the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) protein. Theoretically, the modulation of the uptake affects S1P-signaling in the MR. My thesis specifically assesses the role of CFTR as a microvascular regulator, and how possible interplay between S1P and CFTR governs the MR.

1.5 The Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) channel

CFTR is a transmembrane anion channel that is responsible for primarily the secretion of chloride (Cl\(^-\)) out of the cell, and is a member of the ATP-Binding Cassette (ABC) class of transporters (Tsui and Dorfan 2013, Kim Chiaw et al. 2011, Riordan 2008, Riordan 2005, Sharma et al. 2004, Gadsby and Nairn 1999, Bear et al. 1993). CFTR is predominantly localized in the apical surface of epithelial cells (van Meegen et al. 2013, Bomberger et al. 2011, Guggino and Stanton 2006, Ameen et al. 1999), but is also expressed in tissues of the cardiovascular system, such as the human myocardium (Solbach et al. 2008), cultured cardiac myocytes (Lader et al. 2001), hamster gracilis muscle resistance arteries (Peter et al. 2008), murine cerebral resistance arteries (e.g., PCAs) (Meissner et al. 2012), and murine VSMCs from mesenteric resistance arteries (Meissner et al. 2012). The structure of CFTR (Figure 1-3) is composed of two membrane-spanning domains (MSDs), two nucleotide binding domains (NBDs) that control channel gating, and a regulatory (R)-domain.
Figure 1-3. Proposed model for phosphorylation of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Regulatory (R)-domain.
This figure shows a CFTR protein with its two membrane-spanning domains, two nucleotide-binding domains (NBDs), intracellular C- and N-termini, and the regulatory (R)-domain. ATP
binding and hydrolysis to the NBDs stimulates heterodimerization. Non-phosphorylated or basally phosphorylated R-domain is bound to the C-terminus of CFTR. Phosphorylation of the stimulatory serine residues by Protein Kinase A (PKA) causes the binding between the R-domain and C-terminus to decrease (Chappe et al. 2005). Subsequently, the binding between the R-domain and the N-terminus is enhanced. The conformational change(s) induced may contribute to opening of the channel (Chappe et al. 2005). In contrast, the binding of AMP-activated protein kinase (AMPK) to CFTR, and the subsequent phosphorylation of the inhibitory serine residues may reestablish the interaction of the R-domain to the C-terminus. Here, the conformational changes induced will lead to closing of the channel.
1.5.1 The regulation of CFTR channel function by phosphorylation

The channel function of CFTR is dependent upon ATP in the same manner as the other ABC transporters, where cytosolic ATP acts as the ligand (see reviews by Kim Chiaw et al. 2011, Gadsby et al. 2006, Gadsby and Nairn 1999). ATP binds to CFTR’s nucleotide binding domains, NBD1 and NBD2 (Berger et al. 2005, Ikum and Welsh 2000, Carson et al. 1995, Li et al. 1996, also see review by Gadsby et al. 2006), to trigger a conformational change that causes the NBDs to dimerize in a head-to-tail fashion, and this opens the channel (Rahman et al. 2013, Hwang and Sheppard 2009). However, ATP cannot trigger channel opening unless CFTR’s R-domain is phosphorylated. Thus, the R-domain maintains primary dynamic control of CFTR’s gating via allosteric mechanisms that are predominantly independent of ATP/NBD interaction (Ma et al. 1997, see Gadsby and Nairn 1999 for a comprehensive review).

There are several enzymes, including Protein kinase A (PKA) (Hegedus et al. 2009, Chappe et al. 2005, Csanady et al. 2005), Protein kinase C (PKC) (Chappe et al. 2003), and AMP-activated protein kinase (AMPK) (King et al. 2009, Kongsuphol et al. 2009, Hallows et al. 2003, Hallows et al. 2000), that are known to phosphorylate the R-domain. The enzymes catalyze the addition of a phosphate group to amino acid residues within CFTR’s R-domain. The addition of phosphate is generally thought to promote conformational changes within the target protein (Johnson 2009), possibly by changing the hydrophobicity of protein regions. Indeed phosphorylation of CFTR’s R-domain is thought to increase the number of negative charges (Bozoky et al. 2013), and change the polarity of amino acid residues. The conformational changes alter CFTR’s activity. There are dibasic and monobasic PKA consensus sequences in the CFTR protein. The dibasic sequences contain arginine (Arg, R), lysine (Lys, K), Ser (S) or Thr (T), and the sequences are R-R/K-X-S/T and R-X-X-S/T. The monobasic sequences include Arg and Ser or Thr, and an example is R-X-S/T (Townsend et al.1996, Kennelly and Krebs
1991). In total, there are 14 high-affinity PKA consensus sequences (i.e., preferential phosphorylation by PKA) clustered around CFTR’s NBD1 and the R-domain. There are also numerous potential low-affinity sites, or residues that are less conserved, for PKA-mediated Ser phosphorylation (Gadsby and Nairn 1999, Mathews et al. 1998, Riordan et al. 1989). The existence of these additional residues may explain why a CFTR construct with alanine substitutions at ten consensus PKA-dependent Ser residues, still displays some PKA-dependent channel activation (e.g., approximately 70% less than the WT-CFTR protein), as assessed by patch-clamp electrophysiology and iodide efflux studies in Chinese hamster ovary cells (Chang et al. 1993). At least five of the R-domain Ser residues have been shown to be phosphorylated with amino acid sequencing or mass spectrometry studies of the full-length CFTR protein, or its isolated domains (at positions 660, 700, 737, 795 and 813). Furthermore, the Ser residues at positions 660, 700, 737, and 813 undergo phosphorylation via treatment with cAMP agonists (Cheng et al. 1991) or treatment with purified PKA (Cohn et al. 1992, Picciotto et al. 1992) in cells (e.g., T84 cells), as detected by radioactive labeling (e.g., $^{32}$P). There are at least an additional three Ser residues at positions 712, 753 and 768 shown to be phosphorylated in cells (e.g., HEK-293 cells) expressing the WT-CFTR protein, as detected using radioactive labeling (e.g., $^{32}$P) (King Jr. et al. 2009, summarized in Gadsby and Nairn 1999, Gadsby and Nairn 1994). Although most of the Ser residues stimulate CFTR function, at least two of these residues (S737, S768) inhibit its function when phosphorylated (Wilkinson et al. 1997).

1.5.1.1 Stimulation of CFTR function by PKA and PKC

Biochemical experiments, using purified and reconstituted full-length CFTR, demonstrated that the ability of CFTR to respond to ATP binding is enhanced when it is phosphorylated by PKA (Li et al. 1996). Therefore, PKA-mediated phosphorylation of the R-domain increases the affinity for ATP (see reviews by Gadsby and Nairn 1999, Carson and
Welsh 1993). Furthermore, phosphorylation causes conformational changes that contribute to opening of the channel (Grimard et al. 2003). There is no evidence in the literature that PKA can bind to CFTR directly instead the kinase forms a complex with cytoplasmic proteins, such as ezrin, that may link CFTR’s C-terminus to the actin cytoskeleton of the cell (Sun et al. 2000, also see Guggino and Stanton 2006). Thus, PKA-dependent phosphorylation promotes conformational changes independent of direct interaction(s) with CFTR. Phosphorylation by PKA promotes intramolecular interactions, and interactions between several of CFTR’s domains and the R-domain have been shown (Bozoky et al. 2013), such as the NBDs (Wang et al. 2012, Baker et al. 2007), the amino-terminus (i.e., N-terminus) (Naren et al. 1999), and the carboxyl-terminus (i.e., C-terminus) (Wang et al. 2012). One proposed theory for the effects of these conformational changes on channel gating is based upon experiments with a CFTR construct that lacks the R-domain (Split∆R-CFTR) (Figure 1-3). Chappe and colleagues demonstrated PKA-dependent changes in CFTR protein conformation may promote interactions between the R-domain and the N-terminus (Chappe et al. 2005). Concurrently, interactions between the R-domain and other regions of the protein are reduced, thereby promoting channel activity. PKA-dependent phosphorylation of CFTR’s R-domain is also thought to decrease the number of α-helical structures, and increase the number of β-pleated sheets (Baker et al. 2007). Therefore, phosphorylation may also affect the secondary structures of the R-domain. PKA-dependent phosphorylation may promote channel activity by affecting the secondary structures of the R-domain, and decrease the interactions between the R-domain and the NBDs. Therefore, heterodimerization of NBDs is promoted (Baker et al. 2007). The critical role of PKA is further observed with the ΔF508-CFTR mutation, a genetic deletion of the phenylalanine (Phe)-508 residue from the NBD1. The deletion of the F508 residue leads to errors in protein folding and processing that hinders localization of the mutant protein to the plasma membrane, and PKA-
mediated phosphorylation of the R-domain. This contributes to the decreased channel function, relative to the wild-type (WT) protein (Wang et al. 2000). The mutation affects approximately 70% of all CF patients (Clancy et al. 2012, Chai et al. 2011, Kerem et al. 1989, Cystic Fibrosis Mutation Database).

Although PKA-mediated phosphorylation dominates the activation of CFTR’s function, there are also two residues present in the R-domain that are phosphorylated by PKC (S686 and S790). In-vitro studies have demonstrated that inhibition of PKC causes CFTR to enter a refractory state, and the channel is less responsive to PKA (Liedtke and Cole 1998, Middleton and Harvey 1998, Jia et al. 1997). Conversely, PKC-mediated phosphorylation of the R-domain enhances the ability of PKA to open the channel, by reducing the mean closed time (Jia et al. 1997). Furthermore, patch-clamp studies have demonstrated that phosphorylation by PKC can increase the number of CFTR channels that can be opened by PKA-mediated phosphorylation (Chappe et al. 2003). Thus, PKC is a weak-to-modest activator of CFTR’s function compared to PKA (Chappe et al. 2003, Berger et al. 1993, Tabcharani et al. 1991). PKC may potentiate the effects of PKA or be a prerequisite for subsequent PKA-mediated activation (see reviews by Gadsby et al. 2006, Gadsby and Nairn 1999).

In addition to activating CFTR function, phosphorylation by either PKA or PKC increases the stability of CFTR in the membrane (Lewarchik et al. 2008, Bertrand and Frizzell 2003), and this may contribute to enhanced channel function. One possible explanation is phosphorylation enhances the interaction of CFTR to the actin cytoskeleton, and likely occurs at the PDZ domains of cytoskeletal proteins. PDZ domains are modular 70-90 amino acid domains in proteins, capable of binding to PDZ-interacting domains that are composed of short peptide sequences at the C-terminus (Moyer et al. 1999, also see review by Fanning and Anderson 1999). CFTR’s PDZ-interacting domain comprises the last three amino acids of the C-terminus.
(Moyer et al. 1999). It has been demonstrated that CFTR’s PDZ-interacting domain interacts with the PDZ domains in ezrin-radixin-moesin-binding phosphoprotein 50 (EBP50), a cytoskeletal protein found in the apical membrane of epithelial cells (Haggie et al. 2006, Moyer et al. 1999, Hall et al. 1998, Short et al. 1998, Wang et al. 1998). The binding between CFTR and EBP50 decreases the diffusion of CFTR from the membrane (Haggie et al. 2004), and phosphorylation may stabilize the attachment of CFTR to EBP50 (Sun et al. 2000). A second explanation deals with trafficking from the plasma membrane. PKA and PKC-mediated phosphorylation has also been shown to reduce endocytosis of CFTR (Lukacs et al. 1993), and this increases CFTR expression in the plasma membrane. Thus, phosphorylation of CFTR’s R-domain by PKA, and to a lesser degree by PKC, causes conformational changes that open the channel pore, and can also maintain CFTR expression in the plasma membrane. The outcome is stimulation of channel activity.

Phosphorylation of the R-domain’s inhibitory residues (S737, S768) can decrease CFTR function (Wilkinson et al. 1997, Gadsby and Nairn 1999). Indeed, transfection of a CFTR construct with site-directed mutagenesis of the S737 residue to alanine, in Fischer rat thyroid epithelial cells, has been shown to enhance the transepithelial chloride current (Baldrursson et al. 2000). Similar effects on in-vitro channel function, assessed in Xenopus oocytes, have also been observed after site-directed mutagenesis of the S768 residue (Kongsuphol et al. 2009, Csanády et al. 2005). These studies support the inhibitory role of the two residues. The S737 and S768 residues can be phosphorylated by PKA (Hegedus et al. 2009, Seibert et al. 1999, Wilkinson et al. 1997). An in-vitro study by Csanády and colleagues demonstrated that PKA-dependent phosphorylation of the S737 residue may change the secondary structures of regions within CFTR’s R-domain, as suggested by differences in electrophoretic mobility of R-domain peptides (Bozoky et al. 2013, Csanády et al. 2005, also see Section 6.3). These changes could account for
the inhibitory effect of PKA-dependent S737 phosphorylation. However, an *in-vitro* study by the Riordan laboratory demonstrated that PKA-mediated phosphorylation of S737 or S768 does not inhibit CFTR channel function, as assessed by single channel patch-clamp recordings of membrane vesicles generated from BHK cells stably transfected with WT-CFTR or its constructs (Hegedus *et al.* 2009). Therefore, there are differences in the inhibitory results observed with PKA-dependent phosphorylation of the S737 and S768 residues, and this may be related to the models used to assess CFTR’s channel function (e.g., *Xenopus* oocytes versus patch-clamping of membrane vesicles). It has been reported that *in-vitro* AMPK-mediated phosphorylation of the R-domain at the inhibitory residues inhibit CFTR function (King Jr. *et al.* 2009, Kongsuphol *et al.* 2009), and this inhibition is discussed next.

### 1.5.1.2 Inhibition of CFTR’s function by AMPK

AMPK is a serine-threonine kinase, and its activity is stimulated during energy reduction in cells, where the AMP to ATP ratio is increased, thereby acting as a metabolic sensor (Wang *et al.* 2003). The mammalian AMPK is a heterotrimeric complex (Dolinsky and Dyck 2006), containing α (two isoforms), β (two isoforms), and γ subunits (three isoforms) (Steinberg and Kemp 2009). Alternative splicing of the AMPK mRNA has been shown (Verhoeven *et al.* 1995), and suggests there are additional isoforms of the subunits (Viollet *et al.* 2009). Hence, there are least 12 different combinations of the AMPK enzyme because of different subunit isoforms. The expression of AMPK isoforms in resistance arteries has not been characterized. The α subunits comprise the catalytic domain of the enzyme (Xiao *et al.* 2011), whereas the β-subunits act as a linker between the α and γ subunits (Xiao *et al.* 2011). The γ-subunits form four cystathionine β-synthase (CBS) domains, and together this is called the Bateman domain, which can bind AMP or ATP (Xiao *et al.* 2011, Nagata and Hirata 2010). Under basal
conditions the ratio of AMP to ATP is low, and ATP binds to the Bateman domain, rendering AMPK inactive. Cellular stress can increase the concentration of AMP, then ATP is removed from the Bateman domain, and AMP binds in its place. The binding “unlocks” the γ-subunit and induces a conformational change. The conformational change induced by the unlocking provides access to upstream kinases, such as calcium/calmodulin-dependent protein kinase kinase β (CaMKKβ) (Levine et al. 2007) and liver kinase B1 (Shaw et al. 2004), to phosphorylate AMPK’s α subunit at its Thr-172 (T172) amino acid residue, and AMPK is activated (Xiao et al. 2011, Levine et al. 2007). In contrast, AMPK is inactivated by a combination of phosphatase-dependent dephosphorylation of the T172 residue (Ruiz et al. 2013), and stimulation of its autoinhibitory domain (Chen et al. 2009).

Pharmacologic activation of AMPK reduces the FSK and cAMP-stimulated channel function of CFTR, as assessed with the drug AICAR in polarized T84 cell monolayers (Hallows et al. 2003), or the drug phenformin in Xenopus oocytes (Kongsuphol et al. 2009). AMPK’s α1 and α2 subunits bind to CFTR’s C-terminus, and this was demonstrated using glutathione-s-transferase (GST) pull-down experiments (Hallows et al. 2000). The α1 subunit mRNA and protein have greater tissue expression distribution than the α2 subunit (Stapleton et al. 1996). Additionally, Hallows and colleagues initially identified the α1 subunit in yeast-two-hybrid screening experiments (Hallows et al. 2000). AMPK’s α1 subunit interacts with CFTR’s C-terminus at amino acid residues 1420-1437, and the constitutively active catalytic subunit fragment, AMPK α1-1-312 construct, can phosphorylate immunoprecipitated CFTR. Injection of the α1 subunit into Xenopus oocytes expressing WT-CFTR was shown to inhibit cAMP-stimulated whole cell conductance (Hallows et al. 2000). If the AMPK α1 subunit is mutated to reduce persistent binding to CFTR, but phosphorylation of CFTR can still occur (AMPK α1-1-312 construct) then AMPK is rendered incapable of inhibiting channel function (Hallows et al. 2000).
A similar finding is observed when AMPK catalytic activity is reduced (i.e., ability to phosphorylate CFTR), but binding can still occur (AMPK α1-K45R construct) (King Jr. et al. 2009, Hallows et al. 2003, Hallows et al. 2000). Thus, the inhibitory effect of AMPK is likely the result of the combined allosteric effects of CFTR R-domain phosphorylation and CFTR-AMPK protein interaction. The binding of AMPK to CFTR’s C-terminus may account for the absence of an inhibitory role for the S737 or the S768 residue, in response to PKA-dependent phosphorylation, observed by Hegedus and colleagues (Hegedus et al. 2009). Since there is no evidence in the literature that demonstrates direct CFTR-PKA binding, it is possible that the intramolecular conformational changes promoted by CFTR-AMPK interaction and AMPK-dependent phosphorylation are different from PKA-dependent phosphorylation, of the S737 and S768 residues. One possibility is that the binding of AMPK to CFTR, and the subsequent phosphorylation of its inhibitory sites, could cause conformational changes that decrease the intramolecular interactions between CFTR’s R-domain and N-terminus (Figure 1-3). Hence, AMPK-dependent phosphorylation of CFTR’s R-domain occurs at inhibitory residues, and this is reliant upon AMPK-CFTR binding, and the outcome is decreased channel function.

The ratio of AMP to ATP, and thus the catalytic activity of AMPK, is increased during such events as hypoxia and accumulation of reactive oxygen species (Mungai et al. 2011), and low glucose (McCrimmon et al. 2006). Hallows and colleagues demonstrated that in-vitro treatment with the Staphylococcus aureus α-toxin, to permeabilize the basolateral side of polarized T84 cells, in combination with Na-AMP and the adenylate cyclase inhibitor diadenosine pentaphosphate (Beall et al. 2013, Vanderlijn et al. 1979) presence in the basolateral solution, inhibited the cAMP-stimulated chloride conductance (Hallows et al. 2003). The results suggest that the direct elevation of intracellular AMP concentration inhibits CFTR channel function. However, pharmacologic antagonism of adenosine receptors, with the drug 8-p-(sulphophenyl)-
theophylline (Crosson and Petrovich 1999), did not affect the reduction in channel function (Hallows et al. 2003). Additionally, a study by Schultz and colleagues used mouse L cells transfected with WT-CFTR and patch-clamp recordings, and demonstrated the absence of AMP-dependent inhibition on CFTR’s channel function (Schultz et al. 1995). Schultz et al.’s data and the work from Hallows et al. suggest the elevated intracellular AMP concentration does not directly inhibit CFTR’s channel function. Instead, the elevation in AMP may promote activation of endogenous AMPK, and then the activated AMPK inhibits CFTR’s channel function (Hallows et al. 2003). An additional in-vitro activator of AMPK is S1P through the stimulation of CaMKKβ, as demonstrated by Levine and colleagues in bovine aortic endothelial cells (Levine et al. 2007). This finding suggests S1P is a modulator of CFTR’s channel function, in an AMPK-dependent manner, as proposed in Section 1.6.

1.5.2 The role of CFTR in the MR

Our laboratory assessed the role of CFTR in the MR through pharmacological inhibition of its function with CFTRinh-172 (Peter et al. 2008), a specific cell-permeable thiazolidinone compound. This compound acts as a voltage-independent inhibitor of CFTR function by decreasing the frequency of channel opening, without affecting conductance (Taddei et al. 2004). CFTRinh-172 is unlikely to be a pore blocker (Kopeikin et al. 2010), and instead may bind directly to CFTR at sites within the MSD1 (Caci et al. 2008) near the channel pore. When the CFTR channel is open the binding of CFTRinh-172 closes the channel, and when the CFTR channel is closed the binding stabilizes the closed state and prevents opening of the channel (Kopeikin et al. 2010). Treatment with CFTRinh-172 increased the myogenic vasoconstriction of hamster gracilis muscle resistance arteries (Peter et al. 2008). Though CFTRinh-172 is characterized as an inhibitor of CFTR channel function in cell culture, the reduction in Cl− conductance would lead to hyperpolarization, and in resistance arteries this could possibly
decrease vasoconstriction. Therefore, the enhanced vasoconstriction our laboratory documented appears to be independent of CFTR channel function. Instead the enhancement in the MR may have been caused by abnormal regulation of S1P bioavailability, and this prediction was based upon Boujaoude et al.’s seminal finding that cells expressing WT-CFTR can uptake labeled S1P, whereas cells lacking CFTR cannot (Boujaoude et al. 2001). Hence, our laboratory predicted that CFTR inhibition would enhance S1P’s effects on vasoconstriction (Figure 1-4). Indeed, CFTR$_{inh}$-172 treatment augmented the vasoconstriction to exogenous S1P in arteries transfected with WT-SPP1 (Peter et al. 2008), and this provides support for CFTR’s role as the regulatory bottleneck for S1P degradation in resistance arteries. The uptake of S1P was confirmed to be CFTR-dependent, by demonstrating that cultured VSMCs from the mesenteric arteries of CFTR KO mice cannot uptake fluorescein isothiocyanate-labeled S1P (S1P-FITC), whereas cells from WT mice can (Meissner et al. 2012). Furthermore, isolated and cannulated proximal PCA’s from CFTR KO display greater myogenic reactivity, relative to PCAs from WT-CFTR expressing mice, and the augmentation is dependent upon S1P-signaling (Meissner et al. 2012). CFTR’s critical role leads to the question of how the protein can be regulated to inhibit S1P’s degradation. Furthermore, the importance of CFTR in regulating S1P bioavailability suggests that in diseases where CFTR expression is abnormal there would be enhanced S1P-signaling, and abnormal microvascular function.
Reduced or abnormal expression of CFTR

Under control conditions, vascular smooth muscle cells that express endogenous WT-CFTR can uptake extracellular S1P. In resistance arteries, the CFTR-dependent uptake may increase the likelihood that intracellular S1P phosphohydrolase 1 (SPP1) can bind to, and dephosphorylate S1P. Together this may decrease the interaction between S1P and its G-protein coupled receptors (e.g. S1PRs), and regulate the myogenic response (MR). However, when CFTR expression is reduced there is also a reduction in the uptake of S1P. Then in resistance arteries, the reduction in CFTR-dependent uptake may lead to increased binding of S1P to its S1PRs, and therefore there is enhancement of the myogenic responsiveness.

Figure 1-4. The uptake of sphingosine-1-phosphate (S1P) is dependent upon expression of the wild-type Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) protein.
Abnormal expression and function of the CFTR protein is the cause of the cystic fibrosis (CF) diseases, and assessing cellular and animal models of this disease insight could provide insight to role of CFTR in regulating S1P’s bioavailability, as discussed next. Furthermore, our group has previously described the role of CFTR in an animal model of heart failure (HF), a cardiovascular disease associated with reduced CFTR mRNA and protein expression in microvascular tissue (Meissner et al. 2012), and this is discussed shortly.

1.5.3 CFTR in cystic fibrosis (CF)

CF is the most common and fatal genetic disease (i.e., homozygous recessive) that affects the Caucasian population. In Canada, one in every 3,600 children born within the Caucasian population has the disease (source: Cystic Fibrosis Canada). The CFTR gene is located on chromosome 7 (Riordan et al. 1989), and CF is primarily caused by genetic mutations of this gene. The mutations affect the function and/or expression of the ubiquitously expressed CFTR anion channel (reviewed in Gelfond and Borowitz 2013, Anderso 2010). There are over 1,900 mutations identified in the Cystic Fibrosis Mutation Database, and approximately 70% of CF patients have the F508 mutation (Anderson 2010, Kunzelmann and Nitschke 2008), and over 90% of CF patients have at least one allele of this mutation (Kirby et al. 2013). The defects in the CFTR protein have effects on the ion transport for epithelial cells. In the airways, thickening of the mucus results, which contributes to impaired breathing and increased rate of infection (reviewed in Ratjen 2006). Other organs and physiological systems that are affected by CF include: the sweat glands (Quinton 2007), diagnosed as increased concentration of salt (e.g., NaCl) in the sweat of CF patients (Mishra et al. 2005, Gibson and Cooke 1959); gastrointestinal tract (Gelfond and Borowitz 2013, Anderson 2010, Dodge 1986), observed as mucus buildup and bowel obstruction (Davidson et al. 1987); and the kidneys (Yahiaoui et al. 2009), observed
as renal dysfunction (Nazareth and Walshaw 2013). The wall thickness of the heart’s right ventricle and dimensions of the cavity are abnormal in children with CF. Furthermore, in older children with CF the wall of the right ventricle was shown to be thicker as the severity of the disease increases (Allen et al. 1979). However, the cardiovascular parameters, such as left and right ventricular ejection fractions, and right ventricular systolic and diastolic wall thicknesses, show no significant differences between healthy and CF patients (Panidis et al. 1985). The cardiovascular effects may be secondary to the chronic lung disease. Additionally, there are no published data on the effects of CF on cerebral microvascular function (e.g., the MR), and it is difficult to make a statement on cerebral autoregulation in CF patients. Nevertheless, microvascular studies with CFTR KO mice demonstrate that CFTR is critical to the myogenic reactivity of cerebral resistance arteries via the S1P-signaling pathway (Meissner et al. 2012), described further in Section 1.5.4, and studies with mouse models of CF could verify and provide insight into the role of CFTR in cerebral microvascular function and cerebral autoregulation (see Section 1.6.2).

The genetic deletion of the F508 residue from CFTR’s NBD1 contributes to errors in protein folding, and export from the endoplasmic reticulum (ER) is prevented. Consequently, almost all the ΔF508-CFTR is ubiquinated, and then subsequently degraded by the cytoplasmic proteasomes and other proteases. Thus, the mutant does not transport to the Golgi apparatus (Sharma et al. 2004, Jensen et al. 1995, Ward et al. 1995). The inability of ΔF508-CFTR to escape the ER and enter the Golgi prevents its processing to the complex glycosylated mature form, and decreases expression of the protein in the plasma membrane (Molinski et al. 2013, Okiyoneda et al. 2013, Lukacs and Verkman 2012, Belcher and Vig 2010, Rosser et al. 2008, Sharma et al. 2004). Low-temperature rescue and small-molecule corrector compounds have been shown to enhance processing of the mutant. However, rescued ΔF508-CFTR also displays
decreased channel function relative to WT-CFTR (Kim Chiaw et al. 2010, Van Goor et al. 2006, Hwang et al. 1997, Dalemans et al. 1991), and one reason for this may be that the conformational changes induced by the F508 deletion affect the channel gate (Molinski et al. 2013, Sharma et al. 2004). Hence, the severity of the CF phenotype in patients that express ΔF508-CFTR is due to the effects of the mutation on protein processing and channel function.

Precursors of S1P, such as ceramide, have been shown to be elevated in the lung tissue of CF patients (Wojewodka et al. 2011, Becker et al. 2010). Furthermore, cells expressing ΔF508-CFTR cannot uptake S1P-FITC relative to WT-CFTR cells (Meissner et al. 2012, also see Chapter 4). These findings suggest the genetic F508 mutation affect CFTR’s S1P-uptake function. As previously mentioned, S1P is an upstream regulator of AMPK, and the reduced uptake could explain the enhanced enzymatic function of AMPK in non-differentiated airway epithelial cells isolated from CF patients (Hallows et al. 2006). Inhibition of the S1P and AMPK-signaling pathways may have beneficial effects on ΔF508-CFTR function in cells, and its microvascular function, such as cerebral autoregulation (see Section 1.6.2. and Chapter 4).

1.5.4 CFTR in heart failure (HF)

HF is a multifaceted disease characterized by the heart’s inability to provide sufficient blood in order to meet the metabolic demands of tissues (Kroetsch and Bolz 2013, Guyton et al. 2000), and this leads to progressive damage of multiple organs (Bui et al. 2011). HF is the leading cause of cardiovascular morbidity and mortality, and it affects over 23 million people worldwide (Lloyd-Jones et al. 2010, also see review by Bui et al. 2011). It is important to decipher the pathology and develop effective treatments because of the detrimental effects of HF. Population based studies, like the Framingham heart study (Levy and Larson 1996), have identified some of the most common conditions that can cause HF in humans including
hypertension, cardiomyopathies, coronary artery disease, and congenital heart disease (Mosterd and Hoes 2007). Common clinical symptoms of HF include fatigue, difficulty in breathing (e.g., wheezing), muscle wasting, and confusion or delirium (Mosterd and Hoes 2007). The primary hemodynamic characteristic of the disease is the dramatic drop in cardiac output (CO) that is caused by abnormal contractility of the heart, with minimal impact on the mean arterial pressure (MAP) (Sullivan et al. 1989). The effect on the MAP is small due to increased total peripheral resistance (TPR), as a compensatory mechanism (Lindsay et al. 1996, Sullivan et al. 1989, also see Kroetsch and Bolz 2013). The augmented TPR provides maintenance support by keeping the MAP at approximately normal levels in the initial stages of HF, and is tolerated by the organs (Kroetsch and Bolz 2013). As the HF disease progresses, the TPR reaches a maximum level, and along with the continued drop in CO the MAP dramatically decreases. This is a hallmark of late-stage HF (Rosenblum et al. 2010, Cowie et al. 2000, Chin et al. 1997). Furthermore, rodent studies demonstrate the augmented myogenic responsiveness of resistance arteries accounts for the increase in the TPR (Kroetsch and Bolz 2013, Yang et al. 2012, Hoefer et al. 2010, Gschwend et al. 2003, Ledoux et al. 2003). In the early-stages of HF, a strong MR is critical as it keeps blood flow constant to the tissues, but in the late-stages of HF the continued augmentation compromises tissue perfusion, in an effort to maintain MAP within acceptable limits (Kroetsch and Bolz 2013). In HF mice, several resistance arteries demonstrate enhancement of their MR as a compensatory mechanism, including mesenteric arteries (Hoefer et al. 2010, Xu et al. 2009, Gschwend et al. 2003), proximal PCAs (Meissner et al. 2012, Yang et al. 2012, Hoefer et al. 2010, Georgiadis et al. 2000), and cremaster arteries (Hoefer et al. 2010). Since the augmentation occurs across several vascular beds, this may explain the multitude of clinical consequences of HF including skeletal muscle abnormalities (Schaufelberger et al. 1997) that could be caused by decreased blood flow through the skeletal muscle resistance arteries,
dysfunction of the gastrointestinal system via reductions in blood perfusion (Krack et al. 2005), and decreased CBF (Choi et al. 2006) and the negative consequences on higher order brain function (e.g., impaired cognition, confusion, impaired memory) (Pressler et al. 2010, Sauvé et al. 2009, Gruhn et al. 2001).

Abnormal cognitive function has been reported in 30 to 80% of all HF patients (Vogels et al. 2007, Bennett and Sauve 2003), and the wide range of prevalence in these reports could be due to differences in study designs, severity of the HF disease, and differences in the diagnostic parameters for cognitive dysfunction (Dardiotis et al. 2012). In patients, as the severity of HF increases (e.g., according to New York Hospital Association classification system), CBF has been shown to decrease (Dardiotis et al. 2012, Gruhn et al. 2001), and there are greater incidences of cognitive dysfunction (Zuccala et al. 1997). In humans, HF has been shown to affect global CBF measurements (Choi et al. 2006, Gruhn et al. 2001), or CBF within distinct regions like the posterior cortex (Alves et al. 2005). Thus, the literature demonstrates there are global or local reductions in CBF associated with human HF, and there is a consensus that reduced cerebral perfusion contributes to the cognitive dysfunction (Dardiotis et al. 2012). The cognitive dysfunction affects the quality of HF patients’ lives, because there may be difficulties in adhering to medication and treatment protocols. These patients have also been shown to display an impaired ability to make health care decisions, as assessed with self-reports of their medical history (Malik et al. 2011, also see Dardiotis et al. 2012). Treatments that restore cerebral perfusion in HF patients may have beneficial effects on the abnormal cognitive function, and improve the quality of their lives.

Mice with HF, induced through myocardial infarction via ligation of the left anterior descending coronary (LAD) artery, display reduced CBF measurements in several regions of their brains, such as the anterior and posterior cortex, as measured using magnetic resonance
imaging (MRI) techniques (Yang et al. 2012). Yang and colleagues observed no structural abnormalities in the brains of HF mice, and this suggests that the reduction in CBF is unrelated to changes in brain structure (Yang et al. 2012). Furthermore, there was no correlation between CBF and MAP, but there was a negative correlation between CBF measurements and calculated CVR in HF mice (Yang et al. 2012). In HF mice, the MAP was maintained within the autoregulatory range (e.g., approximate MAP of 80 mmHg versus 90 mmHg for HF and control mice respectively), and the compiled results suggest that the effects on CBF are attributed to abnormal cerebral autoregulation. Our laboratory has previously demonstrated that proximal PCAs from control mice (i.e., sham-operated) exhibit little myogenic reactivity, and the induction of HF in mice enhances the myogenic responsiveness of PCAs (Meissner et al. 2012, Yang et al. 2012, Hoefer et al. 2010). Therefore, the reduction in CBF observed in HF mice could be due to the cerebral autoregulation via enhanced myogenic reactivity of the cerebral microvasculature (e.g., PCAs), rather than systemic hemodynamic parameters. The enhanced myogenic reactivity is dependent upon upregulation of S1P-signaling, and enhancement of this signaling pathway contributes to the reduction of CBF in HF mice (Yang et al. 2012). HF also decreased the expression of CFTR’s mRNA and protein in murine vascular tissue, like the PCAs (Meissner et al. 2012). As already mentioned, PCAs from CFTR KO mice have greater S1P-dependent myogenic reactivity than the PCAs from WT mice (Meissner et al. 2012). The data provides support that CFTR contributes to cerebral autoregulation in mice, by acting as the regulatory bottleneck for S1P’s bioavailability in the cerebral microvasculature. The aforementioned studies in mice suggest CFTR is a therapeutic target to correct the cerebral deficits in HF.
1.6 Specific aims, hypotheses, and thesis organization

S1P is a critical regulator of the Ca\textsuperscript{2+}-dependent and Ca\textsuperscript{2+}-sensitive mechanisms of the MR, and for this reason the bioavailability of S1P must be tightly controlled. The CFTR channel is essential for the uptake of S1P into VSMCs, a prerequisite for S1P’s degradation by the intracellular enzyme SPP1. Previously, our laboratory has shown that CFTR\textsubscript{inh-172} pre-treatment of murine VSMCs, isolated from WT mice, decreases the uptake of S1P-FITC (Meissner \textit{et al.} 2012), and we believe there could be decreased degradation of S1P. Since CFTR\textsubscript{inh-172} also affects CFTR channel function (see Section 1.5.2), the direct modulation of function (i.e., independent of effects on genomic or protein expression) may alter the bioavailability of S1P, as demonstrated by reduced uptake of fluorescently labeled S1P (Meissner \textit{et al.} 2012). Furthermore, in the cerebral microvasculature the reduction in CFTR expression associated with HF contributes to an increase in the pressure-dependent vasoconstriction of proximal PCAs, and the subsequent decrease in CBF. Evidence from our laboratory demonstrates that the effect on the MR is through enhancement of endogenous S1P-signaling. Therefore, the indirect modulation of CFTR function (i.e., dependent upon genomic or protein expression) can enhance S1P’s bioavailability as well, and this has detrimental effects on the cerebral microvasculature. The studies in this thesis set out to answer three important questions: (i) does S1P directly modulate CFTR channel function; (ii) does enhanced S1P-signaling contribute to the expression and/or function defects for one of CFTR’s disease-causing mutants; and (iii) can targeting CFTR directly restore the cerebral microvascular abnormalities in HF mice. \textbf{My overall hypothesis is that S1P modulates CFTR function, and that this modulation affects the microvascular reactivity of cerebral resistance arteries} (Figure 1-5). I test this hypothesis by focusing on three specific aims outlined below with their own sub-hypotheses. In the thesis document each specific aim is formatted as a manuscript.
1.6.1 Specific Aim 1: Is S1P a modulator of CFTR channel function?

Boujaoude and colleagues demonstrated that mock-transfected C127 cells treated with exogenous S1P have greater expression of phosphorylated mitogen activated protein kinase, relative to C127-WT-CFTR cells (Boujaoude et al. 2001). More recently, our laboratory demonstrated that CFTR\textsubscript{inh}-172 treatment enhances the anti-proliferative effect of exogenous S1P in murine VSMC-WT-CFTR cells (Meissner et al. 2012). Together the results suggest that CFTR-dependent uptake inhibits S1PR-mediated signaling pathways. Pharmacologic modulation is intriguing (e.g., CFTR\textsubscript{inh}-172), however I focused on whether a direct relationship between exogenous S1P and CFTR existed. Specifically, I investigated whether S1P directly modulates CFTR channel function, because the assessment of channel function may serve as a surrogate for S1P-uptake function, and provide biochemical insight into the manner by which S1P’s degradation is delayed. Additionally, the modulation could have broad implications in physiology and diseases because of the dual importance of CFTR (e.g., airways, CF), and S1P. One method to evaluate modulation of CFTR is through the assessment of its channel function, and it is not known whether S1P can regulate CFTR’s channel function. As previously described, S1P is an upstream activator of AMPK, and phosphorylation of CFTR’s R-domain by AMPK has been shown to inhibit CFTR’s function. Furthermore, the binding of S1P to the G-protein coupled S1P\textsubscript{1,3}R\textsubscript{s} has been shown to activate AMPK \textit{in-vitro} (Kimura et al. 2010).

Therefore, I hypothesize that S1P is a modulator of CFTR channel function via S1P\textsubscript{1,3}R-AMPK-dependent signaling.

1.6.2 Specific Aim 2: Does the S1P-signaling pathway contribute to ΔF508-CFTR defective channel function?

The ΔF508-CFTR mutation results from the genetic deletion of the F508 residue from NBD1. The deletion is responsible for protein processing errors because the mutant cannot
escape the ER’s quality control mechanism (Rosser et al. 2008, Sharma et al. 2004, Yang et al. 1993). Hence, the ΔF508-CFTR protein does not undergo post-translational processing in the Gogli, and its maturation is abnormal (Lukacs et al. 1994). Therefore, the mutant does not traffic appreciably to the plasma membrane (Okiyoneda et al. 2004, Welsh et al. 1993), and its expression in the plasma membrane is significantly less than the WT protein (Meegen et al. 2013). The deletion of the F508 residue may cause conformational changes in the protein, and this could account for the decrease in ΔF508-CFTR’s channel function, relative to WT-CFTR, observed by *in-vitro* studies (Eckford et al. 2012, Van Goor et al. 2009, Van Goor et al. 2006, Dalemans et al. 1991). Our laboratory and others have demonstrated that cells expressing ΔF508-CFTR cannot uptake S1P (Meissner et al. 2012, Boujaoude et al. 2001), which suggests the accumulated S1P in the extracellular environment could enhance that S1P-signaling pathways. Since S1P is an upstream regulator of AMPK in an S1PR-dependent manner, the enhanced signaling could aggravate the mutant’s phenotype. CFTR KO mice do not display abnormalities in their heart function, assessed from electrocardiogram recordings (Duan 2009). However, the isolated proximal PCAs from CFTR KO mice display enhanced myogenic reactivity via upregulation of the S1P-signaling pathway (Meissner et al. 2012), as previously mentioned. This suggests that CFTR contributes to cerebral autoregulation, and mice with the genetic deletion of the F508 residue may display abnormalities in this autoregulatory mechanism via an increase in CVR related to enhanced myogenic reactivity of proximal PCAs. A literature search reveals only one publication that has assessed the vascular effects of CF in the human CF brain, and no publications with mouse models of CF. For example, Bandyopadhyay and colleagues used the transcranial Doppler technique and observed reversal of diastolic flow in the middle cerebral artery, in a young adult CF patient with severe liver cirrhosis (Bandyopadhyay et al. 2010). Therefore, the assessment of CBF in a mouse model of CF is novel. The assessment
will provide information on the importance of CFTR in cerebral autoregulation, and provide insight into the CNS dysfunction observed in some CF patients (Goldstein et al. 2000), discussed further in Chapter 4. In the cerebral microvasculature, the abnormal expression of the mutant CFTR protein could contribute to enhanced endogenous S1P-signaling. The up-regulation in S1P may augment the myogenic reactivity for murine proximal PCAs, isolated from mice expressing the genetic F508 mutation, and adversely affect the CBF. Then it is also possible that inhibitory signaling pathways, like the proposed mechanism in Aim 1, may contribute to the mutant’s cerebral microvascular phenotype. Therefore, I hypothesize that the S1P1/3R-AMPK-signaling pathway contributes to the defective processing and/or function of ΔF508-CFTR mutant.

1.6.3 Specific Aim 3: Is CFTR a therapeutic target for the abnormal cerebral microvascular reactivity in HF?

Our group has shown that HF in mice indiscriminately augments the myogenic responsiveness for several types of resistance arteries, and this may account for the multitude of clinical effects. Importantly, the reduction in CBF may be responsible for the abnormal higher-order brain functions in HF patients. In mice, the reduction is caused by augmented myogenic responsiveness of proximal PCAs, mediated by enhanced endogenous and exogenous S1P-signaling. Since HF in mice also reduces CFTR expression in a TNFα-dependent manner (Meissner et al. 2012), the decreased CBF in HF could be caused by abnormal regulation of S1P’s bioavailability. Our laboratory has also previously demonstrated that inhibition of TNFα, with the FDA-approved drug Etanercept, can restore CFTR expression and attenuate the MR of PCAs, thereby restoring CBF (Meissner et al. 2012). Etanercept treatment does not have beneficial effects on the systemic hemodynamic parameters in HF mice (Meissner et al. 2012, Yang et al. 2012), or on the clinical outcome of HF patients (Mann et al. 2004, discussed further
in Chapter 5). Although our studies in mice indicate the inhibition of TNFα may have beneficial effects on the cerebral function in HF patients, treatment with Etanercept could have non-specific effects or off-target effects because TNFα is involved in many cellular and physiological processes. Therefore, I assessed a CFTR-specific approach by using a small-molecule corrector compound. High-throughput screening has identified specific small-molecule compounds that can correct expression or potentiate function of CFTR and its mutants (He et al. 2013, Molinski et al. 2012, Van Goor et al. 2011, Yu et al. 2011, Kim Chiaw et al. 2010, Van Goor et al. 2009, Loo et al. 2008, Van Goor et al. 2006). I hypothesize treatment of HF mice with a small molecule corrector compound normalizes the cerebral microvascular effects, in a CFTR-targeted manner.
Figure 1-5. Outline of specific aims.
This figure represents some of the known and unknown signaling pathways that regulate S1P, CFTR, and the myogenic response. The studies presented in the thesis seek to understand the relationship between CFTR and S1P by focusing on three aims: (1) First, the assessment of whether S1P modulates CFTR channel function, and the mechanism involved. (2) Second, to assess whether the modulation contributes to the expression and/or function defects for the ΔF508-CFTR mutant. (3) Finally, the use of in-vivo and in-vitro models of HF to determine whether CFTR is a therapeutic target in the disease.
Chapter 2

2 MATERIALS AND METHODS

2.1 Animals

All animal experiments were done according to the Guide for the Care and Use of Laboratory Animals (NIH, Publication No. 85-23). The care and protocols were approved by the Institutional Animal Care and Use Committees at the University of Toronto and the University Health Network (UHN, Toronto, Canada), and were conducted in accordance with the Canadian animal protection laws. WT C57BL/6N (8 to 12 weeks old) mice were purchased from Charles River Laboratories. The FVB-129 mice that are a backcross of the C57BL/6N and FVB strains, expressing WT and ΔF508-CFTR (8 to 12 weeks old) were purchased from the Cystic Fibrosis Mouse Facility at the Hospital for Sick Children (Toronto, Canada). All the mice were housed under standard 14 hour : 10 hour light dark cycle, were fed normal chow and had access to water continuously. It should be noted that the FVB-129 mice were given coolyte water, since the mutation in CFTR in these mice leads to functional gastrointestinal abnormalities, and the symptoms can be slightly reduced by coolyte water (Saussereau et al. 2013).

2.2 Antibodies

All antibodies with the exception of the phosphosensitive CFTR antibody (67D4) were purchased from commercial suppliers. The 67D4 antibody was a gift from Dr. D. Thomas (McGill University, Montreal, Canada) and Dr. C. Bear (the Hospital for Sick Children, Toronto, Canada). The remaining antibodies are shown in the table below.
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Clone</th>
<th>Species</th>
<th>Epitope</th>
<th>Dilution used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CFTR</td>
<td>Pierce/Thermo Scientific</td>
<td>M3A7</td>
<td>Mouse monoclonal</td>
<td>NBD2</td>
<td>1/2000</td>
</tr>
<tr>
<td>Anti-CFTR</td>
<td>Santa Cruz Biotechnology</td>
<td>H-182</td>
<td>Rabbit polyclonal</td>
<td>Amino-terminus</td>
<td>1/2000</td>
</tr>
<tr>
<td>Anti-GAPDH-HRP</td>
<td>Sigma-Aldrich</td>
<td>GAPDH-71.1</td>
<td>Mouse monoclonal</td>
<td></td>
<td>1/10,000</td>
</tr>
<tr>
<td>Anti-HA</td>
<td>Sigma-Aldrich</td>
<td>HA-7</td>
<td>Mouse monoclonal</td>
<td>YPYDVPDYA</td>
<td>1/2000</td>
</tr>
<tr>
<td>Anti-mouse-HRP</td>
<td>GE Amersham</td>
<td>N/A</td>
<td>Sheep</td>
<td>N/A</td>
<td>1/10,000</td>
</tr>
<tr>
<td>Anti-rabbit-HRP</td>
<td>GE Amersham</td>
<td>N/A</td>
<td>Donkey</td>
<td>N/A</td>
<td>1/10,000</td>
</tr>
<tr>
<td>Biotinylated</td>
<td>EMD Millipore</td>
<td>N/A</td>
<td>Donkey</td>
<td>N/A</td>
<td>1/20,000</td>
</tr>
<tr>
<td>anti-mouse</td>
<td>EMD Millipore</td>
<td>N/A</td>
<td>Donkey</td>
<td>N/A</td>
<td>1/20,000</td>
</tr>
<tr>
<td>Biotinylated</td>
<td>Cell Signaling Technologies</td>
<td>40H9</td>
<td>Rabbit monoclonal</td>
<td>Thr-172</td>
<td>1/2000</td>
</tr>
<tr>
<td>anti-rabbit</td>
<td>Cell Signaling Technologies</td>
<td>23A3</td>
<td>Rabbit monoclonal</td>
<td>α-subunit</td>
<td>1/2000</td>
</tr>
<tr>
<td>p-AMPK</td>
<td>Cell Signaling Technologies</td>
<td>40H9</td>
<td>Rabbit monoclonal</td>
<td>Thr-172</td>
<td>1/2000</td>
</tr>
<tr>
<td>Total AMPK</td>
<td>Cell Signaling Technologies</td>
<td>23A3</td>
<td>Rabbit monoclonal</td>
<td>α-subunit</td>
<td>1/2000</td>
</tr>
<tr>
<td>α-tubulin</td>
<td>Cell Signaling Technologies</td>
<td>DM1A</td>
<td>Mouse monoclonal</td>
<td>Val-440</td>
<td>1/5000</td>
</tr>
</tbody>
</table>

Table 2-1. List of antibodies and dilutions used for Western blotting

2.3 Reagents

All common reagents (e.g., sodium dodecyl sulfate-SDS, sodium chloride-NaCl) were purchased from Sigma-Aldrich or Bioshop Canada. The information for specific reagents is shown in the table below:
<table>
<thead>
<tr>
<th>Common name</th>
<th>Chemical name</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>C18</td>
<td>1-(benzo[d][1,3]dioxol-5-yl)-N-(5-(2-chlorophenyl)[3-hydroxy]pyrrolidin-1-yl)methyl)thiazol-2-yl)cyclopropanecarboxamide</td>
<td>Cystic Fibrosis Foundation Therapeutics (Dr. R. Bridges, Rosalind Franklin University of Medicine and Science)</td>
</tr>
<tr>
<td>Compound C</td>
<td>6-[4-(2-Piperidin-1-ylethoxy)phenyl]-3-pyridin-4-ylyrazolo[1,5-a]pyrimidine</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>cpt-cAMP</td>
<td>8-(4-Chlorophenylthio)adenosine-3',5'-cyclic monophosphorothioate</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Etanercept</td>
<td>N/A</td>
<td>Amgen-Pfizer</td>
</tr>
<tr>
<td>EZ-Link NHS-Biotin</td>
<td>N-hydroxysuccinimidobiotin</td>
<td>Pierce/Thmero-Scientific</td>
</tr>
<tr>
<td>FSK (Forsolin)</td>
<td>7-beta-acetoxy-8,13-epoxy-1-alpha, 6-beta, 9-alpha-trihydroxylabd-14-en-11-one</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>IBMX</td>
<td>3-Isobutyl-1-methylxanthine</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>NeutrAvidin: agarose resin (50% slurry)</td>
<td>N/A</td>
<td>Pierce/Thmero-Scientific</td>
</tr>
<tr>
<td>ODQ</td>
<td>1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Okadaic acid-sodium salt</td>
<td>N/A</td>
<td>Bioshop Canada</td>
</tr>
<tr>
<td>Phenylephrine</td>
<td>L-phenylephrine-hydrochloride</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>S1P</td>
<td>sphingosine-1-phosphate</td>
<td>Biomol International</td>
</tr>
<tr>
<td>S1P-FITC</td>
<td>Sphingosine-1-phosphate-fluorescein isothiocyanate</td>
<td>Echelon Biosciences</td>
</tr>
<tr>
<td>SEW 2871</td>
<td>5-[4-Phenyl-5-(trifluoromethyl)thio-phen-2-yl]-3-[3-(trifluoromethyl)phenyl]1,2,4-oxadiazole</td>
<td>Tocris Biosciences</td>
</tr>
<tr>
<td>SNP</td>
<td>sodium nitroprusside dehydrate</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>VPC 23019</td>
<td>(R)-phosphoric acid mono-[2-amino-2-(3-octylphenyl)carbamoyl]-ethyl ester</td>
<td>Tocris Biosciences</td>
</tr>
</tbody>
</table>

**Table 2–2. List of reagents and sources.**

### 2.4 Cell culture

The cell-lines used were Baby Hamster Kidney (BHK) cells (naïve and stably transfected), and murine vascular smooth muscle cells (VSMCs). The BHK stably transfected cell-lines express the human epithelial isoforms of WT-CFTR and ΔF508-CFTR, and were generously provided by Dr. C. Bear (the Hospital for Sick Children, Toronto, Canada). The CFTR constructs expressed in these cells also have an exofacial triple-HA tag (YPYDVPDYASYPYDVPDYAYPYDVPDYA) in the fourth extracellular loop, and the cells
were originally developed and characterized by the Lukacs laboratory (Pedemonte et al. 2005, Sharma et al. 2004). The BHK stable-cell lines were cultured using Dulbecco’s Modified Eagle Medium Nutrient Mixture F-12 (DMEM/F12, 50/50) (Gibco, Invitrogen Life Technologies), containing 5% (v/v) fetal bovine serum (FBS), 1% (v/v) Anti-Anti (100x antibiotic solution, Gibco, Invitrogen Life Technologies), and 250 µmol/L methotrexate (Bioshop Canada). Naïve BHK cells were cultured in the same media, but in the absence of methotrexate.

VSMCs were cultured from the smooth muscle of murine mesenteric arteries, and our laboratory previously characterized these cells (Meissner et al. 2012, Lidington et al. 2009). Artery segments were isolated from the surrounding connective tissue, and then cut into small pieces. Subsequently, the arteries were digested in a solution containing 0.25% trypsin for 30 minutes at 37°C, and 100% oxygen was gently bubbled into the solution, and after the incubation the supernatant was discarded. The remaining tissue was incubated for 40 minutes with collagenase IV-S (2 mg), papain (1 mg), elastase (70 µL) and dithiothreitol (1.5 mg, DTT) in 1 mL HEPES (4-(2-hydroxyethyl)-1-piperazineneethanesulfonic acid) buffer No. 1 (126 mmol/L NaCl, 6 mmol/L potassium chloride-KCl, 10 mmol/L HEPES, 20 mmol/L Taurine, 20 mmol/L Glucose, 5 mmol/L Pyruvate and 1 mmol/L magnesium chloride-MgCl2). After incubation, the supernatant was removed and the remaining tissue was washed in HEPES buffer No. 2 (141 mmol/L NaCl, 4.7 mmol/L KCl, 1.8 mmol/L calcium chloride-CaCl2, 1.2 mmol/L MgCl2, 10 mmol/L HEPES, 10 mmol/L Glucose). The resulting cell solution was finally washed three times in PBS The VSMCs were then cultured in DMEM supplantied with 10%(v/v) FBS and 1%(v/v) of Anti-Anti. VSMC identity was confirmed by positive PCR results for myocardin and MyoD, and negative PCR results for eNOS to confirm the absence of endothelial cells. All cell-lines were maintained in an incubator kept at 37°C and 5% CO2 (Dr. S.P. Heximer’s laboratory, University of Toronto).
2.5 Reverse transcriptase polymerase chain reaction (RT-PCR)

BHK cells were grown to 95-100% confluency, trypsinized for two minutes at 37°C, and centrifuged at 2000 g for five minutes at room temperature (RT). The supernatant was discarded and the pellet was washed once with ice-cold 1X phosphate buffered saline (PBS). RNA was extracted using the Qiagen RNeasy® Kit, following the manufacturer’s instructions. Total RNA (5 µg) was reverse transcribed using the Superscript III® kit (Invitrogen Life Technologies) and random hexamer primers. The cDNA product was then diluted to a final volume of 280 µL and this was used as the template for a subsequent PCR protocol (40 cycles): denaturation for 30 seconds at 95°C; annealing for 45 seconds at 55°C; and primer extension for 45 seconds at 72°C. The products from the reactions were then separated on a 2.5% agarose gel containing ethidium bromide. The primer sequences were designed against mouse mRNA, and are shown in the table below, and all primers had an annealing temperature of 55°C.

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer Sequence</th>
<th>Amplicon Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Forward: 5’-TTCACCACCATGGAGAAGG-3’&lt;br&gt;Reverse: 5’-CTCGTGTTCAACCCCATC-3’</td>
<td>111 bp</td>
</tr>
<tr>
<td>SphK1</td>
<td>Forward: 5’-ACCCCTGTGTAGCTCCCT-3’&lt;br&gt;Reverse: 5’-TGCAGTTGATGAGCAGGTCT-3’</td>
<td>244 bp</td>
</tr>
<tr>
<td>SPP1</td>
<td>Forward: 5’-ATTTGCTTTTGCTTTTG-3’&lt;br&gt;Reverse: 5’-TGCTAGATGGTGGTGC-3’</td>
<td>203 bp</td>
</tr>
<tr>
<td>S1P1R</td>
<td>Forward: 5’-GAACCTGACTCTGTGCTTTGTCTC-3’&lt;br&gt;Reverse: 5’-TGAAGAGTCAACACTTCTTCCTGG-3’</td>
<td>395 bp</td>
</tr>
<tr>
<td>S1P2R</td>
<td>Forward: 5’-AAAACCAACCCTGGCTGTC-3’&lt;br&gt;Reverse: 5’-CTCTGAGTAAGGCCCA-3’</td>
<td>170 bp</td>
</tr>
<tr>
<td>S1P3R</td>
<td>Forward: 5’-TCAGTATCTTCACCGCATT-3’&lt;br&gt;Reverse: 5’-AATCACTACGGTCCAGAA-3’</td>
<td>137 bp</td>
</tr>
</tbody>
</table>

Table 2–3. List of primer sequences.
2.6 Transfection

2.6.1 S737A-CFTR

Dr. C. Bear’s laboratory generously provided the plasmid for the S737A-CFTR construct (pcDNA 3.1, ampicillin-resistant). A sample of the plasmid was electroporated (25 uFD, 200 Ω, and 1.8 kV) into electrocompetent *E. coli* cells (DH5α), and then incubated at 37°C for one hour. Placement of the mixture onto ampicillin-resistant agar (overnight at 37°C) led to colony generation. The following day colonies were selected, and bacterial culture was grown up in lysogeny broth (LB) media (250 mL) by shaking (overnight at 37°C and 250 rpm) in a 1 L flask. Plasmid was purified using the Qiagen EndoFree® Plasmid Purification (Maxi) kit, following the manufacturer’s instructions, and purity of the plasmid was then determined using a Nanodrop ultra-violet spectrophotometer. Dr. Guillaume Bastin (Dr. S. Heximer’s laboratory, University of Toronto) provided technical help for purification and for the use of the spectrophotometer. The purified plasmid was transfected into BHK naïve cells (plated onto 25 mm plastic coverslips in six-well plates, 40-50% confluency) using Fugene® 6 Transfection reagent in a 3:2 ratio (e.g., 3 µL Fugene : 2 µg DNA), according to the manufacturer’s instructions. Serum-free DMEM/F12 media was used to dilute the Fugene and DNA, however cells were given full serum media to grow. The cells were incubated for 48 hours post-transfection in a cell-incubator (37°C, 5% CO2), and grown until they reached 95-100% confluency.

2.6.2 siRNA

The SMARTpool ON-TARGETplus siRNA against the AMPKα1 (PRKA1-L-005027-00), and the ON-TARGETplus non-targeting control pool siRNA (D-001810-10-05) were purchased from Dhharmacon/Thermo-Scientific. The lyophilized pellet was reconstituted according to the manufacturer’s instructions, and 25 nmol/L of the siRNAs (diluted
from a reconstituted stock was 20 µmol/L) were transfected into the BHK-stable cell-lines using Fugene® 6. For six-well plates, 3 µL of Fugene was used per well. Transfection was performed when cells were 40-50% confluent, and cells were incubated for 48 hours post-transfection in a cell-incubator (37°C, 5% CO2) and grown until they reached 95-100% confluency.

2.7 Preparation of S1P

The lyophilized powder was resuspended in 100% methanol, and heated at 65°C with incremental vortexing until the powder was completely dissolved. Then the solution was rapidly aliquoted in 1.5 mL centrifuge tubes (10 uL per tube), and the methanol was left to evaporate overnight in a fume hood. On the day of its use, 125 uL of 4% BSA (fatty-acid free) was added to a S1P tube, and this made a stock solution of 10^{-4} mol/L. This solution was then heated at 37°C for 30 minutes, and vortexed. For cell experiments, the stock was diluted to a working concentration of 1 umol/L in serum-free media. For vessel experiments, serial dilutions were made in normal MOPS buffer (Section 2.10).

2.8 Iodide efflux assays

2.8.1 Conventional iodide efflux assay

Cells were grown onto 25 mm plastic cover slips in six-well titer plates to a confluency of 95-100%. The cover slips were then rinsed in 1X PBS and incubated with 2 mL of sodium iodide (NaI) uptake buffer (136 mmol/L NaI, 3 mmol/L potassium nitrate-KNO₃, 2 mmol/L calcium nitrate-Ca(NO₃)₂, 11 mmol/L glucose, and 20 mmol/L HEPES, pH 7.2, 300 osm) for one hour at 37°C and 5% CO₂ in a cell culture incubator. Another set of six-well titer plates were prepared containing 2 mL of iodide-free efflux buffer (136 mmol/L sodium nitrate-NaNO₃, 3 mmol/L KNO₃, 2 mmol/L Ca(NO₃)₂, 11 mmol/L Glucose, and 20 mmol/L HEPES, pH 7.2, 300 osm) in the first four wells, and a cAMP stimulation cocktail in the next eight wells. The
cocktail contained: (i) 10 µmol/L FSK to activate adenylate cyclase and promote PKA-mediated phosphorylation; (ii) 1000 µmol/L IBMX to slow channel inactivation by inhibiting phosphodiesterases; and (iii) 100 µmol/L of the cell-permeable cAMP to further increase the intracellular concentration of cAMP. The three compounds were dissolved in 1% (v/v) DMSO and the iodide-free efflux buffer. For acute treatments of S1P, 10^{-4} mol/L of the stock was diluted to 1 µmol/L in the cAMP cocktail, and this was added to the first two cAMP wells. The coverslips were immersed in the wells containing efflux buffer alone (four wells), after incubation with NaI to remove the excess iodide ions, and this was followed by immersion in the cAMP stimulation cocktail wells to stimulate channel activity (Figure 2-1). Incubation in each well was for a total of one minute. For measurements, the plates were gently shaken, and an iodide ion selective probe (Lazar Research Laboratories) was immersed into each well until a stable reading was established. The probe detected voltage (mV), and this corresponded to the amount of iodide ions secreted out of the cells into solution. Then the probe was washed in distilled water until a stable reading was achieved (i.e., equilibration), and immersed into the next well. The post-stimulation mV value that corresponded to the greatest difference was subtracted from the pre-stimulation value (i.e., fourth wash well). This calculated value was converted to concentration values (µmol/L) using a standard curve equation. Measuring the voltage of different concentration of iodide solutions generated the standard curve, and the curve parameters were used in the equation below:

\[
[t^-] = \ln \frac{107.5}{(mV - (-109.4)) - 0.0515}
\]
Figure 2-1. The iodide efflux assay to assess CFTR's channel function.
The steps for the iodide efflux assays are: (1) Cells expressing the CFTR protein (e.g., BHK stable cell-line, murine VSMCs) are loaded with sodium iodide (NaI) containing buffer for one hour at 37°C. Due to the concentration gradient, there is an influx of I⁻ into the cell. (2) The cells are then washed with iodide-free efflux buffer, which removes excess I⁻ by a concentration gradient. (3) Cells are stimulated by a forskolin (FSK) or cpt-cAMP containing solution, and the voltage of the supernatant is measured (mV) with an iodide-selective voltage electrode. The greater the secretion of I⁻ out of the cell, the more negative the voltage.
2.8.2 Continuous iodide efflux assay

This method was published by Dr. C. Bear’s laboratory (Yu et al. 2011 and Kim Chiaw et al. 2010, Wellhauser et al. 2009), and the measurements were performed in their laboratory. BHK cells were grown in 60 mm cell-culture dishes (BD Falcon) to 95-100% confluency, and then incubated with 2 mL of NaI buffer for one hour in a cell-culture incubator (37°C, 5% CO₂). The cells were washed at RT with 1X PBS (2 mL per dish), and then once with iodide-free efflux buffer (2 mL per dish). Then 300 µL of efflux buffer was added to each dish, and the cells were gently scraped and collected into a 1.5 mL centrifuge tube. The samples were centrifuged at 2000 g for five minutes at RT, and the supernatant was discarded. The pellet was then resuspended in 250 µL of efflux buffer and the solution was pipetted into a single well of a 96-well plate. The plate was placed onto a magnetic stirrer, a micro-stir bar was inserted into the well, and then the solution was slowly stirred (120 rpm). An iodide ion selective voltage probe was immersed into the well, and equilibrated for 15 minutes prior to measurement. A baseline tracing of the voltage (mV) was recorded for three minutes with the Digidata 1320A Data Acquisition System and Clampex® 8 software. CFTR channel activity was then stimulated by pipetting 10 µmol/L FSK into the well using a gel-loading tip. A tracing was recorded for eight minutes post-stimulation, and then the cells were lysed with 0.1% (v/v) Triton X-100 to release the total iodide remaining in the cells. A tracing was recorded another five minutes. The mV values were converted into concentration values, and the slope for the steepest part of the curve was calculated, relative to time (nmol/L*sec.).

2.9 SDS-PAGE and Western blotting

Cells were placed on ice, washed once with ice-cold 1x PBS, and lysed on ice for 15 minutes with RIPA-lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, and 2 mmol/L EDTA,
pH 7.3, along with 0.1% SDS, 0.1% Triton X-100, and a protease inhibitor cocktail tablet Complete-Mini®, EDTA-free, from Roche). The cells were then scraped, and samples were collected and spun at 14,000 g for 15 minutes at 4°C. The supernatant was collected and the pellet was discarded. The supernatant was then mixed with 5x Lamelli’s sample buffer (10% SDS, 20% glycerol, 0.05% bromophenol blue, 8 mL of 1 mol/L Tris-HCl pH 6.8, top to 40 mL with distilled water), containing 50 mmol/L DTT, 2% beta-mercaptoethanol, and 1 mmol/L EDTA (i.e., in 500 µL of 5x sample buffer use 25 µL of 1 M DTT, 10 µL beta-mercaptoethanol, and 1 µL of 0.5 mol/L EDTA), in a 5:1 ratio (supernatant:sample buffer). The samples were then loaded onto an 8% Tris-glycine gels. The resolving gel was made using: 4 mL of 30% acrylamide/0.8% bisacrylamide, 7.3 mL of distilled water, 3.7 mL of 4x Tris-Cl/SDS buffer (pH 8.8), 200 µL of 10% ammonium persulfate (APS), and 10 µL TEMED. This was overlaid with saturated iso-butanol (1:1 distilled water and butanol). After the gel had set, the iso-butanol was washed off, and the stacking gel was overlaid. The stacking gel was made using: 3 mL distilled water, 1.3 mL of 4x Tris-Cl/SDS buffer (pH 6.8), 0.9 mL 30% acrylamide/0.8% bisacryl, 80 µL 10% APS, and 5 µL TEMED. The SDS-PAGE was run at 140 V, until the dye front reached the bottom, in SDS-PAGE 1x running buffer (190 mmol/L glycine, 25 mmol/L Tris-base, and 0.1% SDS in distilled water, pH 8.3).

Following SDS-PAGE electrophoresis, the gels were removed from the glass cassettes, the stacking gel was discarded, and the resolving gel was incubated in 1x transfer buffer (190 mmol/L glycine, 25 mmol/L Tris-base and 20% v/v methanol in distilled water), for 15 minutes at room temperature. During the gel incubation, Immobilon-P PVDF-membranes (0.45 µm, EMD-Millipore) were soaked in 100% methanol to hydrate, and then put into 1X transfer buffer. Plastic transfer cassettes were assembled, and the transfer was electrophoresed at 100 V for one hour on ice with gentle stirring (300 rpm).
Following completion of the transfer step, the PVDF membranes were soaked in 100% methanol for one minute, and stained with Ponceau-S (BioRad) for five minutes. The Ponceau-S staining was used to confirm appreciable transfer of proteins to the membranes. The stain was washed five to six times in 1x transfer buffer, and then once in distilled water. The membranes were then blocked for one hour, by gentle shaking, at RT with 5% v/v non-fat milk (Bioshop Canada) in 1x PBS-T (8 g NaCl, 1.44 g Na₂HPO₄, 0.2 g KCl, 0.24 g KH₂PO₄, and 1 mL Tween-20, pH 7.2). The solution was discarded and the membranes were then incubated with primary antibodies diluted in 5% non-fat milk blocking buffer, with the exception of p-AMPK (this was diluted in 5% sterile-filtered BSA blocking buffer), by gentle shaking at room temperature for 15 minutes, and then overnight at 4°C. The antibodies and dilutions utilized are shown in Table 2.1.

The following day, the membranes were warmed by gentle shaking at RT for 30 minutes, and then washed five times, five minutes each wash, with 1x PBS-T. Secondary antibodies were then added, which were diluted in 5% non-fat milk blocking buffer (see Table 2.1 for dilutions). The membranes were incubated in the secondary antibodies for two hours at room temperature with gentle shaking. The secondary antibody solutions were discarded and the membranes were then washed five times, five minutes each wash, with 1x PBS-T. There was an additional incubation step with streptavidin-HRP for 30 minutes at room temperature, for the membranes incubated with the biotinylated secondary antibodies. Development was achieved by incubating the membranes for five minutes in Westar ETA Enhanced Chemiluminescent kit for HRP detection (VPQ Scientific, Toronto, Canada). Films were exposed for various durations (e.g., 30 seconds, one minutes, three minutes, five minutes and ten minutes). The films were scanned and densitometry was then measured using the ImageJ software (NIH).
2.10 Functional assessments of isolated and cannulated murine proximal PCAs

This method is summarized in Figure 2-2. Mice were sacrificed by first exposing them to anesthesia (2%), followed by cervical dislocation. The animal was then decapitated and the brain from removed from the skull, and immediately immersed in ice-cold sterile MOPS (3-(n-morpholino)propanesulfonic acid) buffered saline (145 mmol/L NaCl, 4.7 mmol/L KCl, 3 mmol/L CaCl₂, 1.17 mmol/L MgSO₄•7H₂O, 1.2 mmol/L NaH₂PO₄•2H₂O, 2 mmol/L pyruvate, 0.02 mmol/L EDTA, 3 mmol/L MOPS, and 5 mmol/L Glucose, pH 7.4) in a petri dish. Proximal PCAs were dissected between 0.8 and 1.0 mm in length from the brain tissue, and the surrounding connective tissue was removed. It should be noted only first-order PCAs were isolated, which are 160 to 220 μm in diameter. After isolation, the arteries were cannulated on glass micropipettes. Then the arteries were warmed slowly by increasing the in-bath temperature over 20 minutes until it reached 37°C, followed by stretching of the vessels to their in vivo length, and pressurization to 45 mmHg. The PCAs were washed by exchanging of the MOPS bath for 15 to 20 minutes, and this was followed by functional assessments and experiments that were conducted in normal MOPS at 37°C with no perfusion. The first assessment was for viability, where a baseline reading of the diameter was established, and then 3 μmol/L phenylephrine (PE) was added to the bath. The diameter was measured with microscopy and edge detection software that identified the artery’s luminal diameter. Additional assessments included KCl (60 mmol/L) mediated vasoconstriction, and vasomotor responses to a range of PE concentrations (10⁻⁹ to 10⁻⁵ mol/L). Baseline recordings were established after one minute, the drug was then added, the artery was left to react for three minutes, and the diameter was measured at the end of three minutes. Arteries were washed with fresh MOPS to re-establish baseline diameters with 15 minutes of bath exchanges. To assess the myogenic response (MR),
the arteries were subjected to step-wise increases in transmural pressure in 20 mmHg increments, ranging from 20 to 100 mmHg. The MR was assessed in both normal MOPS and Ca\textsuperscript{2+}-free MOPS (147 mmol/L NaCl, 4.7 mmol/L KCl, 1.17 mmol/L MgSO\textsubscript{4}•7H\textsubscript{2}O, 1.2 mmol/L NaH\textsubscript{2}PO\textsubscript{4}•2H\textsubscript{2}O, 2 mmol/L pyruvate, 1 mmol/L EDTA, 3 mmol/L MOPS, and 5 mmol/L Glucose, pH 7.4). The artery diameter at each pressure step was measured as the steady state diameter after five minutes. Myogenic tone was calculated as the percent constriction in relation to the maximal, at each of the pressure steps. Thus, tone is percent of \( \text{dia}_{\text{max}} = \left( \frac{\text{dia}_{\text{active}} - \text{dia}_{\text{active}}}{\text{dia}_{\text{max}}} \right) \times 100\% \). In this equation, \( \text{dia}_{\text{active}} \) is the vessel diameter at each pressure step in MOPS containing Ca\textsuperscript{2+}, whereas \( \text{dia}_{\text{max}} \) is the artery diameter in Ca\textsuperscript{2+}-free MOPS. The latter is also known as the passive diameter. Vasomotor responses (e.g., PE, KCl) utilized the same equation, except \( \text{dia}_{\text{active}} \) was the diameter of the vessel after treatment once a steady state had been reached (three minutes), and \( \text{dia}_{\text{max}} \) was the passive diameter at 45 mmHg in Ca\textsuperscript{2+}-free MOPS.

All drug pre-treatments, utilized 30 minutes incubation, and were done after control assessments (e.g., control MR). The drugs were diluted in normal MOPS to the following final concentrations: 10 \( \mu \text{mol/L} \) of VPC-23019, 0.6 and 6 \( \mu \text{mol/L} \) of C18. Care was taken to ensure the final DMSO concentration was at or below 0.1% v/v. All diluted drug solutions were kept in a 37°C water bath before they were used.
Figure 2-2. Functional assessment of isolated and cannulated posterior cerebral arteries (PCAs) from mice.
(1) Mice were euthanized with isoflurane and cervical dislocation. (2) They were decapitated and the brain was removed, and put into ice-cold MOP. (3) The proximal PCAs were isolated and dissected, and the connecting tissue was removed. (4) The arteries were then cannulated with glass pipettes in an organ bath containing MOPS, and warmed up slowly to 37°C. Once warmed, the arteries were stretched to their in-vivo length at 45 mmHg. (5) For the assessment of the myogenic response (MR), a baseline reading was established at 45 mmHg (A), and then the transmural pressure was increased in step-wise increments of 20 mmHg (e.g., 20, 40, 60, 80, and 100 mmHg). This causes an initial distension (B), and then the artery constricts in response to the pressure (C). The changes in the inner wall diameter were tracked with a microscope.
2.11 S1P-FITC uptake assay

Cells were plated on six-well plates and grown to 95-100% confluency. Pre-treatments with drugs (e.g., Compound C) were performed for 30 minutes, and the cells were then incubated with S1P-FITC (1 μmol/L, 250 μL/well) (Figure 2-3), in the presence of the drug for one hour at 37°C in a cell culture incubator. Treatment with unlabeled S1P served as controls. The S1P-FITC was prepared in serum-free DMEM cell culture media with 4% BSA (fatty-acid free).

After incubation, cells were washed in ice-cold 1X PBS, and trypsin was added to each well (500 μL per well) to detach the cells. The reaction was quenched in DMEM supplemented with FBS then the samples were transferred to light-resistant 1.5 mL centrifuge tubes, and centrifuged at 2000 g for three minutes at 4°C. The supernatant was discarded, and the pellet was washed by re-suspending in ice-cold 1X PBS and centrifuged again, and the supernatant was discarded. Finally, the pellet was re-suspended in ice-cold 1X PBS (200 μL) and then filtered through tubes with a nylon mesh cap (5 mL, polystyrene with cell-strainer cap, 35 μm nylon mesh, BD Falcon). The samples were put on ice, protected from light, and then analyzed by Ms. Dionne White (Technician, the University of Toronto Flow Cytometry Service) on the Becton-Dickinson FACS Canto and operated by FACS DIVA version 6.1 software. Uptake was assessed as the mean fluorescence intensity at 530 nm, and statistical comparisons were made to the unlabeled controls.
Figure 2-3. The sphingosine-1-phosphate fluorescein thiocyanate (S1P-FITC) uptake assay. The steps for the S1P-FITC uptake assay are: (1) Cells expressing the CFTR protein (e.g., BHK stable cell-line, murine VSMCs) are cultured in six-well tissue culture plates. After treatment of interest, the cells are incubated with either unlabeled S1P or S1P bound to FITC (S1P-FITC) for one hour at 37°C. (2) Following incubation, the cells are washed with ice-cold PBS, detached with trypsin, and centrifuged at low speed. The supernatant is discarded, and the pellet is resuspended in ice-cold PBS, and centrifuged again. After discarding supernatant once again, the pellet is resuspended again in ice-cold PBS. (3) The mean fluorescence intensity at 515 nm is analyzed with a cell-sorter. Uptake of S1P-FITC is detected as an increase in the intensity.
2.12 Magnetic resonance imaging (MRI) of cerebral blood flow

Our laboratory originally described this procedure in Yang et al. (2012). CBF was assessed in mice using a modification of the flow-sensitive alternating inversion recovery (FAIR) MRI technique, established by Kim et al. (1995). These measurements were performed in collaboration with Dr. W. Foltz (Spatio-Temporal Targeting and Amplification of Radiation Response - STTARR facility in the Toronto Medical Discovery Tower, MaRS, Toronto, Canada). The FAIR-MRI technique measures perfusion by detecting the length of time taken for \(T_1\) signal relaxation (i.e., spin-lattice relaxation time) after it is inverted in slice-selective measurements and non-slice selective measurements (i.e., provides information on background signal in tissue). Slice-selective and non-selective slices were taken for the forebrain, midbrain, and hindbrain regions. In each region, CBF measurements were taken from areas corresponding to the cortex, the sub cortex, and whole brain. The \(T_1\) values are put into the following equation to measure the CBF: 

\[
CBF = \lambda (1/T_{1,ss} - 1/T_{1,ns}) \times (mL/(100g\cdot min))
\]

where “ss” and “ns” represents slice-selective and non-selective measurements respectively; and \(\lambda\) is the blood-brain partition coefficient, which is defined as the ratio between the water concentration per gram (g) of brain tissue and per mL of blood. The \(\lambda\) value for mice is 90-mL/100 g. A 7-Tesla (7-T) micro-MRI (BioSpec 70/30 USR, Bruker BioSpin, Ettlingen, Germany) system was used. This system had a B-GA12 gradient insert, a 72 mm inner diameter linear volume resonator (used for transmission of RF), and an anteriorly located head coil for reception of RF. It should be noted the modification of the FAIR-MRI technique used involved an optimization protocol, where a single-shot EPI technique was preceded by adiabatic inversion. The specific parameters were: 11 milliseconds echo time; 17 seconds repetition time; 18 inversion times that ranged from 25-to-6825 milliseconds in 400 milliseconds increments; 3 mm slice-selective inversion slab; 16.8 x 16.8 mm field-of-view (FOV) with 64 x 64 matrix for 263 um in-plane resolution; a slice
thickness of 1 mm; and 10 minutes of 12s data acquisition time. All the acquisitions were repeated in fore, mid, and hindbrain vertical sections, and these corresponded to anterior, mixed and posterior circulations respectively (Yang et al. 2012). The MRI images were evaluated using the MIPAV program (NIH, Bethesda, USA). Regions of interest were taken that corresponded to the sub-hemispheric region and this was called the global measurement. In addition, local regions of interest were taken that corresponded to cortical and sub-cortical parenchyma in the forebrain slices; cortical and paraventricular parenchyma in the mid-brain slices; and cortical and mid-brain parenchyma in hindbrain slices. Using the MIPAV program, the regions-of-interest were drawn directly on the T1 images obtained and this allowed for manual correction. The images were compared to parametric CBF maps to correct for any vessels with high perfusion and as well as meninges. The regression analysis of the T1 values and the CBF calculations was done using the Matlab program (Mathworks, Natick, USA; all calculations done by Dr. W. Foltz).

2.13 Echocardiography

All echocardiography measurements were done with the help of Dr. Roozbeh Sobbi (Dr. P. Backx’s laboratory, University of Toronto, Toronto, Canada). Mice were anesthetized with isoflurane (1.5%), and a 13-MHz linear array probe (Sequoia, Acuson, USA) was used to measure the left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD), left ventricular diastolic anterior and posterior wall thickness, mean ejection fraction, mean stroke volume, and the mean cardiac output (CO). The echocardiography measurements were taken under blinded conditions (i.e., genotype of mouse and/or treatment condition of mouse was not known to the technician). The same mice were then used for terminal blood pressure measurements.
2.14 Terminal blood pressure measurements

All blood pressure measurements were done with the help of Dr. Hangjun Zhang (Dr. S. Heximer’s laboratory, University of Toronto, Toronto, Canada). Mice were anesthetized with isoflurane (1.5%) and placed on a warming pad. An incision was then made in the midline of the neck, and the right common carotid artery was isolated and cannulated. For this a Millar-Mikrotip pressure transducer (1.4F sensor, 2F catheter) was used. Subsequently, hemodynamic tracings were recorded in the common carotid artery and the left ventricle. This technique was used to determine the mean arterial pressure (MAP), heart rate (HR), systolic blood pressure, and diastolic blood pressure. The measurements were taken under blinded conditions (i.e., genotype of mouse and/or treatment condition of mouse was not known to the technician). Mice were sacrificed immediately afterwards and tissue was collected for molecular work (e.g., mRNA, protein detection, S1P levels). The following equation was used for calculating the total peripheral resistance (TPR): TPR = MAP/mean CO.

2.15 Statistics

All statistical analysis used GraphPad Prism® 6.0 (version b) from GraphPad Software Inc. (La Jolla USA) and all data was expressed as mean ± SEM. For experiments that had two treatment groups, a Student’s t-test (two-tailed) was used. A one-way ANOVA was used for experiments with three or more treatment groups, and selected comparisons were made among all groups with the Bonferroni’s multiple comparison post-hoc test. Bonferroni’s is the most commonly used statistical test in the literature for selective comparisons of three or more treatment groups. Standard or repeated-measures two-way ANOVA were used for the functional experiments of resistance arteries, to assess the effect of pressure or vasomotor responses on the % tone, and the effect of the treatment (e.g., in-vitro C18 treatment). Then selected comparisons
were made with the Bonferroni’s multiple comparison post-hoc test. Significance was defined as a p-value of less than 0.05 (i.e., p<0.05) for all statistical tests.
Chapter 3

3 S1P IS A MODULATOR OF CFTR CHANNEL FUNCTION

Note: This chapter includes data presented in a manuscript submitted to PLoS ONE - Firhan A. Malik*, MSc, Anja Meissner*, PhD, Hai H. Bui, PhD, Christine E. Bear, PhD, Darcy Lidington, PhD, and Steffen-Sebastian Bolz, MD, PhD. (2014)
* denotes equal first authorship contributions

3.1 Acknowledgements

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3.2 Overview and rationale

S1P is a major signaling molecule with developmental, homeostatic and immune response roles (Fryst and Saba 2010, Spiegel and Milstien 2003), and SphK1 and SPP1 tightly
regulate the bioavailability of S1P (see Section 1.4). As previously mentioned, SPP1 converts S1P into sphingosine, but its intracellular localization requires import of the phosphorylated sphingolipid (see Section 1.4.4). The CFTR protein contributes to regulating the bioavailability by importing exogenous S1P, and therefore SPP1 gains access to S1P (see Section 1.5.2). Our laboratory has previously demonstrated that the CFTR-dependent uptake controls exogenous S1P’s receptor-mediated effects, like cell proliferation and vasoconstriction of resistance arteries (Meissner et al. 2012). Additionally, inhibiting CFTR’s function with CFTR$_{inh}$-172 enhances the effects of S1P, and this suggests that modulating the function of CFTR can affect S1P-signaling pathways (see Section 1.5.2). Our published vascular work with CFTR$_{inh}$-172 (Meissner et al. 2012, Peter et al. 2008) suggests that CFTR channel function can serve as a surrogate for the S1P-uptake function. It is not known whether S1P affects CFTR channel function, and assessing possible modulation could provide information on the CFTR-dependent regulation of S1P’s bioavailability. Furthermore, linking S1P-signaling to channel function would have wide-ranging implications for our understanding of CFTR regulation in health and disease. As previously mentioned, CFTR channel function is regulated by PKA- and AMPK-dependent phosphorylation (see Section 1.5.1). Additionally, exogenous S1P has been shown to stimulate the phosphorylation of AMPK in-vitro in bovine endothelial cells (Levine et al. 2007), and thus S1P is an upstream activator of AMPK. The activation is dependent upon the G-protein coupled S1P$_1$R and S1P$_3$R, because siRNA transfection against these receptors inhibits S1P-dependent phosphorylation of AMPK in human endothelial cells. Furthermore, siRNA transfection against the upstream kinase Ca$^{2+}$/calmodulin-dependent protein kinase kinase beta (CaMMK$\beta$) abolished the S1P-dependent phosphorylation of AMPK (Kimura et al. 2010). Therefore, we hypothesized that S1P modulates the channel function of CFTR in an S1P$_1$R / S1P$_3$R- and AMPK-dependent manner. In the present study, we use RT-PCR to show that BHK cells stably transfected with the
human epithelial CFTR isoform endogenously express S1P-signalling components (e.g., S1PRs). The stable transfection of CFTR into BHK cells is a well-established model to assess the biochemical regulation of CFTR’s function (Kim Chiaw et al. 2010, Wellhauser et al. 2009, Sharma et al. 2004). These cells are appropriate experimental models because the aim of the first study was establishing the biochemical link between exogenous S1P and CFTR function. CFTR channel function was assessed using iodide efflux techniques (continuous and conventional), and we demonstrate that S1P inhibits the FSK- and cAMP-stimulated iodide efflux. Next we assessed phosphorylation of CFTR and AMPK with phosphosensitive antibodies and Western blotting. We found that S1P’s inhibition of channel function was phosphorylation-dependent via the S1P1R -AMPK signaling pathway.

3.3 Methods

3.3.1 Antibodies and reagents

See Section 2.2 and 2.3.

3.3.2 Cell culture

See Section 2.4.

3.3.3 RT-PCR

See Section 2.5.

3.3.4 Transfections (S737A-CFTR, siRNA)

See Section 2.6.

3.3.5 Preparation of S1P

See Section 2.7.
3.3.6 Iodide efflux assays

See Section 2.8.

3.3.7 SDS-PAGE and Western blotting

See Section 2.9.

3.3.8 Cell-surface biotinylation

Cells were grown to 90 to 95% confluency in 35 mm cell-culture dishes. After the cells were treated, the dishes were placed on ice and washed once with ice-cold 1X PBS (pH 7.2), once with ice-cold 1X PBS containing magnesium chloride and calcium chloride (pH 7.2), and then once with ice-cold 1X PBS containing MgCl₂ and CaCl₂ at pH 8.2. The biotinylation reagent was prepared by dissolving EZ-Link Sulfo NHS-SS Biotin (Pierce, Thermo Scientific) in 1X PBS with MgCl₂ and CaCl₂ buffer at pH 8.2 (1 mg/mL), and 2 mL was added per dish. The cells were incubated for 30 minutes on ice. The reaction was then quenched with 1% BSA (w/v in 1X PBS pH 7.2), with a volume of 3 mL per dish. The quenching incubations were performed for 15 minutes, and then repeated for an additional five minutes. During the BSA incubations, NeutrAvidin beads were prepared (50 µL per sample) in 1.5 mL centrifuge tubes by washing the slurry with 1 mL of RIPA buffer (no SDS, no Triton X-100), centrifuging at 2000 g at RT, and aspirating off the supernatant. This was repeated two more times. Then 25 µL of RIPA cell lysis buffer was added to each tube. The cells were lysed with 120 µL RIPA cell lysis buffer, and by incubating the dishes on ice for 15 minutes. Samples were centrifuged at 14,000 g for 15 minutes at 4°C. After centrifugation, the supernatant was removed, and 25 µL was kept for the whole cell-lysate sample from each 1.5 mL centrifuge tube. The remaining supernatant was incubated with the beads for two hours, with end-over-end mixing, at 4°C. Samples were centrifuged at 14,000 g for five minutes at 4°C, and the supernatant was kept as the “unbound-
to-beads” aliquot. The beads were then washed twice with RIPA cell lysis buffer, and then twice with RIPA buffer. Between the washes, the beads were centrifuged at 2000 g for five minutes at room temperature, and the supernatant was discarded. After the final wash, a 27.5 gauge needle was used to aspirate the excess fluid, and the beads were incubated with 45 µL of warmed 5X sample buffer (1000 µL of 5X Lammeli buffer, 20 µL β-mercaptoethanol, 50 µL of 1 mol/L DTT, and 2 µL of 0.5 M EDTA). The beads were incubated at 59°C for 20 minutes, with occasional gentle flicking of the tube, and this was followed by incubation at RT for 10 minutes. The samples were then centrifuged at 14,000 g for five minutes at RT, and the supernatant was aspirated with gel-loading tips. The samples were electrophoresed in 8% SDS-PAGE gels.

3.3.9 Statistics
See Section 2.15.

3.4 Results
3.4.1 S1P attenuates the CFTR-dependent iodide efflux

We used RT-PCR analysis of BHK cells to demonstrate the endogenous mRNA expression of components of the S1P-signaling pathway, such as Sphk1, SPP1 and S1PR1-3 mRNA (Supplemental Figure 1). Then we assessed the effect of S1P on CFTR channel function with the iodide efflux assays. These assays have previously been demonstrated to be a valid and reliable method to assess CFTR channel function (Yu et al. 2011, Kim Chiaw et al. 2010, Schmidt et al. 2008). It is based on CFTR’s ability to secrete iodide ions as well as chloride, and therefore iodide replaces endogenous chloride. Thus, the measured iodide conductance reflects the behavior of chloride, and allows a direct conclusion regarding the state of the CFTR channel. BHK naïve cells express endogenous Cl⁻ channels, but do not express CFTR. This explains why
the iodide-loaded BHK naive cells do not show iodide efflux in response to FSK, whereas BHK-WT-CFTR cells do (Wellhauser et al. 2009), and therefore the assays are CFTR-specific. The continuous iodide efflux assay was developed and characterized by Wellhauser and colleagues, and provides real-time information on the function of plasma membrane-expressed CFTR (Wellhauser et al. 2009). The assay was originally used to assess potentiation of CFTR’s FSK-stimulated iodide efflux. In the present study, we found that acute treatment with FSK (20 µmol/L) stimulated significant iodide efflux in the BHK-WT-CFTR cells, and a representative tracing is shown in Figure 3-1A. To confirm the CFTR specificity of the efflux, we verified that FSK did not elicit significant iodide efflux in naïve, non-CFTR transfected BHK cells (data not shown). Co-stimulation with FSK and S1P (1 µmol/L) significantly decreased the FSK-stimulated iodide efflux in the BHK-WT-CFTR cells (representative tracing shown in Figure 3-1A). Permeabilization (0.1% Triton X-100) confirmed equal iodide loading between samples (Figure 3-1A). We also found attenuation of FSK-stimulated iodide efflux rate by 200 nmol/L S1P (6.46±2.33 nmol/L*s versus 13.6±2.41 nmol/L*s S1P and control respectively, p<0.05, n=7 cell culture dishes, Figure 3-1B), a S1P concentration that is considered physiologically relevant (Yatomi et al. 1997). CFTR inhibition by 200 nmol/L was maximal as increasing the S1P concentration did not lead to further inhibition (e.g., 4.48±nmol/L*s for 600 nmol/L, p>0.05 relative to 200 nmol/L, n=7 cell culture dishes). For all subsequent experiments, we used a concentration of 1 µmol/L S1P to assure comparability with our previously published results on the S1P uptake function of CFTR (e.g., 1 µmol/L S1P-FITC).

To determine whether S1P’s effect on CFTR function was reliant on cell-surface expression of functional CFTR, we next assessed the effect of S1P on iodide efflux in BHK-ΔF508-CFTR cells. Mutant ΔF508-CFTR is largely retained in the endoplasmic reticulum due to insufficient processing and folding (Molinski et al. 2012, Yu et al. 2011, Sharma et al. 2001,
French et al. 1996). As a consequence, its localization to the plasma membrane is significantly less than the WT protein. Even when the mutant is expressed in the membrane through treatment interventions (e.g., low-temperature incubation), the rescued ΔF508-CFTR has less channel function than WT-CFTR (Van Goor et al. 2006, Hwang et al. 1997). Under control conditions where cells were kept at 37°C, we found FSK stimulated little-to-no iodide efflux in the BHK-ΔF508-CFTR cells. Low temperature incubation (24 hours, 27°C) promotes the integration of ΔF508-CFTR into the plasma membrane (He et al. 2013, French et al. 1996), and conferred the ability for FSK to stimulate iodide efflux in these cells (iodide efflux rate=6.59±2.07 nmol/L*s versus 1.12±0.0457 nmol/L*s for 27°C and 37°C respectively, p<0.05, n_{27°C}=5, n_{37°C}=6 cell culture dishes, Figure 1C). Consistent with our results in Figure 1A, S1P attenuated FSK-stimulated iodide efflux in temperature-rescued BHK-ΔF508-CFTR cells (2.51±1.66 nmol/L*s, p<0.05 relative to FSK + BSA, n=6 cell culture dishes, Figure 3-1C).

Phosphorylation of CFTR’s inhibitory residues has been shown to oppose PKA’s stimulatory sites. Therefore, we postulated that S1P’s attenuation of CFTR’s FSK-stimulated iodide efflux was phosphorylation-dependent. We assessed the S737 amino acid residue because phosphorylation at this site has been shown to inhibit CFTR’s channel function (Wilkinson et al. 1997). Furthermore, S737 phosphorylation, in response to PKA, has been shown to promote distinct conformational changes in the CFTR protein (Csanády et al. 2005), and these changes may inhibit channel function. The 67D4 phosphosensitive antibody recognizes a region of the R-domain that includes the S737 residue, and phosphorylation of the R-domain inhibits binding of the 67D4 antibody to the CFTR protein. Thus, phosphorylation was recognized as a decrease in the densitometry of the 67D4 signal, normalized to a loading control (e.g., GAPDH, α-tubulin) after gel electrophoresis under reducing conditions and Western blotting detection.
Therefore, the 67D4 antibody recognizes CFTR phosphorylation status rather than conformational changes. BHK-WT-CFTR cells were acutely treated with S1P (1 µmol/L) for time points ranging from 30 seconds to 60 minutes, in a cell culture incubator (37°C, 5% CO₂). There was a significant decrease in the normalized 67D4 antibody signal after 30 seconds of treatment with S1P, relative to the untreated control samples (mean ratio of 67D4 signal densitometry to GAPDH = 1.06±0.0453 versus 1.25±0.128 for 30 seconds S1P treatment and untreated respectively, p<0.05, n=all=6 cell culture dishes, Figure 3-1D). Treatment with FSK (30 seconds, 10 µmol/L) served as a positive control for phosphorylation (0.0889±0.0422, p<0.05 relative to untreated samples, n=all=6 cell culture dishes). The five and 15 minutes time points did not decrease the signal further, relative to the 30 seconds time point (0.576±0.158 versus 0.524±0.230 for five and 15 minutes time points respectively, n=all=6 cell culture dishes), and this suggested the phosphorylation response was maximal within 30 seconds of stimulation. After 60 minutes of continuous stimulation with S1P there was a full reversal in the normalized 67D4 signal (0.817±0.168, p>0.05 relative to untreated samples, n=6 cell culture dishes), and this suggested that S1P has a transient effect on the phosphorylation of CFTR.

As an additional experiment, we assessed whether acute treatment with exogenous S1P affects expression of total CFTR, detected with the M3A7 antibody that recognizes an epitope in CFTR’s NBD2. Treatment of BHK-WT-CFTR cells with S1P (1 µmol/L) for various time points did not significantly affect the expression of total CFTR (p>0.05), as detected with the M3A7 antibody (Supplemental Figure 2A). For example the mean ratio of the M3A7 to α-tubulin densitometry was 2.26±0.167 after DMSO treatment (0.1% v/v, n=4 cell culture dishes) and 2.28±0.0924 after 30 seconds treatment with S1P (n=4 cell culture dishes). However, when the same samples were probed with the 67D4 phosphosensitive antibody, there was a significant
reduction in the 67D4 signaling normalized to M3A7 signal after 30 seconds S1P treatment (mean 67D4:M3A7= 0.220±0.0738 versus 0.480±0.0227 for S1P and DMSO treatments respectively, p<0.05, Supplemental Figure 2B). This suggests exogenous S1P treatment promotes phosphorylation of CFTR, in BHK-WT-CFTR cells, and does not affect the total CFTR protein expression. Phosphorylation was confirmed with FSK treatment (30 seconds, 10 µmol/L, mean M3A7:α-tubulin signal= 2.023±0.228, mean 67D4:M3A7 signal= 0.0175±0.00250, n=4 cell culture dishes). Furthermore, normalization of the 67D4 signaling to α-tubulin also demonstrated that 30 seconds S1P promotes phosphorylation of CFTR (mean 67D4:α-tubulin signal= 0.500±0.134 versus 1.08±0.0570 for S1P and DMSO treatments respectively, p<0.05, Supplemental Figure 2C). These results also validate our normalization of the phosphosensitive 67D4 antibody signal to α-tubulin, in order to detect phosphorylation of CFTR in the BHK-WT-CFTR cells.

The cell-surface biotinylation assay was then used to assess whether acute S1P treatment affects the expression of CFTR in the plasma membrane, and we found there was no significant effect of S1P on the expression of biotinylated CFTR, relative to untreated samples (mean ratio of biotinylated CFTR to total CFTR = 0.895±0.179 versus 0.930±0.146 for S1P and control treatments respectively, p>0.05, nS1P=8, ncontrol=7 cell culture dishes, Supplemental Figure 3A). This finding supports phosphorylation of CFTR is associated with a change in iodide conductance, rather than a change in CFTR’s subcellular localization.

We next assessed whether mutating the S737 phosphorylation site (i.e., serine-to-alanine mutation; S737A-CFTR) prevented the inhibitory effect of S1P on FSK-stimulated iodide efflux. Naïve BHK cells were transfected with the S737A-CFTR construct for 48 hours, and channel function was assessed with the conventional iodide efflux assay because the continuous assay did could not appreciably detect efflux in transiently transfected cells. In the absence of S1P, the
BHKS737A-CFTR cells had significantly greater FSK-stimulated iodide efflux (mean concentration of iodide in supernatant= 6.50±0.352 µmol/L, n=7 coverslips, Figure 3-1E) relative to the non-stimulated control treatment group, which had no detectable iodide ions in the supernatant (n=4 coverslips, p<0.05). Therefore, FSK stimulated a comparable iodide efflux for the S737A-CFTR construct as it did for the WT protein in cells. Furthermore, there was no significant effect of the acute S1P treatment on the iodide efflux for S737A-CFTR (6.72±0.285 µmol/L, n=7 coverslips) relative to the no-S1P condition (p>0.05), indicating that the S737 residue is a key molecular target for the S1P-induced intracellular signaling chain to exert an inhibitory effect on CFTR channel function.
Figure 3-1. Sphingosine-1-phosphate (S1P) attenuates CFTR-dependent iodide efflux via phosphorylation of serine 737 (S737).

(A) A representative tracing of iodide efflux in BHK cells stably transfected with wild-type CFTR (WT-CFTR). Forskolin (FSK; 20µmol/L) stimulates CFTR-dependent iodide efflux; more iodide is released in the vehicle control (4% BSA), compared to cells treated with 1µmol/L S1P. Permeabilization with 0.1% Triton X-100 (TX-100) confirms that iodide loading was similar. (B) In BHK cells stably expressing WT-CFTR, the full inhibitory effect is observed at S1P concentrations as low as 200µmol/L, which is a physiological level (n=7). (C) Iodide efflux is essentially absent in BHK cells stably expressing the ΔF508 mutant of CFTR (ΔF508-CFTR; a mutant that does not integrate into the plasma membrane). Low temperature rescue (27°C for 24h), which allows plasma membrane integration of CFTR, restored FSK-stimulated iodide efflux responses, could be inhibited with 1µmol/L S1P (n=6). (D) Western blots utilizing an antibody that exclusively binds to the non-phosphorylated regulatory domain (R-domain) of CFTR (67D4) indicate that WT-CFTR is rapidly (within 30 seconds) phosphorylated in response to 1µmol/L S1P (n=5). This response is transient in nature and phosphorylation is significantly lessened after 60 minutes (i.e., p<0.05 comparing 0.5 and 60 minutes). (E) In BHK transfected with a plasmid encoding a mutated CFTR protein containing a serine-to-alanine substitution as amino acid 737 (S737A-CFTR), S1P failed to inhibit FSK-stimulated iodide efflux. * denotes p<0.05 relative to control, one-way ANOVA and Bonferroni’s selected comparisons.
3.4.2 S1P stimulates phosphorylation of CFTR and inhibits iodide efflux in an AMPK-dependent manner

As already mentioned, the S737 inhibitory residue can be phosphorylated by AMPK (see Section 1.5.1.2), and S1P has been shown to be an upstream activator of AMPK (Levine et al. 2007). We therefore proposed S1P’s inhibitory effect on CFTR’s channel function was AMPK-dependent. Treatment with S1P (three minutes, 1 µmol/L) in the BHK-WT-CFTR cells significantly increased the ratio for the densitometry of phosphorylated AMPK to total AMPK, relative to the untreated control samples (mean p-AMPK:AMPK= 1.47±0.216 versus 0.460±0.0874 for S1P and untreated respectively, p<0.05, n=3 cell culture dishes, Figure 3-2A). The rapid phosphorylation of AMPK by S1P was in line with Levine et al.’s study, and confirmed that S1P is an upstream activator of AMPK. We also found that pre-treatment of the cells with Compound C (30 minutes, 80 µmol/L), a cell-permeable competitive-inhibitor of AMPK that forms an inactive analogue of AMP, prevented the phosphorylation of AMPK (0.647±0.153, p>0.05 relative to untreated samples, n=3 cell culture dishes). Compound C treatment also blocked the phosphorylation of CFTR by S1P (mean 67D4:GAPDH ratio= 5.38±0.716 for DMSO treatment, 1.67±0.664 for 30 seconds S1P, and 4.09±0.513 for Compound C + S1P, n_all=6 cell culture dishes, Figure 3-2B). Furthermore, Compound C prevented the S1P-dependent attenuation of FSK-stimulated iodide efflux (mean iodide efflux rate= 19.4±2.49 nmol/L*s for FSK + BSA, n=6; 3.65±1.35 nmol/L*s for FSK + S1P, n=7; and 39.8±7.53 nmol/L*s for FSK + Compound C + S1P, n=9 cell culture dishes, Figure 3-2C).

We also assessed acute treatments with the fluorescently-labeled S1P and found that relative to BSA treatment (30 seconds, 4% v/v), S1P-FITC treatment (30 seconds, 1 µmol/L) significantly increased the phosphorylation of AMPK (mean p-AMPK: total AMPK= 0.550±0.00453 versus 0.0522±0.00820 for S1P-FITC and BSA treatments respectively, p<0.05,
n_{S1P}=6, n_{BSA}=4 cell culture dishes, Supplemental Figure 3B). This suggests that S1P-FITC is bioactive and can promote AMPK phosphorylation. Therefore, assessing uptake directly would be difficult because the molecule enhances a known inhibitory pathway for CFTR’s function. The results support our use of the iodide efflux assay as a surrogate to assess modulation.

Compound C can have non-specific effects (Emerling et al. 2007), therefore we complimented the use of Compound C with a second, more specific AMPK-inhibitory strategy: siRNA transfection in BHK-WT-CFTR cells. Western blots confirmed that siRNA transfection targeting AMPK (48 hours, 25 nmol/L) significantly reduced AMPK protein expression (normalized to GAPDH), relative to transfection of negative control pool siRNA (mean ratio of AMPK to GAPDH= 0.331±0.0635 versus 1.07±0.110 for AMPK and Neg. siRNA respectively, p<0.05, n_{all}=12 cell culture dishes, Figure 3-2D). We also found that transfection with the AMPK siRNA prevented the S1P-stimulated phosphorylation of CFTR (mean 67D4:GAPDH= 2.22±0.262 versus 2.09±0.146 for AMPK siRNA + S1P, and DMSO treatments respectively, p>0.05, n_{AMPK}=6, n_{DMSO}=5 cell culture dishes, Figure 3-2E). The effect of siRNA transfection appeared to be specific to AMPK, because FSK was still able to stimulate phosphorylation of CFTR (0.0855±0.0286, p<0.05 relative to DMSO treatment, n=6 cell culture dishes). Furthermore, transfection with Neg. siRNA did not block the S1P-stimulated CFTR phosphorylation (0.371±0.124 versus 1.63±0.230 for Neg. + S1P, and DMSO treatments respectively, p<0.05, n_{NEG}=6, n_{DMSO}=5 cell culture dishes). Next we assessed the effect of AMPK siRNA transfection on the S1P-mediated attenuation of CFTR’s channel function, with the conventional iodide efflux assay, and found that the attenuation was abolished (mean iodide in supernatant= 14.8±1.08 µmol/L versus 13.9±0.579 µmol/L for S1P and no-S1P treatments respectively, p>0.05, n_{all}=6 coverslips, Figure 3-2F). The non-stimulated treatment condition had a low concentration of detectable iodide ions (0.900±0.900 µmol/L, n=6 coverslips).
Transfection with Neg. siRNA did not prevent the attenuation of the FSK-stimulated iodide efflux mediated by S1P (1.51±0.352 µmol/L versus 6.53±0.46 µmol/L for S1P and no-S1P treatments respectively, p<0.05, n_all=6 coverslips). The siRNA experiments confirmed the AMPK-dependency for S1P’s effect on the phosphorylation and channel function of CFTR. We also observed that the FSK-stimulated iodide efflux was significantly greater with the AMPK siRNA than with the Neg. siRNA (p<0.05, Figure 3-2F), suggesting a basal activity of AMPK.
Figure 3-2. Sphingosine-1-phosphate (S1P) inhibits CFTR by an AMP-activated protein kinase (AMPK)-dependent mechanism.

In BHK cells stably expressing WT-CFTR, the chemical AMPK inhibitor compound C (CdC; 20µmol/L, 60min pre-treatment) prevented S1P (1µmol/L)-stimulated (A) AMPK phosphorylation (n=3), (B) CFTR S737 phosphorylation (assessed by phospho-sensitive 67D4 antibody; n=6) and (C) attenuation of cAMP-stimulated iodide efflux (n=6-9). In (B), elevated CFTR phosphorylation was observed after 30 seconds stimulation, but not after 60 minutes (i.e., a transient response); CdC treatment did not alter CFTR phosphorylation after 60min S1P stimulation. (D) Transfecting siRNA targeting the AMPK α1 subunit effectively reduced AMPK α1 protein expression in BHK cells stably expressing WT-CFTR; control siRNA had no effect on AMPK α1 expression (n=12). Relative of siRNA controls, AMPK α1 siRNA treatment abolished S1P-stimulated (1µmol/L, 30 seconds) (E) CFTR S737 phosphorylation (n=4-5) and (F) the attenuation of FSK/cAMP-stimulated iodide efflux (n=6). * denotes p<0.05 relative to control, one-way ANOVA and Bonferroni’s selected comparisons.
3.4.3 The effect of S1P relies on the S1P$_1$R

Since S1P is an upstream activator of AMPK, and the effects of exogenous S1P are receptor-dependent, we therefore determined the S1PR receptor responsible for the effects of S1P on CFTR phosphorylation and channel function. Of the five known S1PRs, only S1P$_1$R and S1P$_3$R have been linked to AMPK-signaling (Kimura et al. 2010), and our RT-PCR experiments demonstrated expression of both receptors in the BHK cells. Next we pre-treated BHK-WT-CFTR cells with the S1P$_1$ / S1P$_3$ receptor antagonist VPC 23019 (VPC; 30 minutes, 4 µmol/L), and found that the treatment decreased, but did not prevent S1P-stimulated AMPK phosphorylation (mean p-AMPK:total AMPK= 0.0471±0.00885 for DMSO control, n=5; 0.878±0.290 for S1P, n=4; and 0.504±0.102 for VPC + S1P, n=4 cell culture dishes Figure 3-3A). VPC 23019 prevented CFTR phosphorylation (mean 67D4:α-tubulin= 4.58±0.363 for DMSO control; 1.27±0.219 for S1P; and 2.35±0.145 for VPC + S1P, n=all=9 cell culture dishes, Figure 3-3B). Pre-treatment with VPC 23019 also abolished the S1P-dependent decrease in the FSK-stimulated iodide efflux (mean iodide in supernatant = 12.7±1.10 µmol/L for the control; 2.12±0.494 µmol/L for S1P; and 12.2±1.72 µmol/L for VPC + S1P, n=6 coverslips, Figure 3-3C). The phosphorylation and iodide efflux data with VPC 23019 suggested that S1P inhibits CFTR’s channel function, in a S1PR and AMPK dependent manner. VPC 23019 is an antagonist against both the S1P$_1$R and the S1P$_3$R. However, Davis et al. demonstrated that the affinity of VPC 23019 for S1P$_1$R is ten-fold greater than the affinity for S1P$_3$R, in S1PR transiently transfected HEK-293 cells (Davis et al. 2005), we also confirmed the involvement of the S1P$_1$R by using the S1P$_1$ receptor-specific agonist SEW 2871. We assessed the effect of acute treatment with SEW 2871 (10 µmol/L) on both AMPK (Figure 3-3D) and CFTR phosphorylation (Figure 3-3E). Treatment with SEW 2871 (30 seconds) led to significant
phosphorylation of CFTR relative to the DMSO (0.1% v/v) control treatment (mean 67D4:α-tubulin = 1.05±0.221 versus 1.89±0.166 for SEW 2871 and DMSO treatments respectively, p<0.05, n=all=4 cell culture dishes). Acute SEW 2871 treatment also significantly decreased the FSK-stimulated iodide efflux relative to the DMSO control treatment (mean concentration of iodide in supernatant= 2.74±0.254 μmol/L versus 11.6±1.10 μmol/L for SEW 2871 and control treatments respectively, p<0.05, n=6 coverslips, Figure 3-3F). Treatment with CFTRinh-172 also significantly decreased the FSK-stimulated iodide efflux relative to control (1.08±0.422 μmol/L, n=3 coverslips), and this served as control for CFTR channel inhibition. Although we cannot completely rule out a role for the S1P3R, the finding that the S1P1R-specific agonist promoted phosphorylation of AMPK and CFTR, and reduced CFTR iodide efflux, strongly suggests S1P stimulates the phosphorylation of CFTR in an S1P1R-AMPK-dependent manner, and this causes the reduction in CFTR channel function.
Figure 3.3. Sphingosine-1-phosphate (S1P) mediates its effects via the S1P₁ receptor (S1P₁R) subtype.

In BHK cells stably expressing WT-CFTR, the chemical S1P₁ / S1P₃ receptor antagonist VPC23019 (VPC; 4µmol/L, 30min pre-treatment) prevented S1P (1µmol/L)-stimulated (A) AMPK phosphorylation (n=6), (B) CFTR S737 phosphorylation (assessed by phospho-sensitive 67D4 antibody; n=6) and (C) attenuation of PKA-stimulated iodide efflux (n=6). Stimulating WT-CFTR-expressing BHK cells with the S1P₁ receptor-specific agonist SEW2871 (SEW; 10µmol/L) increased both (D) AMPK phosphorylation, (E) CFTR phosphorylation. (F) SEW co-stimulation attenuated FSK/cAMP-dependent iodide efflux to a similar extent as S1P (n=6 for control and SEW; n=3 for S1P). * denotes p<0.05 relative to control, + denotes p<0.05 relative to S1P treatment, one-way ANOVA and Bonferroni’s selected comparisons.
3.5 Discussion

3.5.1 S1P is a modulator of CFTR channel function

CFTR is a tightly regulated anion channel, and in the present study we found that S1P is a novel modulator of CFTR’s channel function, which was assessed with the iodide efflux assays. Specifically, we found that acute treatment with S1P inhibits the FSK-stimulated iodide efflux of the human epithelial isoform of CFTR, in a stably transfected BHK cell line. Additionally, the effective concentration for the inhibition was 200 nmol/L and this can be considered physiological because it corresponds well to in vivo S1P plasma levels (Yatomi et al. 1997). We also found that the S1P-dependent inhibition of the iodide efflux was reliant on CFTR expressed in the plasma membrane, because non-temperature rescued BHK-ΔF508-CFTR did not respond to S1P, but temperature-rescued ΔF508-CFTR did show attenuation in response to S1P.

We observed an association between the phosphorylation of CFTR’s S737 amino acid residue, which resides within the R-domain, and the attenuation of PKA-stimulated CFTR’s channel activity (measured as iodide efflux in response to either FSK, or a cocktail containing FSK, IBMX, and cpt-cAMP). We found that phosphorylation of CFTR was rapidly induced (i.e., within 30 seconds of treatment) by S1P at the S737 residue, and the phosphorylation response was transient in nature (i.e., phosphorylation declines to baseline levels after approximately 60 minutes). These results corresponded well with observations described by Levine and co-workers for AMPK in endothelial cells. They showed significant phosphorylation of AMPK within one minute of treatment with S1P, and a gradual decline to basal levels after 30 minutes of treatment (Levine et al. 2007). Furthermore, acute S1P treatment did not affect expression of WT-CFTR in the plasma membrane, even though CFTR phosphorylation has been linked to endocytosis and trafficking (see Section 1.5.1.1.). Thus, acute treatment with S1P...
decreases CFTR’s channel function, and does not affect CFTR localization in the plasma membrane.

3.5.2 The S1P₁R-AMPK signaling pathway is responsible for the effect of S1P on CFTR channel function

AMPK is thought to inhibit CFTR channel function by causing conformational changes that make it resistant to PKA-dependent activation, but AMPK does not prevent phosphorylation of CFTR by PKA (King et al. 2009, see Section 1.5.1.2.). The S737 and S768 residues are two known inhibitory sites in the R-domain of CFTR, and work by Kongsuphol and co-workers demonstrated that AMPK phosphorylates both residues. Their study employed an in-vitro assay with recombinant AMPK and R-domain proteins, and they found that mutation of the amino acid corresponding to the S768 residue of CFTR abrogates almost all AMPK-stimulated R-domain phosphorylation. The finding suggests that AMPK has a distinct preference for the S768 residue (Kongsuphol et al. 2009). Similar results have been shown for the S768A-CFTR construct by King Jr. and co-workers (King et al. 2009). Their study used purified recombinant AMPK to stimulate the in-vitro phosphorylation of CFTR in HEK-293 cells, and found that mutation of S768 prevented AMPK-dependent phosphorylation (King et al. 2009). The authors also used voltage clamping in Xenopus oocytes to demonstrate that the S768A-CFTR was insensitive to the effect on AMPK on PKA-stimulated channel function. The S768A-CFTR construct has also been shown to have greater open probability (i.e., the length of time a channel remains open relative to when it remains closed) (Csanady et al. 2005, Vais et al. 2004), and channel conductance (King et al. 2009, Kongsuphol et al. 2009) than the WT protein. King Jr. and colleagues propose this is indicative of tonic inhibition of CFTR channel function mediated by AMPK.
It is possible that the phosphorylation of both S737 and S768 is required for the S1P-mediated attenuation of PKA-stimulated CFTR channel activity, and S1P may also induce phosphorylation of the S768 residue. In the present study, we show that mutation of the S737 residue abolished the inhibitory effect of S1P on CFTR’s channel function. Our data suggest that the CFTR S737 phosphorylation site may be largely insensitive to constitutive (i.e., metabolically-regulated) AMPK activity under non-stimulated conditions. Therefore, we postulate the S737 residue is primarily involved in regulating CFTR channel function in response to extracellular stimuli, such as exogenous S1P.

Nonetheless, it is unlikely that CFTR S737 phosphorylation alone is sufficient to mediate the inhibitory effect, since the S737 phosphorylation site is also rapidly phosphorylated in response to PKA activation (e.g., FSK treatment). Thus, the inhibitory effect is the result of the combined allosteric effects of CFTR R-domain phosphorylation and CFTR-AMPK protein interaction, as previously described (see Section 1.5.1.2). This would explain why PKA-mediated phosphorylation of S737 alone does not inhibit CFTR channel function. In addition to blocking the inhibitory effect of S1P, we found that the inhibition of AMPK with Compound C or siRNA transfection resulted in a clear enhancement of PKA-stimulated CFTR channel activity, relative to control samples (e.g., FSK alone, Neg. siRNA). Similar enhancements have been shown by other studies (King et al. 2009, Kongsuphol et al. 2009), and it is thought to occur because AMPK has high baseline activity (Kongsuphol et al. 2009). Furthermore, AMPK is thought to already exert a degree of inhibition on CFTR channel function, and this may explain the enhanced iodide conductance observed in our study with Compound C or AMPK siRNA transfection.

Extracellular S1P binds to five distinct G-protein-coupled receptors (S1P1-5R) that have a cell type-specific expression pattern and couple to multiple G-proteins and downstream effectors
The BHK cells express mRNA for S1P\textsubscript{1,3}R, and in the present study we found that exogenous S1P inhibits CFTR channel function via the S1P\textsubscript{1}R, because pre-treatment with VPC 23019 prevented S1P-dependent phosphorylation of AMPK and CFTR, and prevented the inhibition of the FSK-stimulated iodide efflux. We also found that the S1P\textsubscript{1}R agonist, SEW 2871, mimicked the phosphorylation and iodide efflux effects of S1P, and this confirmed the S1P\textsubscript{1}R dependence. Our observation of the S1P\textsubscript{1}R -AMPK link is consistent with the S1P\textsubscript{1}R / S1P\textsubscript{3}R -mediated AMPK activation observed by Kimura and co-workers in the distinctly separate endothelial cell model (Kimura et al. 2010). Given the ubiquitous nature of the two signaling pathways, this consistency (Kimura et al. 2010, Levine et al. 2007) suggests that the S1P/AMPK connection is pertinent in a variety of cellular and physiological processes, such as metabolism (Kowalski et al. 2013), and the immune response (Mayer et al. 2008).

### 3.6 Conclusion

S1P is a ubiquitous lipid-signaling mediator that directs a diverse array of biological processes involved in virtually every aspect of cellular homeostasis (e.g., development, growth and differentiation, motility and survival)(Spiegel and Milstien 2011, Spiegel and Milstien 2003). The present investigation bridges the S1P signaling pathway to the regulation of CFTR, by demonstrating that S1P is a negative modulator of CFTR channel function. The modulation is through the S1P\textsubscript{1}R-AMPK-signaling pathway. Given the highly-integrative nature of S1P signaling and the multitude of stimuli that converge onto this pathway (Spiegel and Milstien 2011, Spiegel and Milstien 2003), this is seminal to characterizing CFTR as an effector in pathways not yet associated with CFTR channel function.
Chapter 4

4 The S1P₁R-AMPK signaling pathway aggravates the cellular ΔF508-CFTR phenotype

Note: This chapter includes data presented in two manuscripts – (1) Firhan A. Malik*, MSc, Anja Meissner*, PhD, Hai H. Bui, PhD, Christine E Bear, PhD, Darcy Lидington PhD, and Steffen-Sebastian Bolz, MD, PhD. (2014), submitted to PLoS ONE. (2) Anja Meissner, PhD*, Firhan A. Malik, MSc*, Jeffreyy T. Kroetsch, MSc, Meghan Sauvé, MSc, Jessica Fares, Danny D. Dinh, Abdul Momen, MD, Mansoor Husain, MD, Christine E Bear, PhD, Darcy Lидington PhD, and Steffen-Sebastian Bolz, MD, PhD. (2014), submitted to Circulation. *denotes these authors contributed equally.

4.1 Acknowledgements

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data. Post Doctoral fellow Dr. Roozbeh Sobbi of Dr. Peter Backx’s laboratory (University of Toronto) assisted in the echocardiography of the mice, and assisted in data analysis. Technologist Dr. Hangjun Zhang of Dr. Scott Heximer’s laboratory performed the terminal blood pressure measurements of the mice, and assisted in data analysis.

4.2 Overview and rationale

As discussed in Section 1.5.3, Cystic fibrosis (CF) is the most common fatal genetic disease (i.e., homozygous recessive) that affects the Caucasian population, and the most common disease causing mutation is the genetic deletion of the F508 residue from CFTR’s NBD1. The pathways that exacerbate or contribute to ΔF508-CFTR’s defective processing and/or function are not well understood. Hallows and colleagues observed that non-differentiated airway epithelial cells from CF patients have increased AMPK activity, relative to cells from non-CF patients (Hallows et al. 2006). However, direct inhibition of WT-CFTR with CFTR\textsubscript{inh-172} treatment did not affect the enzymatic activity of AMPK. Therefore, the group suggested that abnormal localization and/or function of CFTR contributes to enhancing AMPK’s activity via upregulation of upstream kinases or pathways that regulate AMPK (Hallows et al. 2006). As previously mentioned, S1P is an upstream activator of AMPK (Levine et al. 2007). Indeed, in Chapter 3 we demonstrated that acute S1P treatment decreases the FSK-stimulated iodide efflux of temperature rescued ΔF508-CFTR. Furthermore, we demonstrated the effect of S1P on WT-CFTR was dependent upon the S1P\textsubscript{1R} -AMPK signaling pathway. Additionally, BHK-ΔF508-CFTR cells are unable to uptake exogenous S1P (Meissner et al. 2012), and this suggests there is increased bioavailability of S1P. This may stimulate the S1P\textsubscript{1R}-AMPK through a positive feedback loop. Therefore, we hypothesize that signaling pathways that activate AMPK, like S1P and S1P\textsubscript{1R}, contribute to the defective processing and/or function of the ΔF508-CFTR mutant.
protein. Channel function was assessed using the conventional iodide efflux technique in BHK-ΔF508-CFTR cells, and we show that antagonism of the S1P₁R (e.g., VPC 23019), or inhibition of AMPK (e.g., Compound C, siRNA transfection) enhances the FSK-stimulated iodide efflux, and the S1P-uptake function. The enhanced channel function suggested reestablishment for the regulation of S1P bioavailability, and we then tested cerebral microvascular reactivity because this is a physiological parameter that is affected by abnormal S1P-signaling (Meissner et al. 2012, Yang et al. 2012). We assessed cerebral microvascular reactivity of FVB-129-ΔF508-CFTR mice, and found that these mice have decreased CBF compared to WT mice. However, the mutant mice did not show significant differences in their systemic hemodynamic parameters (e.g., CO, MAP, TPR) relative to WT mice. Therefore, the genetic deletion of the F508 residue contributes abnormal cerebral autoregulation in mice. In addition, their isolated PCAs show enhanced endogenous (i.e., myogenic reactivity) and exogenous-S1P signaling that is dependent upon the S1P₁R.

4.3 Methods

4.3.1 Animals

See Section 2.1.

4.3.2 Antibodies and Reagents

See Sections 2.2 and 2.3.

4.3.3 Cell culture

See Section 2.4.

4.3.4 siRNA transfection

See Section 2.6.2.
4.3.5 Conventional iodide efflux assay

See Section 2.8.1.

4.3.6 Membrane fractionation

The membrane fractionation procedure was originally published by our laboratory in Meissner et al. (2012), and in the present study BHK-ΔF508-CFTR cells were grown to 95-100% confluency in 60 mm tissue culture dishes. The cells were treated with Compound C (20 μM) for 10, 30, and 60 minutes, and kept in a cell-culture incubator (37°C, 5% CO₂) during treatment. The cells were scraped into a 1.5 mL centrifuge tube and lysed (six freeze-thaw cycles using liquid nitrogen followed by RT vortexing) in a buffer containing 10mM Tris (pH 7.3), 140mM NaCl, 1% Triton-X-100, 5mM EDTA, 1mM DTT and 25µg/ml protease inhibitor cocktail. The samples were centrifuged (30 minutes at 15,000g; 4°C) to remove the insoluble material. The supernatant was collected and the pellet was discarded. The supernatant was then overlayed 1:1 with 3% sucrose and incubated for five minutes at RT and then five minutes at 30°C. The samples were centrifuged again for five minutes at 2000g and 4°C. After centrifugation two phases were evident: (i) a clear phase containing the intracellular fractions, and (ii) a translucent phase containing the plasma membrane fraction. The cytosolic fraction (clear phase) was mixed 1:1 with buffer containing 10mM Tris (pH 7.3), 140mM NaCl, 1.5mM KCl, 5mM EDTA, 1mM DTT, and 25µg/ml protease inhibitor cocktail, heated to 65°C for 10 minutes and then centrifuged (30 minutes at 15,000g; 4°C) to remove insoluble material. The membrane fraction was not processed further. Samples were then electrophoresed on 8% SDS-PAGE gels.

4.3.7 Functional assessments of isolated and cannulated PCAs

See Section 2.10.
4.3.8 S1P-FITC uptake assay

See Section 2.11.

4.3.9 MRI imaging, echocardiography, and terminal blood pressure measurements

See Sections 2.12 to 2.14

4.3.10 Statistics

See Section 2.15

4.4 Results

4.4.1 Antagonism of the S1P$_1$R or inhibition of AMPK enhances the iodide efflux of BHK-ΔF508-CFTR cells

The conventional iodide efflux was used to assess the channel function of BHK-ΔF508-CFTR cells, and for these experiments cells were cultured at 37°C and 5% CO$_2$. We found cells that were pre-treated with DMSO (24 hours, 0.1% v/v) had an FSK-stimulated iodide efflux of 0.276±0.148 µmol/L (n=11 coverslips, Figure 4-1A). Treatment with the S1P$_1$R antagonist VPC 23019 (24 hours, 4 µmol/L) significantly increased the iodide efflux by almost 12-fold to 3.52±0.636 µmol/L (p<0.05 relative to DMSO, n=6 coverslips, iodide efflux profile shown in Figure 4-1A, and maximal response shown in Figure 4-1B). This demonstrated that antagonism of the S1P$_1$R enhances the channel function of ΔF508-CFTR. We also treated cells with the known CFTR small-molecule corrector compound C18 (He et al. 2013, Holleran et al. 2013) recently patented by Vertex Pharmaceuticals. C18 promotes the maturation of ΔF508-CFTR, and as a consequence enhances mutant channel function (Wong et al. 2012), and this served as a treatment to assess processing and maturation of the mutant protein. We found that C18 treatment (24 hours, 6 µmol/L) significantly increased the FSK-stimulated iodide efflux by almost 19-fold compared to the DMSO control (5.18±0.558 µmol/L, p<0.05, n=12 coverslips,
There was no significant difference in the iodide efflux for the C18 treatment, relative to the VPC 23019 treatment (p>0.05). As a negative control for the iodide efflux assay we used DMSO-treated cells that were not activated (i.e., not exposed to the stimulation cocktail), and we found these cells had no detectable iodide ions in the supernatant, and thus no FSK-stimulated iodide efflux (n=3 coverslips, Figure 4-1A).

We next assessed the effects of AMPK inhibition on the FSK-stimulated iodide efflux of ΔF508-CFTR using the cell-permeable inhibitor Compound C or AMPK siRNA transfection. For this experiment, a new set of cells were plated and grown to 95-100% confluency at 37°C and 5% CO₂. Cells pre-treated with DMSO (24 hours, 0.1% v/v) had no detectable iodide ions in the supernatant, and thus had no FSK-stimulated iodide efflux (n=5 coverslips, Figure 4-1B). Treatment with Compound C (24 hours, 1 μmol/L) or transfection of AMPK siRNA (24 hours, 25 nmol/L) significantly increased the FSK-stimulated iodide efflux to 3.46±0.260 μmol/L for Compound C (n=5 coverslips) and 5.19±0.408 μmol/L for AMPK siRNA (n=6 coverslips, p<0.05 relative to DMSO treatment, iodide efflux profile shown in Figure 4-1C, and maximal response shown in Figure 4-1E). In contrast, we found that transfection with the negative control pool siRNA resulted in no detectable iodide ions in the supernatant, and thus the FSK-stimulated iodide efflux was not significantly different relative to the DMSO treatment (p>0.05, n=6 coverslips, Figure 4-1C,E). These findings suggest that inhibition of AMPK enhances the channel function of ΔF508-CFTR. Some treatments that partially restore ΔF508-CFTR’s channel function at 37°C show an additive effect at 27°C, because low-temperature incubation is a more permissible processing and folding condition for the mutant (see review by Farinha et al. 2013). Therefore, cells were also treated with Compound C or transfected with siRNA at 27°C (i.e., following a temperature rescue protocol). We found that low temperature incubation of
DMSO treated cells (24 hours at 27°C, 0.1% v/v) significantly increased the FSK-stimulated iodide efflux (4.78±0.792 µmol/L, n=6 coverslips), relative to the DMSO treatment at 37°C (p<0.05, Figure 4-1D,E). This finding supports the view of previously published work by others that low-temperature incubation improves processing and maturation (Wilke et al. 2012, Sharma et al. 2004, Drumm et al. 1996, Denning et al. 1992), and channel function of the ΔF508-CFTR mutant is improved as a result. Additionally, acute treatment with S1P (1 µmol/L) significantly decreased the FSK-stimulated iodide efflux of low-temperature rescued ΔF508-CFTR (1.36±0.172 µmol/L, p<0.05, n=6 coverslips), and this confirmed our results presented in Chapter 3 that S1P inhibits the channel function of the rescued ΔF508-CFTR. Compound C treatment performed under low-temperature conditions (24 hours at 27°C, 1 µmol/L) did not significantly increase the FSK-stimulated iodide efflux (6.19±0.356 µmol/L, n=6 coverslips, Figure 4-1D,E) relative to the low-temperature DMSO treatment group (p>0.05). However, the iodide efflux for the Compound C treatment group at 27°C was significantly greater than the treatment at 37°C, and this suggests an additive effect of Compound C with the low temperature incubation. Transfection with the AMPK siRNA under low temperature conditions (24 hours at 37°C, then 24 hours at 27°C, 25 nmol/L) significantly increased the FSK-stimulated iodide efflux (9.86±1.23 µmol/L, n=6 coverslips) relative to the low-temperature DMSO treatment (p<0.05, Figure 4-1E). Furthermore, the iodide efflux observed with AMPK siRNA transfection at 27°C was greater than that observed at 37°C (p<0.05). Thus, low temperature incubation led to an additive effect of the AMPK siRNA transfection on the channel function of ΔF508-CFTR. Transfection with the negative control pool siRNA (24 hours at 37°C, then 24 hours at 27°C, 25 nmol/L) did not significantly increase the FSK-stimulated iodide efflux (5.37±1.23 µmol/L, n=6
coverslips), relative to the 27°C DMSO treatment (p>0.05). Overall, the data demonstrates that inhibition of the S1P1R-AMPK signaling pathway enhances the function of ΔF508-CFTR.
Figure 4-1. Antagonism of the S1P₁ receptor (S1P₁R) subtype or inhibition of AMP-activated protein kinase (AMPK) enhances the iodide efflux of BHK-ΔF508-CFTR cells. Represented above are (A) conventional iodide efflux assay profiles and (B) the maximum iodide efflux after treatment at 37°C for 24 hours with DMSO (0.1% v/v), VPC 23019 (4 µmol/L), or C18 (6 µmol/L). VPC 23019 treatment (n=11 coverslips) significantly enhanced the FSK/cAMP-stimulated iodide efflux of BHK-ΔF508-CFTR cells, relative to the DMSO control (n=6 coverslips). The C18 positive control also significantly increased the iodide efflux relative to DMSO (n=12 coverslips). *denotes p<0.05 relative to DMSO control. (C), (D) Conventional
iodide efflux profile and (E) the maximum iodide efflux after treatment for 24 hours with the cell-permeable inhibitor of AMPK, Compound C (1 µmol/L) or AMPK siRNA transfection for 48 hours (25 nmol/L). Controls were DMSO treatment (0.1% v/v) and a negative control pool siRNA (25 nmol/L). Inhibition of AMPK with either Compound C (n=5 coverslips) or AMPK siRNA (n=6 coverslips) significantly increased the FSK-stimulated iodide efflux relative to DMSO control (n=5 coverslips). The Neg. siRNA did not have appreciable iodide efflux (n=6 coverslips). An additive effect of low-temperature incubation was also assessed with the treatments conducted at 27°C. DMSO treatment (n=6 coverslips) and Neg. siRNA (n=6 coverslips) had significantly greater iodide efflux relative to treatments at 37°C. Cmpd C treatment (n=6 coverslips) and AMPK siRNA (n=6 coverslips) had an additive effect at 27°C relative to their respective treatments at 37°C. *denotes p<0.05 relative to 37°C DMSO control. #denotes p<0.05 relative to Cmpd C at 37°C. $denotes p<0.05 relative to AMPK siRNA at 37°C. @denotes p<0.05 relative to DMSO control at 27°C following one-way ANOVA with Bonferroni’s selected comparisons post-hoc test.
4.4.2 Inhibition of AMPK enhances the uptake of S1P-FITC for BHK-ΔF508-CFTR cells

In addition to its anion channel function, the CFTR protein can uptake S1P (see Section 1.5.2), and therefore CFTR is a regulator of S1P bioavailability. Our laboratory has previously shown that treatment of CFTR expressing cells with the selective channel inhibitor CFTRinh-172 decreases the uptake of S1P-FITC (Meissner et al. 2012), and this suggests a link between channel function and S1P-uptake function. In the present study, the enhanced iodide flux observed after inhibition of S1P1R or AMPK suggests that the CFTR-dependent regulation of S1P’s bioavailability is reestablished. Therefore, we first assessed the link between the channel function and S1P-uptake function by using G551D-CFTR mutant, which has processing and glycosylation traits that are comparable to the WT protein (Eckford et al. 2012, Yu et al. 2011, Bompadre et al. 2007, Zegarra-Moran et al. 2002), but the mutant displays abnormal channel function due to defective gating of CFTR’s NBD1 (Taddei et al. 2004). BHK naïve cells were transiently transfected with the G551D-CFTR construct that contained a His-tag. Then 24 hours later the cells were incubated with S1P-FITC (60 minutes, 1 µmol/L). The fluorescence activated cell-sorting (FACS) technique was used to assess uptake of S1P-FITC, which was determined by the geometric mean of fluorescence intensity at 530 nm, and the mean value for each treatment group was compared to unlabeled controls (i.e., cells incubated with non-FITC labeled S1P). We observed that unlabeled samples for the BHK-G551D-CFTR cells had significantly greater fluorescence intensities, relative to BHK-WT-CFTR cells (mean intensity at 530 nm = 371±13.6 versus 138±32.8 for G551D and WT-CFTR respectively, p<0.05, nG551D=4, nWT-CFTR=6 cell culture dishes, Figure 4-2A). The difference in background intensities may have been caused by increased autofluorescence caused by the transfection, and we therefore normalized the intensity values of the S1P-FITC values to the values for the unlabeled samples,
and assessed the fold difference. The BHK-G551D-CFTR cells had significantly less FITC normalized fluorescence intensity relative to the BHK-WT-CFTR cells (1.02±0.0394 versus 2.29±0.0940 for G551D-CFTR and WT-CFTR respectively, p<0.05, n_{G551D}=7, n_{WT-CFTR}=9 cell culture dishes). The CFTR_{inh}-172 treatment (30 minutes, 1 µmol/L) significantly decreased the normalized intensity for BHK-WT-CFTR cells (1.36±0.165, p<0.05 relative to S1P-FITC alone, n=4 cell culture dishes), and the treatment had no effect in the BHK-G551D-CFTR cells (1.19±0.0589, p>0.05 relative to S1P-FITC alone, n=3 cell culture dishes). These results suggest that channel function is linked to the S1P-uptake function.

Our group and others have previously shown that cells expressing ΔF508-CFTR cannot uptake labeled S1P (Meissner et al. 2012, Boujaoude et al. 2001), but low-temperature rescue enhances the uptake (Meissner et al. 2012), and thus membrane localization of CFTR is also linked to the S1P-uptake function. In the present study, we found that BHK-ΔF508-CFTR cells pre-treated with DMSO (60 minutes, 0.1% v/v, at 37°C and 5% CO₂) did not significantly uptake S1P-FITC, relative to the unlabeled control samples (2110±34.8 arbitrary units versus 1963±49.8 arbitrary units for labeled and unlabeled respectively, p>0.05, n=8-10 cell dishes, Figure 4-2B), and this confirmed our previous work. We next assessed whether the inhibition of AMPK stimulates the uptake of S1P-FITC in BHK-ΔF508-CFTR cells. Treatment with Compound C (60 minutes, 20 µmol/L) significantly increased S1P-FITC uptake (5857±149 arbitrary units, p<0.05, n=7 cell dishes), relative to the DMSO treatment group. This demonstrates that inhibition of AMPK stimulates the uptake function of ΔF508-CFTR.

We next assessed whether inhibition of AMPK, by acute treatment with Compound C, affects the plasma membrane expression of ΔF508-CFTR, and if this may contribute to the enhanced S1P-FITC uptake observed. We used the membrane fractionation approach previously
published by our laboratory in Meissner et al. (2012), and found that treatment of BHK-ΔF508-CFTR cells with Compound C (60 minutes, 20 µmol/L, at 37°C and 5% CO₂) significantly increased the expression of ΔF508-CFTR in the plasma membrane fraction, relative to the untreated control group (mean ratio of CFTR in membrane fraction to total protein= 1.06±0.125 versus 0.605±0.039 for 60 minutes Compound C and control respectively, p<0.05, n=3-4 cell culture dishes, Supplemental Figure 4). Accordingly, the expression of ΔF508-CFTR in the intracellular fraction was significantly decreased by Compound C treatment (mean ratio of CFTR in intracellular fraction to total protein= 0.677±0.0750 versus 1.76±0.0700 for 60 minutes Compound C and control respectively, p<0.05), in contrast to the effect in the plasma membrane fraction. Therefore, inhibition of AMPK promotes expression of the ΔF508-CFTR mutant in the plasma membrane.
Figure 4-2. The uptake of S1P-FITC requires a functional CFTR channel, and inhibition of AMP-activated protein kinase (AMPK) enhances the S1P-FITC uptake of BHK-ΔF508-CFTR cells.

(A) BHK cells transiently transfected with G551D-CFTR (n=7 cell culture dishes) had significantly less uptake of S1P-FITC than BHK-WT-CFTR stably transfected cells (n=9 cell culture dishes). Uptake was assessed as an increase in the mean fluorescence intensity at 515 nm, normalized to control samples (unlabeled S1P). CFTRinh=172 treatment (1 μmol/L, 30 minutes) significantly reduced the uptake for BHK-WT-CFTR cells (n=3 cell culture dishes), and not the BHK-G551D-CFTR cells (n=3 cell culture dishes). *denotes p<0.05, #denotes
p<0.05 relative to BHK-WT-CFTR + S1P-FITC treatment following one-way ANOVA and Bonferroni’s selected comparisons post-hoc test. (B) BHK-ΔF508-CFTR cells display no appreciable S1P-FITC uptake (n=10 cell culture dishes) relative to unlabeled control (n=8 cell culture dishes). Acute treatment with Cmpd C (20 μmol/L, 1 hour, 37°C, n=7 cell culture dishes) significantly enhanced the uptake. *denotes p<0.05 relative to the unlabeled control group following one-way ANOVA and Bonferroni’s selected comparison post-hoc test.
4.4.3 FVB-129-ΔF508-CFTR mice show abnormal cerebral microvascular reactivity and decreased CBF

The S1P-signaling pathway is important in regulating the MR for proximal PCAs (Meissner et al. 2012, Yang et al. 2012). Previously, our laboratory has demonstrated that PCAs from CFTR KO mice have augmented myogenic responsiveness due to enhanced S1P-signaling (Meissner et al. 2012). The molecular work presented above suggests S1P-signaling through the S1P$_1$R and AMPK exacerbates the functional and expression defects of the ΔF508-CFTR. We hypothesized that the PCAs from the ΔF508-CFTR would display augmented myogenic responsiveness, and that signaling through the S1P$_1$R would be responsible for it. There are different models of the CF disease in mice, and for the current study we used the FVB-129-ΔF508-CFTR strain of mice first developed and characterized by van Doornick and colleagues. These mice have a relatively mild CF phenotype with little to no airway abnormalities, but there are significant obstructions of their gastrointestinal tract (Wilke et al. 2011, Davidson and Wolfe 2001, French et al. 1996, van Doornick et al. 1995). We used this strain because the mice have a lower mortality rate than other CF mouse strains (Davidson and Wolfe 2001), and the FVB-129 mice can be bred reliably. To understand the microvascular consequences of the ΔF508-CFTR mutation, we isolated and cannulated the proximal PCAs from the brains of the mutant mice and the WT mice. The passive diameters at 45 mmHg were not significantly different between the two genotypes (dia$_{max}$ = 173±3.40 μm versus 174±5.15 μm for ΔF508 and WT respectively, p>0.05 unpaired t-test), nor were the baseline tone values (tone = 3.20±0.791% versus 2.80±0.630% for ΔF508 and WT respectively, p>0.05 unpaired t-test). The PCAs from both genotypes exhibited robust vasomotor responses to PE. We normalized the responses to the baseline tone, and of the five concentrations of PE, the PCAs from ΔF508-CFTR mice had significantly stronger responses than the WT PCAs to treatments of 10$^{-7}$ mol/L PE (tone=
15.9±3.77% versus 5.63±2.56% for ΔF508 and WT respectively, p<0.05), and 10^{-6} mol/L PE (tone = 37.52±1.62% versus 29.0±2.35% for ΔF508 and WT respectively, p<0.05, n_{ΔF508}=8, n_{WT}=6 arteries, Figure 4-3A). This data suggests that the ΔF508 mutation affects contractility of the PCAs. To assess whether the depolarization mechanism was altered, constriction to KCl (60 mM) was also assessed in the same arteries. KCl promotes vasoconstriction through depolarization, and stimulates Ca^{2+} release from intracellular stores. We found the ΔF508-CFTR mutation does not affect vasoconstriction of PCAs to KCl, relative WT PCAs (tone = 46.2±2.47% versus 38.1±3.67% for ΔF508 and WT PCAs respectively, p>0.05, Figure 4-3B).

Proper CFTR expression contributes to regulating the MR of PCAs (Meissner et al. 2012), and we therefore tested whether the ΔF508-CFTR PCAs display augmented myogenic responsiveness. We assessed the MR in the same arteries from above, using a protocol where the transmural pressure was incrementally increased, in 20 mmHg steps (representative tracing shown in Supplemental Figure 5). We found the MR was stronger for the ΔF508-CFTR PCAs, than the WT PCAs at the 80 mmHg pressure step (myogenic tone = 9.23±1.64% versus 4.47±1.81%), and the 100 mmHg pressure step (myogenic tone = 11.2±1.45% versus 4.70±1.57% for ΔF508 and WT PCAs respectively, p<0.05, Figure 4-3C). The passive diameters at the same pressure steps were not significantly different between the two genotypes (p>0.05, Supplemental Figures. Figure 4-S2). Since S1P is a critical regulator of the MR (see Section 1.3.), the stronger MR observed suggests that the regulation of endogenous S1P is abnormal in the PCAs from ΔF508-CFTR mice. We also assessed exogenous S1P-signaling, and found that the dose-dependent S1P vasoconstriction was significantly greater for ΔF508-CFTR PCAs than WT PCAs at 10^{-8} mol/L (tone = 17.7±2.39% versus 6.53±2.33%), 10^{-7} mol/L (tone = 28.8±2.53% versus 14.8±2.21%), and 10^{-6} mol/L S1P (tone = 35.3±2.56% versus
25.4±2.40% for ΔF508 and WT PCAs respectively, p<0.05, Figure 4-3D). Area under the curve analysis supports that overall responsiveness to S1P is greater in the ΔF508 PCAs (area under the curve = 78.5±8.55 %tone*log mol/L versus 38.9±7.88 %tone*log mol/L for ΔF508 and WT PCAs respectively, p<0.05).

We have previously shown that abnormalities in the cerebral MR can contribute to reductions in the CBF of mice (Yang et al. 2012). Based upon the results of our present study we proposed that the ΔF508-CFTR mice would display decreased CBF, relative to WT mice, because of the enhancement in their PCAs’ myogenic tone. We assessed CBF with the FAIR-MRI approach, and found that the ΔF508-CFTR mice had significant reductions in their CBF, relative to the WT mice (Figure 4-4A,B). The reductions were localized in the forebrain regions of the cortex (122.5±11.06 mL/100g*min versus 183.8±11.48 mL/100g*min), subcortex (77.60±8.728 mL/100g*min versus 129.1±8.036 mL/100g*min) and whole brain slices (94.62±8.486 mL/100g*min versus 147.3±8.628 mL/100g*min for ΔF508 and WT mice respectively, p<0.05, n_{ΔF508}=10, n_{WT}=11 mice). However, changes in CBF can be caused by abnormalities in the systemic circulation, such as mean arterial pressure (MAP), cardiac output (CO), and total peripheral resistance (TPR), and therefore we next assessed the systemic hemodynamics in a separate cohort of mice. There were no significant differences in the MAP (75.8±3.06 mmHg versus 85.8±3.81 mmHg), the CO (18.5±0.800 mL/min versus 22.0±1.62 mL/min), and the TPR (4.18±0.370 mmHg*min/mL versus 3.97±0.282 mmHg*min/mL for ΔF508 and WT mice respectively, p>0.05, n_{all}=5 mice, Figure 4-5). The absence of differences in these three parameters suggests the decreased CBF found in the mutant mice is not caused by abnormal cardiac function, or by abnormalities in the peripheral circulation. Furthermore, in the same mice we also found no significant differences in the heart rate (540±21.1 bpm versus
544±31.8 bpm), systolic blood pressure (95.6±4.47 mmHg versus 107±4.13 mmHg), diastolic blood pressure (66.0±2.43 mmHg versus 75.2±3.79 mmHg), and mean ejection fraction (65.8±2.15 % versus 68.4±1.37% for ΔF508-CFTR and WT respectively, p>0.05). The stroke volume was significantly lower in the mutants than the WT (36.0±1.44 µL versus 44.0±2.28 µL), and the body weight as well (24.7±0.750 g versus 31.6±2.26 g for ΔF508-CFTR and WT mice respectively, p<0.05). However, normalization of the stroke volume to body weight demonstrated the values were not significantly different (1.46±0.0383 µL/g versus 1.41±0.0720 µL/g for ΔF508-CFTR and WT mice respectively, p>0.05).

Our laboratory has preliminary quantitative PCR (qPCR) data that shows the WT-CFTR mRNA expression to be significantly higher in cerebral arteries than in skeletal muscle cremaster arteries, from WT C57/Bl6 mice (mean ratio of CFTR mRNA to glucose-6-phosphodehydrogenase mRNA = 5.62±1.19 versus 1.00±0.404 for cerebral and cremaster respectively, p<0.05 unpaired t-test, n_all=6, Supplemental Figure 6A). Since skeletal muscle resistance arteries are an important regulator of TPR (Henrion 2005), the low CFTR expression in skeletal tissue from control WT mice may explain the lack of systemic effects of the genetic F508 deletion, and TPR could be regulated in a CFTR-independent manner.
Figure 4-3. The proximal posterior cerebral arteries (PCAs) from FVB-129-ΔF508-CFTR mice show augmented myogenic responsiveness and vasomotor responses to sphingosine-1-phosphate (S1P).

Relative to WT-CFTR PCAs (n=6 arteries, dia_max=173±5.98 µm), the PCAs from ΔF508-CFTR mice (n=8 arteries, dia_max=174±3.71 µm) display (A) enhanced vasomotor responses to phenylephrine, (C) enhanced myogenic vasoconstriction, and (D) greater vasomotor responses to exogenous S1P. *denotes p<0.05 following two-way ANOVA analysis. (B) The constriction to 60 mmol/L KCl was not significantly different (p>0.05 unpaired t-test).
Figure 4.4. FVB-129-ΔF508-CFTR mice have reduced cerebral blood flow (CBF) relative to FVB-129-WT-CFTR mice.

(A) FAIR-Magnetic Resonance Imaging of the fore-, mid-, and hind-brain was used to assess CBF in predefined local (cortical and subcortical brain) and global (sub-hemispheric) regions of interest. Shown are representative perfusion maps from WT-CFTR and ΔF508-CFTR.
The CBF was significantly less in the forebrain regions of the ΔF508-CFTR mice (n=10) relative to their WT littermates (n=11). *denotes p<0.05 following one-way ANOVA and Bonferroni's selected comparisons post-hoc test.
Figure 4-5. The systemic hemodynamic parameters are not significantly different in FVB-129-ΔF508-CFTR mice relative to FVB-129-WT-CFTR mice.
Relative to WT-CFTR mice (n=5), the mice expressing the ΔF508-CFTR genetic mutation (n=5) display no significant differences (p>0.05, unpaired t-test) in their (A) cardiac output, (B) mean arterial pressure, (C) total peripheral resistance, and (D) normalized stroke volume.
4.4.4 Antagonism of the S1P₁R decreases the myogenic responsiveness of ΔF508-CFTR PCAs

In the present study, we observed that inhibition of the S1P₁R, with VPC 23019 treatment, enhanced the iodide efflux of BHK-ΔF508-CFTR cells, suggesting endogenous S1P-signaling is elevated as a consequence of the mutation. Indeed, we found that the ΔF508-CFTR PCAs had greater myogenic tone than WT PCAs, and this was indicative of enhanced endogenous S1P-signaling. Thus, we next assessed the effect of receptor antagonism in the PCAs from ΔF508-CFTR expressing mice. qPCR with cerebral arteries showed that the mRNA expression of the S1P₁R was approximately 20-fold greater than either the S1P₂R or the S1P₃R (mean ratio of receptor to GAPDH mRNA = 19.3±8.90 for S1P₁R, 1.00±0.487 for S1P₂R, and 1.32±0.599 for S1P₃R, p<0.05 one-way ANOVA and Friedman’s multiple comparison post-hoc test, n_all=7 tissue samples, Supplemental Figure 6B). The expression ratio suggests the S1P₁R is the best candidate for regulating endogenous S1P-signaling, and we predicted the S1P₁R contributes to the augmented myogenic responsiveness of the ΔF508-CFTR PCAs. We assessed the myogenic responsiveness of ΔF508-CFTR PCAs with the multi-step MR protocol, before and after acute in-vitro VPC-23019 treatment (30 minutes, 10 µmol/L). We found VPC 23019 treatment significantly reduced the MR at the 60 mmHg (tone = 16.6±3.59% vs. 9.88±2.44% for before and after VPC-treatment respectively), and the 80 mmHg pressure steps (tone = 17.4±3.34% vs. 7.86±1.68% for before and after VPC-treatment respectively, p<0.05, dia_max = 185±6.06 µm, n=5 arteries, Figure 4-6A). In the same arteries, acute VPC 23019 treatment did not significantly affect the vasomotor response to PE (p>0.05 at all PE concentrations, Figure 4-6B), and this suggests that the receptor antagonism did not affect general contractility of the PCAs. Thus, the data attributes the effect of endogenous S1P-signaling to the S1P₁R, for the ΔF508-CFTR PCAs.
We also assessed the role of the S1P₁R in exogenous S1P-signaling for ΔF508-CFTR PCAs. Pilot-work showed that the PCAs from ΔF508-CFTR mice had time-dependent, enhanced vasoconstriction to exogenous S1P (representative tracing shown in Supplemental Figure 7). This result prohibited us from conducting the dose-dependent S1P vasoconstriction responses, before and after VPC 23019, in the same artery. Instead, we pre-treated one artery with VPC 23019, and paired it with a contemporaneous time control artery from the same mouse. The passive diameters at 45 mmHg were not significantly different (dia\textsubscript{max} = 189±12.1 µm versus 174±4.57 µm for VPC 23019 and time control arteries respectively, p>0.05 unpaired t-test). We found that acute VPC 23019 treatment significantly decreased the vasoconstriction to 10⁻⁸ mol/L S1P (tone = 12.9±3.42% versus 28.8±2.16%) and 10⁻⁷ mol/L S1P (20.3±3.38% versus 35.6±5.03% for VPC 23019 and time control respectively, p<0.05, n\textsubscript{VPC} = 5, n\textsubscript{TIME} = 4 arteries, Figure 4-6C). Area under the curve analysis for the S1P vasomotor responses suggests that antagonism of the S1P₁R decreases the overall constriction responsiveness (area under the curve = 35.8±9.08 %tone*log mol/L versus 75.0±6.61 %tone*log mol/L for VPC 23019 and Time Control PCAs respectively, p<0.05). Additionally, the pre- and post-vasomotor responses to a single-dose of PE (10⁻⁶ mol/L) were not significantly different (tone = 24.2±1.25% versus 23.6±2.14% for pre and post-VPC 23019 treatments respectively, p>0.05, Figure 4-6D). This supports the specificity of the VPC 23019 treatment on exogenous S1P-signaling, rather than a general loss in contractility.
Figure 4-6. Antagonism of the S1P<sub>1</sub> receptor (S1P<sub>1</sub>R) subtype attenuates the enhanced myogenic responsiveness of ΔF508-CFTR posterior cerebral arteries (PCAs) and the vasomotor responses to exogenous sphingosine-1-phosphate (S1P).

(A) Acute treatment with the S1P<sub>1/3</sub>R-antagonist VPC 23019 (10 µmol/L, 30 minutes) significantly decreased the myogenic responsiveness for the PCAs from ΔF508-CFTR mice and did not affect (B) the vasomotor responses to phenylephrine (n=5 arteries, dia<sub>max</sub>=168±5.68 µm).

(C) In a separate set of ΔF508-CFTR PCAs, the acute treatment with VPC 23019 significantly decreased the vasoconstriction to exogenous S1P (n=5 arteries, dia<sub>max</sub>=189±12.1µm) relative to time control arteries (n=4, dia<sub>max</sub>=177±4.39 µm), and did not affect the (D) the vasomotor response to a single dose of phenylephrine (10<sup>-5</sup> mol/L). *denotes p<0.05 following two-way ANOVA repeated measures for the MR, and standard two-way ANOVA for S1P vasomotor responses.
4.5 Discussion

4.5.1 The S1P$_1$R-AMPK signaling pathway contributes the defect in ΔF508-CFTR channel function

The iodide efflux assay was used to assess the channel function of CFTR, which is difficult to do in isolated vessels. DMSO treated BHK-ΔF508-CFTR cells had very little FSK-stimulated iodide efflux, and inhibiting the S1P$_1$R-AMPK signaling axis (e.g., chronic VPC 23019 treatment, AMPK siRNA transfection) enhanced the iodide efflux of these cells. Additionally, low-temperature rescued BHK-ΔF508-CFTR cells had greater iodide efflux relative to cells grown at 37°C, and inhibition of AMPK (e.g., Compound C, siRNA transfection) had an additive effect on the iodide efflux at 27°C. Low-temperature incubation is thought to provide a kinetically favourable folding environment (Farinha et al. 2013, Qu and Thomas 1996), and reduce the proteasome-dependent degradation of ΔF508-CFTR (Gomes-Alves et al. 2009). The additive effect observed in our study suggests that the mechanism of action is different for AMPK inhibition, than it is for low-temperature incubation. Furthermore, we used the membrane fractionation approach to demonstrate that Compound C treatment at 37°C increased the expression of ΔF508-CFTR in the membrane fraction (i.e., plasma membrane). This suggests that AMPK-dependent phosphorylation of ΔF508-CFTR may contribute to locking the protein in the intracellular environment. We also found that acute treatment with S1P decreased the iodide efflux in the temperature-rescued cells, and this demonstrated that plasma membrane-expressed ΔF508-CFTR is sensitive to negative modulation by S1P, like the WT-CFTR protein is (see Chapter 3). The data suggests that S1P-signaling exacerbates the channel function defect of the ΔF508 mutation. We also assessed the S1P uptake function of BHK-ΔF508-CFTR cells with the FACS technique, and found the inhibition of AMPK with
Compound C enhanced the uptake of S1P-FITC. This data suggests that the channel and uptake functions of the mutant are enhanced by inhibition of the S1P-signaling pathway.

The iodide efflux assays utilized chronic, 24 hours and 48 hours treatments with S1P1R-AMPK inhibitors. Typically, effects on protein conformation and processing require chronic intervention, and therefore it is possible that inhibition of S1P1R-AMPK-signaling with VPC23019, Compound C, or AMPK siRNA transfection indirectly enhances channel function of ΔF508-CFTR by promoting processing and/or maturation of the protein. In a 2012 study, Trzinska-Daneluti and colleagues demonstrated the pharmacologic inhibition of ERK1/2, which is a kinase that regulates the S1P-signaling pathway (see Section 1.4.1), partially corrects the processing defect of ΔF508-CFTR stably expressed in epithelial cells, and channel function was enhanced (Trzcinska-Daneluti et al. 2012). This data provides support for our view that processing of ΔF508-CFTR was enhanced through inhibition of the S1P1R-AMPK-signaling pathway.

It is also possible that ΔF508-CFTR was released from sub-membrane compartments (e.g., endosomes), as suggested by our results with acute treatment of Compound C and the membrane fractionation of BHK-ΔF508-CFTR cells. After 60 minutes of treatment, we observed that Compound C increased expression of the mutant protein in the plasma membrane fraction, and a concurrent decrease of the expression in the intracellular fraction. The intracellular ΔF508-CFTR protein has been shown to undergo degradation to a greater degree than the WT protein (Sharma et al. 2004, Sharma et al. 2001). In the present study, inhibition of AMPK may stabilize intracellular ΔF508-CFTR, diverting it from degradation, and therefore the protein is trafficked to the plasma membrane. In support of this, Sharma and colleagues demonstrated that a conformational defective CFTR, which lacks the last 70 amino acids (Δ70-CFTR) of the C-terminus, has impaired recycling relative to the WT protein, and undergoes
ubiquitination more readily than the WT protein (Sharma et al. 2004). Similar results were observed with low-temperature rescued ΔF508-CFTR protein (rΔF508-CFTR). The findings demonstrated that instabilities in the CFTR protein divert the cytoplasmic protein from recycling to degradation, and this could be where inhibition of AMPK has its effect. Of course, the membrane fractionation technique is not without its limitations, and further experiments (e.g., fluorescence microscopy, cell-surface biotinylation) are required to assess this finding in greater detail.

Since the uptake of exogenous S1P is reduced in cells expressing ΔF508-CFTR, the bioavailability of S1P may benefit because of the decreased degradation. Under endogenous conditions this may create a positive feedback loop for the activation of AMPK. Therefore, inhibition of S1P₁R or AMPK disrupts the feedback mechanism, and the membrane expression and function of ΔF508-CFTR is enhanced.

4.5.2 ΔF508-CFTR PCAs may have abnormal endogenous and exogenous S1P signaling

In the present study, we found that the proximal PCAs from ΔF508-CFTR mice have augmented myogenic responsiveness compared to PCAs from WT mice. We also demonstrated that antagonism of the S1P₁R with VPC 23019 attenuates the enhanced myogenic responsiveness of ΔF508-CFTR PCAs. These results suggest that the ΔF508-CFTR mice have enhanced endogenous S1P₁R -S1P-signaling. Coupled with our group’s previous finding with CFTR KO PCAs (Meissner et al. 2012), the data presented also verified the importance of CFTR in regulating the effects of endogenous S1P-signaling. In addition to the augmented myogenic tone, the ΔF508-CFTR PCAs also displayed increased vasoconstriction to exogenous S1P. The enhanced vasoconstriction observed suggests that degradation of S1P is impaired because ΔF508-CFTR cannot uptake it. Treatment with VPC 23019 decreased vasoconstriction, and did
not affect pre and post vasoconstriction to PE, and this indicates that the treatment does not affect overall contractility of the arteries. A caveat is that VPC 23019 also antagonizes the S1P3R (Salomone et al. 2008, Davis et al. 2005), however we detected 20-fold higher mRNA concentration of S1P1R than S1P3R in murine cerebral tissue, and thus we propose that the S1P1R is the best candidate.

In the present study, we used the FVB-129-ΔF508-CFTR mouse strain because it is more viable compared to other ΔF508-CFTR strains (Wilke et al. 2011, Guilbault et al. 2007). Tissue from the mutant FVB mice has been demonstrated to express a residual amount of the mature fully glycosylated Band C form, though the expression has only been documented in the oviducts (French et al. 1996), and intestinal epithelium (Wilke et al. 2012). The residual expression may explain the residual channel activity, observed with Ussing chamber studies, in gastrointestinal tissues from the mutant mice (van Doornick et al. 1995). It also lends credence to our acute in-bath treatments of the isolated PCAs, and supports our theory that inhibition of the S1P1R-AMPK-signaling pathway may affect trafficking of the ΔF508-CFTR from sub-membrane compartments to the plasma membrane. Alternatively, inhibition of the signaling pathway may stabilize protein that is already present in the membrane.

4.5.3 Clinical consequences

Real-time PCR of human brain tissues has demonstrated that CFTR is expressed in the central neurons, but not in glial cells, of several regions of the brain including the cerebellum, hippocampus, frontal lobe, parietal lobe, and occipital lobe (Guo et al. 2009). However, the expression of CFTR in the cerebral tissue examined in Guo et al’s study was approximately 100-fold less than the expression of CFTR in the epithelial cells from the small intestine (Guo et al. 2009). The authors suggested that abnormal neural expression of CFTR could explain the abnormal neurological function (e.g., seizures), observed in some CF patients after lung
transplantation as documented in patient review studies (Goldstein et al. 1998, Vaughn et al. 1996). In a 2000 publication, Goldstein et al. reviewed records for 21 CF patients after the patients had undergone lung transplantation surgery, and found that over half had CNS dysfunctions such as diagnosed seizures and severe headaches (Goldstein et al. 2000), and this supported the earlier studies. Goldstein and colleagues excluded nutritional status and hypertension as contributing factors because there were no significant differences in these parameters between the patients with and without CNS dysfunctions. The seizures could be related to the loss in electrolyte regulation caused by CFTR dysfunction (Bradbury et al. 1994), but electrolyte balance was not a contributing factor in the Goldstein et al. 2000 study. The role of CFTR in the nervous system remains unclear, though CFTR has been shown to regulate the ATP-dependent flux of glutathione (GSH) (Kogan et al. 2003), which is an antioxidant that protects brain cells (e.g., astrocytes) from reactive oxygen species (Dringen and Hirrlinger 2003). CFTR may import GSH to offset the accumulation of reactive oxygen species. Additionally, CFTR contributes to neuropeptide transport because of its association with clathrin-coated vesicles (Bradbury et al. 1994). In CF patients, accumulation of amyloid precursor protein could also contribute to the dysfunction in the CNS (Cochran et al. 1991). The frequent seizures observed in some CF patients (Castilla-Guerra et al. 2006) could be explained by the amyloid accumulation, and CFTR could be involved in the export of amyloids to prevent their accumulation and detrimental effects on brain tissue.

In the present study, we found that genetic deletion of the F508 amino acid residue contributes to decreasing the CBF in mice. However, the mutation does not affect systemic hemodynamic parameters, such as CO and TPR. Thus, ΔF508-CFTR mice display a cerebral microvascular phenotype that is independent of effects on the heart. There is no documented evidence in the literature on whether the ΔF508-CFTR mutation affects the brain function or
behaviour of the FVB-129 mice, and these parameters were not assessed in the current study. The CBF values for the mutant mice were approximately 35-40% less than the values from the WT mice, across all the brain regions assessed (e.g., cortex, subcortex, whole brain). However, these values are above the approximate 60% CBF reduction that is the threshold at which impairment of neuronal function occurs, as documented or described in several mammalian CBF studies (e.g., monkeys, rodents) (Baron 2001, Marchal et al. 1999, Marchal et al. 1996, Matsumoto et al. 1975). Thus, we postulate that the ΔF508-CFTR mice are unlikely to have abnormalities in their brain function or in their behavior, relative to the WT-CFTR mice, at least at 12 weeks of age. Nonetheless, the effects on cerebral microvascular reactivity and cerebral autoregulation are intriguing because the ΔF508-CFTR mutation is the most common CF-disease causing mutation in the human patient population. The abnormal microvascular reactivity observed in the ΔF508-CFTR mice could help explain the CNS dysfunction observed in some CF patients, already described. Neurons are highly metabolic and rely heavily on oxygen (Bélanger et al. 2011), and the blood in the brain supplies oxygen to the neurons via the vascular tissue capillary beds. Decreased CBF via enhanced CVR may affect the amount of oxygen that is received by neurons, and over time it may lead to neural damage, and if extensive enough brain function could be affected. An important consideration with our studies is we used mice younger than 12 weeks, and because there is documented evidence in mice for an age-related reduction in CBF (Katsimpardi et al. 2014), it is possible that as the ΔF508-CFTR mice age, neuronal complications may occur. Humans have also been demonstrated to display an age-related reduction in CBF from 60 years onwards (Stoquart-Elsankari et al. 2007), but the median age of survival for CF patients in Canada is 48 years (Canadian Cystic Fibrosis Foundation). Assuming our mouse data can be correlated to human CF patients, a CF patient will have a CBF value that is closer to the threshold for neuronal impairment than a WT patient. The CF patient
may be at greater risk for neuronal impairment as they age. Therefore, abnormalities in the cerebral myogenic reactivity could contribute to a greater susceptibility for age-related reductions in CBF, and also neuronal impairment, in human CF patients.

4.6 Conclusion

The CFTR protein is an important regulator of S1P bioavailability, and the S1P-signaling pathway is negative modulator of CFTR’s channel function. In the present study, we demonstrate that S1P-signaling through the S1P$_R$ and AMPK exacerbates the expression and function defects of the ΔF508-CFTR mutant. Furthermore, the signaling pathway is responsible for the enhanced microvascular reactivity of PCAs from ΔF508-CFTR mice. Targeting abnormal expression and/or function of CFTR directly, could restore the regulation of S1P-signaling, and could also normalize the cerebral dysfunction.
Chapter 5

CFTR IS A THERAPEUTIC TARGET FOR THE CEREBROVASCULAR EFFECTS OF HF IN MICE

Note: This chapter includes data presented in a submitted manuscript - Anja Meissner, PhD*, Firhan A. Malik, MSc*, Jefffrey T. Kroetsch, MSc, Meghan Sauvé, MSc, Jessica Fares, Danny D. Dinh, Abdul Momen, MD, Mansoor Husain, MD, Christine E Bear, PhD, Darcy Lidington PhD, and Steffen-Sebastian Bolz, MD, PhD. (2014), submitted to Circulation. *denotes these authors contributed equally.

5.1 Acknowledgements

The work presented in this chapter was an equal effort between our laboratory’s former Post Doctoral fellow Dr. Anja Meissner and myself. Specifically, Dr. Meissner isolated and cultured the murine VSMCs from mesenteric resistance arteries, performed the Western blotting and analysis of the effects of TNFα and C18 on the expression of CFTR in VSMCs, the uptake of S1P-FITC, data analysis, and she assisted in the same experiments with the BHK-WT-CFTR cells. Dr. Meissner also performed the Western blotting for the cerebral tissue of the HF and sham in-vivo C18 treatments. Technologist Ms. Dionne White of the University of Toronto Flow Cytometer Service provided technical assistance with the uptake assay. Graduate student Ms. Meghan Sauvé isolated and cannulated the PCAs from HF and sham mice for the in-vitro and in-vivo C18 experiments, assisted in the functional assessments of the arteries, and data analysis. Further assistance in the isolation and cannulation of PCAs from HF mice for the in-vivo C18 experiments, was provided by former Technologist Dr. Jingli Yang. Graduate student Mr. Jeffrey Kroetsch isolated and cannulated the PCAs from the FVB-129 mice, assisted in the
functional assessments of the arteries and the analysis of the data. Senior Research Associate Dr. Darcy Lidington assisted in all data analysis and preparation of figures. Technologist Ms. Catherine Luk of Dr. Christine Bear’s laboratory assisted in care, handling, and genotyping of the FVB-129 mice. Research Associate Dr. Abdul Momem from Dr. Mansoor Husain’s laboratory (Toronto General Hospital) performed the LAD surgery to generate HF mice and sham mice. Researcher Dr. Warren Foltz of the STTARR Innovation Centre (University Health Network) performed the MRI imaging of the HF and sham mice for in-vivo C18 treatments, and analyzed the data. Post Doctoral fellow Dr. Roozbeh Sobbi of Dr. Peter Backx’s laboratory (University of Toronto) performed the echocardiography of the mice, and assisted in data analysis. Technologist Dr. Hangjun Zhang of Dr. Scott Heximer’s laboratory (University of Toronto) performed the terminal blood pressure measurements of the mice, and assisted in data analysis. Members of Dr. Bear’s laboratory provided technical expertise with the use of the C18 corrector compound.

5.2 Overview and rationale

Heart failure (HF) is caused by the heart’s inability to provide sufficient blood in order to meet the periphery’s metabolic demands (see Section 1.5.3). The primary physiological characteristic of HF is the dramatic reduction in CO, but the MAP is only marginally reduced because of compensation by increased TPR. The augmentation of the myogenic responsiveness of resistance arteries is the primary mechanism underlying enhanced TPR (Meissner et al. 2012, Yang et al. 2012, Hoefer et al. 2010). Initially, the role of the augmented MR is to maintain CO and systemic blood pressure, but as HF progresses the continued augmentation leads to detrimental effects, and this may explain the diverse clinical symptoms of the disease, such as reduced CBF (see Section 1.5.3). Our laboratory has previously demonstrated that CFTR’s
vascular mRNA and protein expression (e.g., isolated PCAs) is down-regulated in HF mice, and the myogenic tone of their proximal PCAs is enhanced in an S1P-dependent manner (Meissner et al. 2012).

We have previously established TNFα as an upstream regulator of the HF-mediated effects on CFTR’s mRNA and protein expression (Meissner et al. 2012). Consequently, we found inhibition of TNFα with Etanercept treatment in mice restored CFTR’s protein expression, and normalized the myogenic responsiveness of HF PCAs (Meissner et al. 2012), and Etanercept also normalized the CBF (Yang et al. 2012). Etanercept (also known as Enbrel®) specifically inhibits TNFα by sequestering it, and prevents binding of TNFα to its receptors (e.g., TNFR1 and 2) (Harraoui and Bykerk 2007). Unfortunately, Etanercept treatment in human HF patients has shown no beneficial effect on mortality or length of hospital stay (e.g., the RENEWAL study by Mann et al. 2004), which were the primary end-points of the 24-week studies. The studies were prematurely stopped because of a trend towards increased risk of death (Coletta et al. 2002), and enthusiasm was dampened for the application of Etanercept in HF patients. One common symptom observed in HF patients, in the placebo group, was dizziness (Mann et al. 2004), a symptom associated with reductions in regional CBF (Wang et al. 2003). Etanercept treatment had no significant effect on the number of patients that reported dizziness as a symptom, and mortality associated with treatment may be independent of adverse effects on the brain. Etanercept treatment significantly increased the number of other adverse events, such as bronchitis and constipation. However, causes of death were not assessed in the clinical trials and the increased mortality cannot be fully explained. Another clinical study that utilized short-term treatment with a chimeric antibody against TNFα, infliximab, also found no beneficial effect on the clinical status of the HF condition in patients (ATTACH trial by Chung et al. 2003). In this 28-week study, significant increases in mortality and in hospitalization were observed for HF
patients given infliximab treatment (Mann et al. 2004, Chung et al. 2003). TNFα is an important component of the immune response (Pasparakis et al. 1996, also see reviews by Croft et al. 2013, Aggarwal et al. 2012), and host defenses against infection (Mehrad et al. 1999, Skerrett et al. 1997, Williams et al. 1990). Therefore, anti-TNFα treatments can have severe side effects, such as suppression of the immune system (Aggarwal et al. 2012). Alternatively, TNFα-signaling may be a regulator of microvascular function, as suggested by Yang et al. (2012) and unpublished data from our laboratory, and chronic inhibition or antagonism of TNFα may worsen the HF disease in patients (Mann et al. 2004).

CFTR’s critical role in the CF diseases has spearheaded research into novel therapeutics. High throughput screens have identified small-molecule organic compounds that have beneficial effects on protein expression and/or processing (i.e., correctors), or compounds that enhance function (i.e., potentiators) of CFTR’s mutants (Van Goor et al. 2011, Van Goor et al. 2009, Van Goor et al. 2006). Most of the compounds have been tested in cell-culture models, however some of the compounds have entered human clinical trials (Davies et al. 2013, Clancy et al. 2012, Flume et al. 2012, Ramsey et al. 2011, also see review by Cai et al. 2011). We hypothesize treatment of HF mice with a corrector compound will normalize the MR of PCAs, and the CBF for HF mice, in a CFTR-dependent manner. In the present study, we used C18 (also known as VRT-534 or CF-106951), a small-molecule corrector-compound recently patented by Vertex Pharmaceuticals Inc. C18 treatment in cell-culture has been shown to have a positive effect on the processing, maturation, and trafficking of ΔF508-CFTR (Holleran et al. 2013, Okiyoneda et al. 2013, Snodgrass et al. 2013, Wong et al. 2012), and C18 may also function as a potentiator (Eckford et al. 2014, Lui and Sheppard 2011-Abstract), thus the dual roles make it an ideal compound to use. We show that C18 treatment partially restores the down-regulated protein expression of CFTR, in a simulated model of HF (i.e., chronic TNFα
treatment in murine VSMCs), and fully restores the decreased FSK-stimulated iodide efflux and S1P-FITC uptake. We then assessed cerebral microvascular reactivity of HF mice, and demonstrated that in-vivo C18 treatment normalizes the CBF relative to saline-injection. Finally, we use isolated HF PCAs and show normalization of the myogenic responsiveness after in-vitro or in-vivo treatments with C18.

5.3 Methods

5.3.1 Animals
See Section 2.1.

5.3.2 Antibodies and reagents
See Section 2.2 and 2.3.

5.3.3 Cell culture
See Section 2.4.

5.3.4 Conventional iodide efflux assay
See Section 2.8.1.

5.3.5 Functional assessment of isolated and cannulated PCAs
See Section 2.10.

5.3.6 S1P-FITC uptake assay
See Section 2.11.

5.3.7 Ligation of the left anterior descending (LAD) coronary artery to induce HF

The HF mouse model in this study utilized experimental induction of myocardial infraction (MI), by surgical ligation of the left anterior descending (LAD) coronary artery, and this procedure was described previously by our laboratory (Meissner et al. 2012, Yang et al.)
2012, Hoefer et al. 2010). Briefly, C57/Bl6 mice were anaesthetized with isoflurane (1.5% v/v), intubated with a 20-gauge angiocatheter, and ventilated with room air. The thorax and pericardium were opened under sterile conditions, and the LAD artery was permanently ligated with a 7-0 silk suture (Deknatel). In Sham-operated controls, the thorax and pericardium were opened, but the LAD artery was not ligated. The chest was then closed, and the mice were extubated upon spontaneous respiration. Proximal PCAs were isolated six-to-eight weeks post-MI.

5.3.8 MRI imaging, echocardiography, and terminal blood pressure measurements.

See Sections 2.12 to 2.14

5.3.9 Statistics

See Section 2.15.

5.4 Results

5.4.1 C18 treatment enhances the expression of CFTR in murine VSMCs

First, we assessed whether C18 treatment alone affects the endogenous expression of CFTR in cultured mesenteric VSMCs from WT mice (C57/Bl6). We found C18 treatment (6 µmol/L) significantly increased the expression of CFTR protein, which was expressed as the ratio of the densitometry of CFTR protein to total protein, starting at three hours of treatment. After three hours of treatment with C18 the expression of CFTR was increased by approximately three-fold relative to untreated (mean ratio of CFTR to total protein expression = 0.574±0.0452 versus 0.203±0.0334 for three-hours and untreated samples respectively, p<0.05, n_all=4 cell culture dishes, Figure 5-1A). Then at nine hours of C18 treatment, the ratio was significantly increased by eight-fold (1.60±0.139, p<0.05, n=4 cell culture dishes), relative to the untreated
samples. Enhanced processing of the WT protein, or a proteostatic effect on the stability of the protein, by C18 treatment could cause the increased expression of endogenous CFTR. We also found that C18 treatment significantly increased the ratio of CFTR protein to total protein expression in the stably transfected BHK-WT-CFTR cells, from three hours to nine hours of treatment. These cells express the human epithelial isoform of CFTR. After nine hours of treatment the ratio was increased by approximately two-fold (mean ratio = 1.54±0.0779 versus 0.889±0.0466 for nine hours treatment and untreated respectively, p<0.05, n_all=4 cell culture dishes, Supplemental Figure 7A). Overall, the effect of C18 treatment on the ratio of CFTR to total protein expression was less drastic in BHK-WT-CFTR, in contrast to the murine VSMCs. The difference in magnitude is likely due to the artificial promoter used in the BHK-WT-CFTR cell-line that allow for stable expression of the protein.

5.4.2 C18 treatment partially restores the TNFα-dependent down-regulation of CFTR’s protein expression, and fully restores CFTR’s function under simulated HF conditions.

Our laboratory has previously shown that chronic TNFα treatment (e.g., 24-48 hours) in murine VSMCs significantly decreased the CFTR protein expression, and therefore simulates the HF condition in cells (Meissner et al. 2012). In the present study, we used the HF simulation model to assess whether C18 reverses the TNFα-dependent reduction in CFTR’s protein expression. Chronic TNFα treatment (24 hours, 10 ng/mL) significantly decreased the expression of CFTR by two-fold relative to control (mean ratio = 0.948±0.0361 versus 1.875±0.0325 for TNFα treatment and control respectively, p<0.05, n_{TNFα}=4, n_{con}=3 cell culture dishes, Figure 5-1B), and this confirmed our previously published results. C18 (24 hours, 6 µmol/L) co-treatment with TNFα significantly increased the expression of CFTR protein relative to TNFα alone (mean ratio for TNFα and C18 co-treatment = 1.38±0.0577, p<0.05, n=4 cell-
culture dishes), but the expression was significantly lower than control. We also assessed a treatment protocol that utilized the subsequent addition of C18 after TNFα-mediated down-regulation of CFTR (i.e., 24 hours TNFα, followed by 24 hours of TNFα and C18). This protocol had a similar effect on CFTR expression as the co-treatment protocol (ratio for subsequent C18 = 1.32±0.0302, p<0.05 relative to TNFα alone, p<0.05 relative to control, n=4 cell culture dishes). Thus, in-vitro chronic C18 treatment partially restores the TNFα-dependent down-regulation in CFTR’s protein expression.

Decreased expression of CFTR is related to decreased function, such as the uptake of S1P-FITC (Meissner et al. 2012), therefore we assessed whether C18 could restore VSMC-WT-CFTR function. We used the FACS technique, and found chronic TNFα treatment significantly decreased the uptake of S1P-FITC (1 µmol/L) in murine VSMCs by approximately two-fold, relative to control (mean fluorescence intensity at 515 nm= 152±6.71 versus 279±4.90 for TNFα and control respectively, p<0.05, n_{TNF}=6, n_{con}=5 cell culture dishes, Figure 5-1C). As predicted, the subsequent addition of C18, for another 24 hours, restored uptake of S1P-FITC (312±14.6, p<0.05 relative to TNFα alone, n=6 cell culture dishes).

Next we assessed the channel function of CFTR in VSMCs, with the conventional iodide efflux assay. TNFα treatment (48 hours, 10 ng/mL) significantly decreased the channel function by six-fold relative to the DMSO (48 hours, 0.1% v/v) treatment (mean iodide in the supernatant = 1.48±0.452 µmol/L versus 8.73±0.856 µmol/L for the TNFα and DMSO treatments respectively, p<0.05, n_{TNF}=6, n_{con}=7 coverslips). These findings demonstrated that the murine vascular CFTR is an anion channel, and TNFα down-regulates channel function. Chronic treatment with C18 (24 hours) has been shown to rescue the channel function of ΔF508-CFTR in HEK-293 cells (Holleran et al. 2013), and in cultured CF human bronchial cells (Snodgrass et
In the present study, we found that C18 treatment (24 hours TNFα, followed by 24 hours TNFα and C18) restored VSMC-WT-CFTR’s channel function (8.29±0.426 µmol/L, p<0.05 relative to TNFα alone, p>0.05 relative to DMSO, n=6 coverslips, Figure 5-1D).

We also replicated all the above findings in the BHK-WT-CFTR cell-line that stably expresses the human epithelial isoform of CFTR (Supplemental Figure 8B-D). Furthermore, we found that Etanercept (10 µg/mL) treatment, a drug that inhibits TNFα by binding to it and sequestering it from TNFα-receptors, restores the iodide efflux for BHK-WT-CFTR cells (Supplemental Figure 9A). This confirms that the simulation of HF in cells inhibits CFTR’s channel function in a TNFα-dependent manner.
Figure 5-1. C18 treatment partially reverses the tumor necrosis factor-alpha-(TNFα)-dependent down-regulation in CFTR's protein expression and normalizes CFTR's function in murine VSMCs from WT mice.

(A) C18 treatment (6 µmol/L) significantly increased the endogenous expression of the CFTR protein (normalized to total protein) after three to nine hours incubation in the murine VSMCs (n=3-4 cell culture dishes). *denotes p<0.05 relative to time point 0 hours. (B) Co-incubation with C18 (6 µmol/L) and TNFα (10 ng/mL) for 24 hours (n=4 cell culture dishes) increased CFTR’s protein expression (normalized to total protein) relative to TNFα treatment alone (n=4 cell culture dishes). However, the expression was still significantly less than the untreated control (n=3 cell culture dishes). Subsequent C18 treatment after 24 hours TNFα-dependent down-regulation had a similar effect on CFTR’s protein expression (n=4 cell culture dishes).
*denotes p<0.05 relative to untreated control. +denotes p<0.05 relative to TNFα alone. (C) Coincubation with C18 (6 μmol/L) and TNFα (10 ng/mL) for 24 hours (n=6 cell culture dishes) significantly increased the fluorescence intensity of S1P-FITC relative to TNFα alone (n=6 cell culture dishes) and normalized the value relative to the untreated control (n=5 cell culture dishes). *denotes p<0.05 relative to untreated control. (D) Subsequent C18 treatment after 24 hours TNFα-dependent down-regulation, significantly increased the FSK-stimulated iodide efflux (n=6 coverslips) relative to TNFα alone (n=6 coverslips), and normalized the efflux relative to the DMSO control treatment (24 hours, 0.1% v/v, n=7 coverslips). *denotes p<0.05 relative to DMSO control.
5.4.3 Acute *in-vitro* C18 treatment normalizes the MR of proximal PCAs from HF mice

In the present study, we found C18 treatment increased CFTR’s protein expression, and normalized CFTR’s function under a simulated HF condition. Next we assessed whether C18 treatment normalizes the MR for HF PCAs. HF and sham mice were sacrificed six-to-eight weeks post ligation of the LAD artery, and the proximal PCAs were isolated and dissected from the brains, and cannulated. The passive diameters of the PCAs at 45 mmHg were not significantly different (diameter = 228±17.7 µm versus 193±20.8 µm for HF and sham respectively, p>0.05 unpaired t-test). We found that the HF PCAs had significantly greater myogenic tone than the sham PCAs at the 80 and 100 mmHg pressure steps (myogenic tone at 80 mmHg = 17.8±0.848% versus 9.40±2.24%, and myogenic tone at 100 mmHg = 19.7±2.46% versus 11.2±1.74% for HF and sham respectively, p<0.05, n_HF=7, n_sham=8 arteries, Figure 5-2A). These results confirmed previous findings that HF in mice enhances the effects of endogenous S1P (Meissner *et al.* 2012). In the same arteries, the normalized vasomotor responses to all doses of PE were not significantly different between HF PCAs and the sham PCAs (e.g., tone at 10^{-8.5} mol/L = 0.171±0.117% versus 1.41±0.745% for HF and sham respectively, p>0.05, Figure 5-2B). Acute *in-vitro* C18 treatment (30 minutes, 6 µmol/L) significantly decreased the myogenic tone of the HF PCAs, relative to the pre-C18 condition (p<0.05, Figure 5-2A), and the MR was normalized in comparison to sham PCAs. There were no significant differences in the pre- and post-C18 vasomotor responses to PE, in the HF PCAs (p>0.05, Figure 5-2B). Additionally, C18 treatment did not significantly affect the MR of sham PCAs at any of the pressure steps (p>0.05, Figure 5-2A). The normalized pre-and post-vasomotor responses to PE were significantly decreased for the sham PCAs at PE concentrations of 10^{-6.5} to 10^{-5} mol/L (e.g., tone at 10^{-6.5} mol/L= 16.6±1.96% versus 10.7±1.45% for pre- and post-C18 respectively, p<0.05, Figure 5-
2B). The 6 μmol/L concentration of C18 may have non-specific effects in the isolated sham PCAs; therefore we assessed the specificity of C18.

The specificity of C18 was assessed using PCAs from FVB-129-ΔF508-CFTR mice, because C18 was originally characterized as a corrector of the ΔF508-CFTR mutant protein. We found that the passive diameters at 45 mmHg were not significantly different between the two genotypes (\( \text{dia}_{\text{max}} = 186\pm1.73 \, \mu m \) versus \( 173\pm8.96 \, \mu m \) for ΔF508-CFTR and WT-CFTR PCAs respectively, \( p>0.05 \) unpaired t-test). In Chapter 4, we demonstrated that ΔF508-CFTR PCAs have greater myogenic responsiveness than the WT-CFTR PCAs. Indeed, in the present study we observed enhanced myogenic tone for ΔF508-CFTR PCAs relative to WT-CFTR PCAs at the 60 to 100 mmHg pressure steps (myogenic tone at 60 mmHg = 20.0±2.65% versus 11.0±2.12%; myogenic tone at 80 mmHg = 22.7±2.38 versus 12.8±1.69; myogenic tone at 100 mmHg = 21.9±2.79% versus 12.6±0.938% for ΔF508 and WT PCAs respectively, \( p<0.05, n_{\text{ΔF508}}=6, n_{\text{WT}}=4 \) arteries, Figure 5-2C). Acute C18 treatment with 6 μmol/L concentration affected the MR and the vasomotor response to PE (data not shown), and then we tried a concentration that was ten-fold less (30 minutes, 0.6 μmol/L). At this concentration, we found the myogenic tone for ΔF508-CFTR PCAs was significantly decreased at the 60 to 100 mmHg pressure steps relative to pre-C18 treatment (myogenic tone= 9.62±2.25% at 60 mmHg; 10.2±2.30% at 80 mmHg; 8.68±2.07% at 100 mmHg, \( p<0.05 \) relative to the same pressure steps under the pre-C18 condition, Figure 5-2C). Importantly, the normalization of the responses in comparison to the untreated WT-CFTR PCAs occurred at a physiologically relevant pressure range (e.g., in humans 60-130 mmHg, see review by Lidington et al. 2013). We also found that in-vitro C18 treatment significantly decreased the myogenic tone for WT-CFTR PCAs at the 40 to 100 mmHg pressure steps (e.g., myogenic tone at 40 mmHg= 4.63±0.748% versus 8.93±1.92% for C18 and no-C18
respectively, p<0.05, data not shown). Furthermore, C18 treatment significantly decreased the vasomotor responses to PE for ΔF508-CFTR PCAs (at PE concentrations of 10^{-6} and 10^{-5} mol/L, p<0.05), and WT-CFTR PCAs (at PE concentration of 10^{-6} M, p<0.05, Figure 5-2D). Vasomotor responses at all other concentrations of PE were unaffected (p>0.05).
Figure 5-2. *In-vitro* C18 treatment normalizes the myogenic responsiveness of proximal posterior cerebral arteries (PCAs) from HF and ΔF508-CFTR mice, but also has an effect on the vasomotor responses to phenylephrine (PE).

(A) Acute C18 treatment (6 µmol/L, 30 minutes) significantly decreased the myogenic tone in PCAs from isolated from HF mice (6 weeks post LAD-ligation, n=7 arteries, \( \text{dia}_{\text{max}} = 228 \pm 17.7 \mu m \)), but does not alter myogenic tone in PCAs isolated from sham-operated mice (n=8 arteries, \( \text{dia}_{\text{max}} = 193 \pm 20.8 \mu m \)).

(B) In the same arteries, C18 treatment did not affect the phenylephrine-stimulated vasoconstriction is attenuated for HF PCAs, but did significantly attenuate vasoconstriction in the sham PCAs. * denotes \( p<0.05 \) following two-way repeated measures ANOVA analysis.

(C) The acute C18 treatment significantly decreased the myogenic tone in PCAs from ΔF508-CFTR mice (n=6 arteries, \( \text{dia}_{\text{max}} = 186 \pm 1.73 \mu m \)) and the responses were normalized relative to untreated WT-CFTR PCAs (n=4 arteries, \( \text{dia}_{\text{max}} = 173 \pm 8.96 \mu m \)). The treatment also (D) significantly attenuated the phenylephrine-stimulated vasoconstriction. * denotes \( p<0.05 \) following two-way repeated measures ANOVA analysis.
5.4.4 In-vivo C18 treatment normalizes the CBF in HF mice, and attenuates the MR of their proximal PCAs

The primary hemodynamic characteristics of HF are the drastic reduction in CO, and the increase in TRP as a compensatory mechanism to maintain the MAP (see Section 1.5.3). We assessed the effect of chronic in-vivo C18 treatment (48 hours, 0.8 mg per kg body weight, intraperitoneal injection) on the CO of HF-mice using echocardiography, and found that C18 did not significantly affect CO relative to saline-injected mice (4.40±0.317 mL/min versus 4.94±0.304 mL/min for C18 and saline-injection respectively, p>0.05, n_all=8 mice, Figure 5-3A), and therefore did not normalize CO relative to the sham mice (6.94±0.137 mL/min, n=6 mice).

In the same mice, the C18 treatment did not significantly affect the TPR relative to saline-injected mice (mean TPR = 9.07±0.904 mmHg*min/mL versus 9.76±0.628 mmHg*min/mL for C18 and saline-injected mice respectively, p>0.05, Figure 5-3B), and thus TPR was not normalized to the sham mice (6.14±0.359 mmHg*min/mL). The HF mice injected with saline showed no significant difference in their MAP relative to sham (mean MAP = 58.3±1.28 mmHg versus 66.3±1.41 mmHg for HF and sham mice respectively, p>0.05, Figure 5-3C), and C18 injection had no effect (60.2±3.27 mmHg). The absence of an effect of C18 on CO and TPR suggests the treatment does not have a systemic effect, and this could be due to the reduced expression of endogenous CFTR protein in the peripheral resistance arteries (e.g., cremaster skeletal muscle resistance arteries, see Chapter 4). Therefore, we next assessed the effect of C18 on the CBF of HF and sham mice. We found that HF-mice injected with saline had significantly lower global CBF than the sham mice (mean CBF= 134±4.34 mL/100g*min versus 176±8.57 mL/100g*min for HF and sham mice respectively, p<0.05, n_HF=8, n_sham=6 mice, Figure 5-3D, E). Injection with C18 significantly increased CBF in the HF mice (162±7.92 mL/100g*min,
p<0.05, Figure 5-3E), relative to the saline injected mice, and CBF was normalized in comparison to the sham mice.

Since C18 did not affect the CO of HF mice, we predicted the normalization of the CBF was due to effects on the MR for the PCAs. To test this hypothesis a new cohort of HF and sham were developed. Six-to-eight weeks post surgery, C18 (48 hours, 0.8 mg/kg, i.p.) or saline were injected into the mice. The proximal PCAs were isolated and cannulated, and the MR was assessed. The PCAs from saline-injected HF mice had greater vasoconstriction at the 60 to 100 mHg pressure steps, relative to the PCAs from the saline-injected sham mice (myogenic tone at 60 mmHg = 9.46±1.49% versus 2.88±0.800%; myogenic tone at 80 mmHg= 16.2±2.37% versus 4.92±1.74%; myogenic tone at 100 mmHg= 18.7±1.43% versus 8.62±2.36% for HF and sham respectively, p<0.05, n_{HF}=5, n_{sham}=6 arteries, Figure 5-3F). The PCAs from C18-injected HF mice showed attenuation in the myogenic responsiveness at the 80 and 100 mmHg pressure steps, relative to the HF PCAs from saline-injected mice (myogenic tone at 80 mmHg= 7.35±1.48% at 80 mmHg; myogenic tone at 100 mmHg= 10.2±1.90% for HF mice injected with C18, p<0.05 relative to same pressure steps for saline-injected HF PCAs, n=7 arteries). The passive diameters at 45 mmHg for the PCAs were not significantly different, between the HF mice injected with C18 and those injected with saline (dia_{max} = 160±7.93 μm versus 153±8.25 μm for C18 and saline respectively, p>0.05 unpaired t-test). Furthermore, the vasomotor responses at all doses of PE were unaffected by the in vivo C18 treatment, and this suggests the effect is specific to the MR. Importantly, C18 normalized the myogenic responsiveness for HF PCAs in comparison to the saline-injected sham PCAs (Figure 5-3F).

In contrast to the PCAs from the HF mice, there was no significant effect of C18 on the myogenic responsiveness for the PCAs from the sham mice, at all pressure steps (e.g., myogenic tone at 80 mmHg = 6.37±1.56% versus 4.92±1.74% for C18 and saline injection respectively,
p>0.05, n_{C18}=5, n_{saline}=6 arteries, Figure 5-3F). In the same arteries, the passive diameters at 45 mmHg between the two treatment groups were not significantly different for these PCAs (\text{dia}_{\text{max}} = 128\pm9.75 \ \mu m \ \text{versus} \ 147\pm7.51 \ \mu m \ \text{for} \ C18 \ \text{and} \ \text{saline} \ \text{injection} \ \text{respectively}, \ p>0.05 \ \text{unpaired t-test}). C18 also did not affect the vasomotor responses at all concentrations of PE for sham PCAs, relative to saline injection.

HF in mice decreases CFTR’s protein expression in cerebral tissue (Meissner et al. 2012), and this contributes to the enhancement of the MR of PCAs. Next we assessed the effect of \textit{in vivo} C18 treatment on CFTR’s protein expression in cerebral tissue. We found that the tissue from saline-injected HF mice had significantly less CFTR protein expressed than the tissue from sham mice (ratio of CFTR protein to total protein = 1.02\pm0.102 \ \text{versus} \ 1.53\pm0.147 \ \text{for} \ HF \ \text{and} \ \text{sham} \ \text{respectively}, \ p<0.05, \ n=6 \ \text{mice, Figure 5-3G}). C18 injection in the HF mice (48 hours, 0.8 mg/kg i.p.) significantly increased CFTR’s cerebral protein expression relative to saline-injected HF mice (ratio of CFTR protein to total protein = 1.57\pm0.105, n=6 mice). Importantly, the protein expression was normalized in comparison to the sham mice (p>0.05). Hence, \textit{in vivo} C18 treatment normalizes CFTR’s protein expression, and this contributes to the normalization for the PCAs’ myogenic responsiveness and the CBF in HF mice.
Figure 5-3. In-vivo C18 treatment normalizes the cerebral blood flow (CBF) and myogenic responsiveness of HF mice, and down not affect systemic hemodynamic parameters.

Relative to sham-operated controls (n=6), mice with heart failure (HF; 6 weeks post-LAD ligation) have (A) reduced cardiac output (n=8), (B) elevated total peripheral resistance (n=6) and (C) normal mean arterial pressure (n=6). C18 treatment in vivo (0.8mg/kg BW daily for 2 days) does not significantly alter these systemic parameters. (D) Representative FAIR-Magnetic Resonance Imaging perfusion maps display the heart failure-stimulated reduction in cerebral perfusion, which is graphically summarized in (E). In contrast to the systemic parameters, (D-E) C18 treatment significantly improves cerebral perfusion in mice with HF (sham: n=6; HF: n=8; HF+C18: n=8). The improved cerebral perfusion following C18 treatment...
is associated with (F) attenuated myogenic tone (HF dia$_{\text{max}}$=149±8µm, n=6; HF+C18 dia$_{\text{max}}$=155±8µmol/L, n=8; P=N.S., unpaired comparison) and (G) the normalization of PCA CFTR protein expression (n=6, all groups). For panels A,B,C,E and G, * denotes P<0.05 for unpaired comparisons following one-way ANOVA; For panel F, * denotes P<0.05 following two-way ANOVA.
5.5 Discussion

5.5.1 C18 partially restores the TNFα-dependent down-regulation of CFTR’s protein expression, and normalizes CFTR’s function

Chronic TNFα treatment in murine VSMCs from WT mice, and in BHK-WT-CFTR cells, decreased the protein expression of CFTR, and these in-vitro treatments were used to simulate the HF condition in cells. TNFα treatment was first shown by Nakamura and colleagues to decrease CFTR’s mRNA stability in HT-29 cells (Nakamura et al. 1992). Additionally, our laboratory demonstrated that TNFα treatment (24 hours, 10 ng/mL) decreases CFTR’s mRNA expression in murine VSMCs from WT mice (Meissner et al. 2012). The results from the primary VSMC cell-line and from intact arteries of HF mice dramatically extends the reach and significance of the TNFα-dependent reduction in CFTR’s expression. The reduction in mRNA stability and expression may lead to down-regulation of the translation of CFTR’s mRNA to the protein, and over time protein expression is decreased. The downstream effect on protein expression could be exacerbated by the high turnover rate of the CFTR protein. In the present study, we did not assess the effect of C18 on CFTR’s mRNA expression, because there is no evidence in the literature that small-molecule corrector compounds affect the transcription or translation of CFTR.

Acute TNFα treatment (i.e., three hours incubation) has been shown by our laboratory to significantly decrease the protein expression of CFTR in the plasma membrane fractions from murine VSMCs (Meissner et al. 2012), and this suggests TNFα affects the peripheral trafficking of CFTR. The peripheral trafficking of CFTR is a tightly regulated mechanism (Sharma et al. 2004), and its endocytosis occurs within minutes (Lukacs et al. 1997, Prince et al. 1994). The
internalized WT-CFTR efficiently recycles back to the plasma membrane whereas the mutant proteins, like the ΔF508-CFTR, traffic to the lysosome and are degraded (Sharma et al. 2004). Clathrin-coated vesicles (CCVs) are primarily responsible for regulating CFTR’s endocytosis (Bradbury et al. 1999, Lukacs et al. 1997), but there is no literature that suggests TNFα has an effect on CCVs. Instead, TNFα may destabilize WT-CFTR in the plasma membrane, and in contrast C18 has the opposite effect. In support of this, we found that co-treatment with C18 and TNFα partially inhibited the down-regulation of CFTR’s protein expression in cells, and also completely prevented the loss of channel function and S1P-uptake function. Previously, we have also shown that three-hours incubation with TNFα does not effect CFTR’s protein expression in the cytosolic fractions. This finding suggests the internalized protein is degraded, because the decrease in membrane CFTR did not lead to a corresponding increase of CFTR’s protein expression in the cytoplasm. Thus, the internalized CFTR is not recycled back to the plasma membrane, and C18 may enhance the stability of the internalized protein and promote its recycling back to the plasma membrane. To support this we found that subsequent C18 treatment (i.e., 24 hours TNFα, then 24 hours TNFα/C18) partially reversed CFTR’s down-regulated protein expression. Additionally, the same C18 treatment protocol normalized the FSK-stimulated iodide efflux and the uptake of S1P-FITC. Possible mechanisms of C18 will be discussed in greater detail below.

5.5.2 C18 treatment normalizes the myogenic responsiveness of PCAs and the CBF of HF mice

We found that the proximal PCAs from HF mice display enhanced myogenic constriction, relative to PCAs from Sham mice. This data replicates our laboratory’s previous findings (Meissner et al. 2012, Yang et al. 2012, Hoefer et al. 2010). Acute in-vitro treatment with C18 normalized the myogenic responsiveness of the HF PCAs, in comparison to the sham
PCAs. However, the 6 µmol/L concentration of C18 also affected the vasomotor responses to PE. To better assess the specificity of C18 we used PCAs from ΔF508-CFTR mice, and found that acute in-vitro with a lower concentration of C18 (0.6 µmol/L) normalized the myogenic responsiveness of these arteries, in comparison to PCAs from WT-CFTR mice. Yet, the vasomotor responses to PE for ΔF508-CFTR PCAs were also decreased, at the two highest concentrations of PE, and thus in-vitro C18 treatment could have non-specific effects in isolated PCAs.

In-vivo treatments with C18 were delayed until six-to-eight weeks post LAD coronary artery ligation, because at this time point the pathological changes of HF are more complete (Yang et al. 2012). We found that in-vivo C18 treatment in the HF mice did not normalize the CO or the TPR, and therefore C18 does not affect the systemic hemodynamic parameters. The in-vivo C18 treatment normalized CFTR’s protein expression in cerebral vascular tissue from HF mice, the myogenic responsiveness of HF PCAs, and the CBF of HF mice. Furthermore, in-vivo C18 treatment in the HF mice did not significantly affect the vasomotor responses to PE for their isolated PCAs. The reversal in CFTR’s protein expression and the positive effect on the MR suggest that CFTR’s ability to regulate S1P’s bioavailability and endogenous S1P-signaling are restored.

5.5.3 Possible mechanism of actions for C18

A recent study by Okiyoneda and colleagues provided mechanistic classification of various corrector compounds, depending upon their interactions with the domains of the ΔF508-CFTR mutant (Okiyoneda et al. 2013). A Class 1 corrector influences co-translational processing of the protein by primarily affecting the interaction between NBD1 and MSD1, and may also affect the interaction between NBD1 and MSD2. Class 2 correctors preferentially
affect the post-translational processing, and aid in proper assembly of NBD2. Finally, Class 3 correctors have some effect on the co-translational processing, but their primary target is the energetic defect of CFTR’s mutants.

The effect of C18 on WT-CFTR remains unclear in the literature, and for that reason we will discuss possible mechanisms of C18 by using the published ΔF508-CFTR studies. C18 is a Class 1 corrector and binds directly to ΔF508-CFTR, therefore it may act as a chaperone for protein processing (Okiyoneda et al. 2013, Wong et al. 2012). He and colleagues demonstrated that low-temperature incubation has an additive effect on C18-dependent enhancement of Band C, expression in stably transfected BHK-ΔF508-CFTR cells (He et al. 2013). Hence, C18 appears not to correct the thermal defect, and its mechanism of action is different than low-temperature incubation. Since low-temperature rescued ΔF508-CFTR in CHO epithelial cells has been shown to have less stability in the plasma membrane than WT-CFTR (Lukacs et al. 1993), C18 may improve the interaction between NBD1 and the MSDs. The improved interaction could enhance ΔF508-CFTR stability in the plasma membrane. Alternatively, Holleran and colleagues showed that C18 treatment (three hours, 37°C) in low-temperature rescued HEK-293-ΔF508-CFTR cells, decreases the co-localization between ΔF508-CFTR and the lysosome (Holleran et al. 2013). Thus, C18 also acts indirectly on the peripheral quality control mechanism, and the C18-dependent stabilization of the rescued ΔF508-CFTR may divert it from degradation to recycling. In the present study, the normalization of the MR for ΔF508-CFTR PCAs, with acute in-vitro C18 treatment, provides vascular support for C18-dependent stabilization.

The WT-CFTR protein is more stable in the plasma membrane (Lukacs et al. 1993), has reduced turnover (Sharma et al. 2001), and is less susceptible to proteasome-dependent
degradation than low-temperature rescued ΔF508-CFTR (Sharma et al. 2004). Our compiled murine VSMCs data (e.g., co-treatment with C18 protocol), and murine HF PCA data (e.g., acute in-vitro C18 treatment) suggest C18 is having a protective effect on membrane expressed CFTR. The in-vivo effects of C18 could be due to either enhanced processing or the aforementioned proteostatic effect.

5.5.4 Correction of CFTR as a therapeutic approach for the cerebral effects of HF

The reduction in CBF is caused by an enhancement in cerebral vascular resistance, and this may account for the abnormalities in brain function observed in HF patients, including memory loss, slowing of psychomotor responses, and abnormal executive function (e.g., reasoning, problem solving) associated with HF (see Section 1.5.3, and also Pressler et al. 2010, Sauvè et al. 2009, Choi et al. 2006.). The cerebral dysfunction affects patient quality of life (Loncar et al. 2011). The intriguing effects seen in the cerebral microvasculature, and the absence of an effect on the CO or TPR, suggests C18 could alleviate the cerebral dysfunction in human HF patients without having a deleterious systemic effect. Of course before these assessments can be done, the correction of CFTR should first be assessed in human resistance arteries, discussed in greater detail in Limitations and Future Directions (see Chapter 6).

Promising results have been generated from human clinical trials using other corrector compounds in CF patients. For example, a recent study by Clancy and colleagues used the CFTR corrector compound VX-809, an analogue of C18 (He et al. 2013, Okiyoneda et al. 2013), in a Phase II clinical trial with CF patients expressing the ΔF508-CFTR mutation. Their study found that patients given VX-809 (orally for 28 days) had a dose-dependent reduction in the amount of sweat chloride, relative to the placebo group. However, there were no beneficial effects on lung function, CFTR channel function in the nasal epithelium, or CFTR protein
maturation in rectal biopsy tissue samples (Clancy et al. 2012). A recent Phase II clinical trial was conducted by Vertex Pharmaceuticals used a combination of the VX-809 corrector and the FDA-approved potentiator VX-770 (also known as Ivacaftor or Kalydeco), and the trial demonstrated improved lung function, and CFTR channel function for CF patients expressing the ΔF508-CFTR mutation. The company is currently conducting a Phase III clinical trial of the combined treatment. C18 is the parent compound of VX-770, but VX-770 treatment alone does not affect the lung function or CFTR channel function in CF patients that express the ΔF508 mutation. VX-770 has only been shown to benefit G551D-CFTR CF patients (Flume et al. 2012) because VX-770 lacks corrector-function. Nonetheless, the compound may be re-purposed as a therapeutic for HF, because the HF disease does not abolish the plasma membrane expression of CFTR. Therefore, VX-770 may enhance the function of the remaining CFTR protein, and normalize the myogenic tone of isolated PCAs from HF. This would validate our acute treatments with C18. Importantly, in-vivo treatments with VX-809 or VX-770 may normalize the CBF for HF mice, and confirm that CFTR is a drug target for the cerebral effects of the disease.

5.6 Conclusion

The worldwide prevalence of the HF disease is approximately 2% (Mann and Chakinala in Long et al. 2012), and in Canada there are approximately 50,000 new patients diagnosed every year (Ross et al. 2006). The severe symptoms associated with the disease demand better therapeutic strategies. In summary, our results provide insight into the importance of CFTR in regulating the microvascular reactivity in HF, such as endogenous S1P-signaling in the MR. We identify CFTR as a novel drug target for the correction of cerebral microvascular dysfunction.
associated with HF, and the results of the study lead to the possibility of re-purposing small-molecule correctors and potentiaters.
Chapter 6

6 GENERAL DISCUSSION

6.1 Overview

CFTR is a regulator of S1P bioavailability and degradation, and therefore regulates the endogenous and exogenous S1P-signaling pathways for resistance arteries. In the HF, the abnormal expression and/or function of CFTR contributes to the S1P-dependent enhancement of the MR in proximal PCAs, which augments resistance to blood flow and reduces CBF of mice. We were interested in how the degradation mechanism could be regulated, and used CFTR channel function as a marker for modulation by S1P. This thesis set out to answer three questions: (i) does S1P modulate CFTR function and what is the regulatory mechanism for this modulation; (ii) does the mechanism contribute to the expression and/or function defects of the ΔF508-CFTR mutant; and (iii) can therapeutic targeting of CFTR with corrector compounds restore the cerebral microvascular abnormalities in HF mice. The results presented in this thesis lay out the biochemical and physiological bases for the modulation of CFTR function by S1P (Figure 6-1).

6.2 Major findings

6.2.1 Aim 1: S1P is a modulator of CFTR channel function

The first study of this thesis assessed whether acute S1P modulates CFTR channel function. Then we assessed whether the modulation was caused by S1P;R -AMPK-dependent phosphorylation of a known inhibitory amino acid residue (S737), present in CFTR’s R-domain. We assessed channel function with the iodide efflux assays, in stably transfected BHK cells that express the human epithelial isoform of CFTR. Furthermore, we evaluated the S1P;R receptor, a known upstream activator of AMPK, with an antagonist and agonist approach. The major
findings of the first study were: (1) S1P inhibits WT-CFTR’s FSK-stimulated iodide efflux, and mutation of the S737 residue abolishes the effect of S1P; (2) S1P phosphorylates the region encompassing the S737 residue in a S1P₁R and AMPK-dependent manner; (3) Inhibition of the S1P₁R (e.g., VPC 23019) or AMPK (e.g., AMPK siRNA) abolishes the effect of S1P on phosphorylation of CFTR and AMPK, and channel function was restored.

6.2.2 Aim 2: The S1P₁R-AMPK signaling pathway contributes ΔF508-CFTR’s defective function

The second study of this thesis assessed whether the S1P₁R-AMPK-signaling pathway contributes to the defects of the ΔF508-CFTR mutant (BHK-ΔF508-CFTR cells). We assessed channel function with the conventional iodide efflux assay, the S1P-FITC uptake function with the FACS technique, and the plasma membrane expression of the mutant with the membrane fractionation procedure. Then FVB-129 mice expressing WT-CFTR or ΔF508-CFTR were used to evaluate the role of genetic mutation in cerebral microvascular reactivity, such as the CBF and the MR of PCAs. The major findings of the second study were: (1) Inhibition of the S1P₁R (e.g., VPC 23019) or AMPK (e.g., AMPK siRNA) enhanced ΔF508-CFTR’s FSK-stimulated iodide efflux of cells. There is an additive effect of AMPK inhibition and low-temperature incubation; (2) Inhibition of AMPK (e.g., acute treatment with Compound C) enhances ΔF508-CFTR-dependent uptake of S1P-FITC; (3) FVB-129-ΔF508-CFTR mice have reduced CBF relative to WT mice, and this was independent of the heart (e.g., CO, HR) or the peripheral circulation (e.g., TPR); (4) ΔF508-CFTR PCAs display greater reactivity to endogenous S1P (e.g., the MR) and exogenous S1P than WT-CFTR PCAs; (5) Inhibition of the S1P₁R (e.g., acute in-vitro treatment with VPC 23019) decreases the reactivity of ΔF508-CFTR PCAs to endogenous S1P and exogenous S1P. Overall, the findings demonstrate that the S1P₁R-AMPK-
signaling pathway contributes to ΔF508-CFTR channel defect, and the abnormal cerebral microvascular reactivity of ΔF508-CFTR mice.

6.2.3 Aim 3: CFTR is a therapeutic target for the cerebral microvascular effects of HF in mice

HF in mice demonstrates TNFα-dependent down-regulation of CFTR vascular protein expression, and this contributes to the abnormal cerebral microvascular reactivity observed, such as enhanced myogenic responsiveness of PCAs and reduced CBF. Since there are small-molecule compounds that partially correct the abnormal expression of CFTR, we predicted that CFTR is viable drug target in HF. To test the prediction we used treatments with the C18 corrector compound in a cell-culture simulation of HF (i.e., chronic TNFα treatment in murine VSMCs from healthy WT mice), and a mouse model of HF (i.e., ligation of the LAD coronary artery). The major findings of the third study were: (1) Chronic TNFα treatment down-regulated CFTR protein expression in murine VSMCs, and decreased the uptake of S1P-FITC and FSK-stimulated iodide efflux; (2) Treatment with C18 (24 hours) partially restored CFTR protein expression, and fully restored the S1P-uptake function and channel function; (3) Acute in-vitro C18 treatment (30 minutes) or chronic in-vivo (48 hours, i.p. injection) normalized the myogenic responsiveness of HF PCAs in comparison to sham; (4) Acute in-vitro C18 treatment also normalized the myogenic responsiveness of ΔF508-CFTR PCAs, demonstrating CFTR-specificity; (5) Chronic in-vivo C18 normalized the CBF of HF mice, but did not affect the CO or TPR. Overall, the findings of this study established CFTR as a target for normalizing the HF-dependent cerebral microvascular function defects, and this leads to the potential of re-purposing the CFTR correctors that are in human CF clinical trials, or the FDA-approved potentiator VX-770.
Figure 6-1. Summary of the thesis aims and major findings.

This figure represents some of the known and unknown signaling pathways that regulate S1P, CFTR, and the myogenic response. The studies presented in this thesis on the interplay between S1P and CFTR, and their role in the myogenic response: (1) We found that S1P inhibits CFTR’s channel function (i.e., FSK-stimulated iodide efflux) in an S1P1R-AMPK-dependent manner. (2) The S1P1R-AMPK-signaling pathway aggravates the expression and/or function defects for the ΔF508-CFTR mutant. (3) The in-vivo and in-vitro models of HF were used to show that CFTR is a therapeutic target in HF. The C18 small-molecule corrector compound has positive effects on the cerebrovascular behavior in HF mice.
6.3 S1P modulates CFTR channel function

Our original interest was assessing the modulation of CFTR’s S1P-uptake function, but we found that S1P-FITC treatment phosphorylates AMPK, a known inhibitor of CFTR’s function. Therefore, we assessed channel function of CFTR with the iodide efflux assays in cells expressing the human epithelial isoform, an established Cl⁻ channel. The iodide efflux assays demonstrated that S1P inhibits CFTR’s channel function through AMPK-dependent phosphorylation of CFTR’s R-domain.

The non-phosphorylated R-domain is in close contact with NBD1, and this creates a sterical block (Bozoky et al. 2013). Phosphorylation of the R-domain’s stimulatory Ser residues removes the sterical hindrance, and this causes heterodimerization of the NBDs (Baker et al. 2007). Chappe and colleagues work with the Split∆R-CFTR construct (see Section 1.5.1.1), suggest PKA-dependent phosphorylation promotes interaction between the R-domain and CFTR’s N-terminus. Consequently, the interactions between the R-domain and other regions of the protein are decreased, and this contributes to opening of the channel. However, phosphorylation of the R-domain’s inhibitory residues is thought to fix CFTR in a PKA-insensitive state, and the open probability of the channel is decreased, and closing of the channel remains unaffected (King Jr. et al. 2009, Vais et al. 2004). The inhibitory effect of AMPK on CFTR channel function requires binding of AMPK to the carboxyl-terminus of CFTR, and the phosphorylation of the inhibitory residues (see Section 1.5.1.2). The combination of binding and phosphorylation may cause conformational changes that re-establish or strengthen the interaction between the R-domain and the amino-terminus, and therefore channel opening by PKA is inhibited. Furthermore, AMPK-dependent phosphorylation is thought to stabilize CFTR’s closed state, whereas PKA-dependent phosphorylation is thought to de-stabilize the closed state (King Jr. et al. 2009, Winter and Welsh 1997). These differences may be due to the direct versus
indirect binding to CFTR by AMPK and PKA respectively, as previously mentioned. Others have extensively studied the effects of phosphorylation-dependent conformational changes, and the contribution to CFTR’s channel function (Rahman et al. 2013, Chappe et al. 2005, Grimard et al. 2004, also see Bussolati et al. in Winkle 1999). We can only suggest that S1P-signaling modulates CFTR’s function by affecting protein conformation or domain interactions, because we have no direct evidence for it.

Biochemical studies with a “Quad” mutant of CFTR, where four R-domain Ser residues that are known PKA sites are substituted with alanine, have provided some insight into the importance of certain phosphorylation sites on CFTR’s channel function. Chang and colleagues mutated the Ser residues at positions 660, 737, 795 and 813 to alanine, transfected the construct into Chinese hamster ovary cells, and found a 40% reduction in the FSK-dependent iodide efflux, relative to cells expressing WT-CFTR (Chang et al. 1993). A later study by Baldursson and coworkers, used Fischer rat thyroid epithelial cells transfected with the “Quad” mutant and demonstrated an approximate 50% reduction in the FSK-stimulated transepithelial chloride current, relative to cells transfected with WT-CFTR (Baldursson et al. 2000). The mutation of the Ser residues was likely limited to functional effects, rather than effects on protein expression, because there was an increased sensitivity to greater concentrations of FSK, and an alteration in the time course of the transepithelial chloride current activation (Baldursson et al. 2000).

Furthermore, the expression of the mature fully glycosylated Band C form of CFTR is unaffected in cells transfected with of the “Quad” mutant (Chang et al. 1993). Site-directed mutagenesis of the Ser residues at position 660 or at position 813, while keeping Ser residues at all other positions, lead to a 40-50% reduction in the FSK-stimulated chloride current (Baldursson et al. 2000). This suggested the S660 and S810 are important stimulatory residues. Site-directed mutagenesis of only the S795 residue did not significantly affect FSK-stimulated chloride
current, relative to cells transfected with the WT-CFTR protein. The S795A construct did display an approximate 15-20% reduction in the cAMP-dependent chloride current (i.e., absence of FSK and IBMX). Wilkinson and colleagues found the S795 is a stimulatory residue in electrophysiological studies with *Xenopus* oocytes (Wilkinson et al. 1997). Nevertheless, Baldursson et al. ’s study suggests the S795 residue is less important for CFTR’s channel activation, than the other three residues assessed. This group also showed that re-introduction of a Ser residue at position 737 did not cause a further reduction in FSK-stimulated chloride current, relative to cells transfected with the “Quad” mutant. They next attempted site-directed mutagenesis of the S737 residue to alanine, while keeping Ser residues at the three other positions, and found that cells transfected with this S737A-CFTR construct displayed an approximate 100% enhancement in the transepithelial chloride current, relative to cells expressing WT-CFTR (Baldursson et al. 2000). These results suggest the S737 is an inhibitory residue, and may require other Ser residues in the “Quad” to perform its function. Therefore, the inhibitory role of S737 relies on interaction with other Ser residues within CFTR’s R-domain (Baldursson et al. 2000).

Csanády and colleagues used the purified R-domain of WT-CFTR, and showed that a peptide containing the S768 residue was *in-vitro* phosphorylated by PKA and a low concentration of $^{32}$P-labelled Mg-ATP (1 μmol/L). This suggested S768 was basally phosphorylated in a PKA-dependent manner (Csanády et al. 2005), relative to R-domain peptides that contained other Ser residues, and the S768 residue may be the most readily phosphorylated residue within CFTR’s R-domain. Site-directed mutagenesis of the S768 residue to alanine (S768A-CFTR) was shown to enhance CFTR’s resting conductance in *Xenopus* oocytes (Csanády et al. 2005). The results suggest the S768 residue is responsible for inhibiting CFTR’s channel function under basal conditions. The S737A-CFTR construct displays either
enhanced FSK-dependent channel current in Fisher thyroid epithelial cells and *Xenopus* oocytes (Kongsuphol *et al.* 2009, Baldursson *et al.* 2000, Wilkinson *et al.* 1997), or no change in cell-patches (Winter and Welsh 1997). Thus, there are model-specific differences in the channel function studies of the S737 residue, and the effect of phosphorylating the S737 may be qualitatively different than phosphorylation of S768 (Csanády *et al.* 2005). Indeed, Csanády and colleagues showed that the R-domain peptide containing both the S737 and S768 residues showed a large electrophoretic mobility shift, after treatment with PKA and 1 μmol/L $^{32}$P-Mg-ATP. The S737 residue was the only additional serine phosphorylated, and site-directed mutagenesis of this residue prevented the mobility shift (Csanády *et al.* 2005). It is important to note this study did not assess the effect of S737 mutation on CFTR’s resting conductance. Changes in protein conformation can affect the mobility of peptides during gel electrophoresis (Goldenberg and Creighton 1984), and the results of Csanády *et al.* suggest a distinct effect of S737 phosphorylation: significant changes in CFTR’s protein conformation, at least in response to PKA. The changes in protein conformation may be dependent upon secondary structure propensity (Bozoky *et al.* 2013).

The Hallows laboratory assessed the AMPK-dependency of the S737 and S678 residues in isolated and purified CFTR proteins. They demonstrated an absence in the AMPK-dependent phosphorylation of an immunoprecipitated CFTR construct, with re-introduction of a Ser residue at position 737, while keeping alanine substitutions at nine other sites (e.g., CFTR-10-SA-A737S). Yet, a construct where a Ser residue is re-introduced at position 768 displayed AMPK-dependent phosphorylation (King Jr. *et al.* 2009). Furthermore, *Xenopus* oocytes expressing the S768A-CFTR construct showed greater whole cell conductance in response to FSK, relative to oocytes expressing WT-CFTR. A limitation of this study is the group did not assess the effects of re-introducing Ser residues into other positions, especially in light of the results with the
“Quad” CFTR mutant (Baldrusson et al. 2000). In other words, phosphorylation of S737 by AMPK may require the presence of nearby Ser residues in the R-domain. King Jr. and colleagues also did not assess the effect of S737A-CFTR on the whole cell conductance of Xenopus oocytes. A study by Kongsuphol and colleagues, published a few months prior to the work from the Hallows laboratory, used purified R-domain peptides and $^{32}$P-labeling, and demonstrated AMPK-dependent phosphorylation of the S737 and S768 residues, but phosphorylation appeared to be greater at the S768 residue (Kongsuphol et al. 2009). Xenopus oocytes expressing the S737A-CFTR construct or the S768A-CFTR construct demonstrated enhanced whole cell conductance relative to oocytes expressing WT-CFTR, in response to FSK, but the conductance observed with S768A-CFTR was greater than the S737A-CFTR construct. Pre-treatment with phenformin, a pharmacologic activator of AMPK (Zhang et al. 2007) significantly reduced the FSK-stimulated conductance of WT-CFTR expressing oocytes (Kongsuphol et al. 2009). Furthermore, conductances of oocytes expressing either S737A-CFTR or S768A-CFTR were unaffected by phenformin, suggesting the two residues inhibit CFTR’s channel function in an AMPK-dependent manner. Taken together the data from the literature suggests it is difficult to make concluding statements on the preferential phosphorylation of CFTR’s Ser residues. There are differences depending upon the constructs and cell models used. In Chapter 3, we found that mutation of the S737 residue prevented the inhibitory effect of S1P on CFTR’s iodide efflux, but this does not exclude a role for the S768 residue. S1P-S1P$_1$R-AMPK-dependent phosphorylation of both inhibitory residues could be occurring, and there are accumulated conformational effects. Future experiments can focus on the interplay of the two residues.

The phospholipid lysophosphatidic acid (LPA), which is structurally similar to S1P, was shown by Li and colleagues to modulate CFTR channel function in Calu-3 epithelial cells. They
demonstrated that acute pre-treatment with LPA inhibits adenosine-stimulated iodide efflux (Li et al. 2005). The group also used cell-attached single channel recordings to demonstrate that infusing the pipette solution with FSK (10 µmol/L), or a high concentration of cpt-cAMP (200 µmol/L) prevented the effect of LPA pre-treatment. Since, these treatments contribute to a global increase in the [cAMP], the results demonstrated that LPA inhibited the compartmentalized production of cAMP. Li and colleagues also tested pre-treatment with S1P and found no effect on CFTR’s channel function, but the data were not shown. This is surprising because of the structural and functional similarities between LPA and S1P. There is no published S1PR expression data for Calu-3 cells, and it is possible that Calu-3 cells do not express the S1P1R. In Chapter 3, we demonstrated that S1P inhibits the iodide efflux of WT-CFTR using the continuous iodide efflux assay (i.e., co-treatment with FSK), and the conventional iodide efflux assay (i.e., co-treatment, S1P present in the FSK/IBMX/cpt-cAMP cocktail). Our results suggest that S1P inhibits the channel function independent of cAMP.

In Chapter 4, we showed that G551D-CFTR transfected BHK cells cannot uptake S1P-FITC. As previously mentioned, the CFTR mutant display reduced channel function relative to the WT protein, and the uptake data suggests a functional protein is required. We believe the data links channel function to the uptake function, and it supports our use of the iodide efflux assays to assess S1P-dependent modulation of CFTR’s function. The modulation observed contributes to our understanding for the role of CFTR in S1P-signaling, and the inhibition of CFTR’s channel function may lead to reduced S1P degradation. Therefore, the bioavailability of S1P could be enhanced, and the S1P1R-AMPK signaling pathway is upregulated.
6.4 The S1P$_1$R-AMPK signaling pathway and the ΔF508-CFTR mutant

In Chapter 3, we demonstrated that inhibition of AMPK (i.e., Compound C, AMPK siRNA) enhanced the FSK-stimulated iodide efflux of BHK-WT-CFTR cells; as previously discussed, this was in agreement with other studies. We also found that acute S1P treatment inhibits the FSK-stimulated iodide efflux of low-temperature rescued BHK-ΔF508-CFTR cells. This led us to postulate that S1P-signaling contributes to the functional defects associated with the mutant protein. Indeed, we demonstrated in Chapter 4 that antagonism of the S1P$_1$R (i.e., VPC 23019 treatment), or the chronic inhibition of AMPK (i.e., Compound C, AMPK siRNA) enhanced the FSK-stimulated iodide efflux of BHK-ΔF508-CFTR cells, and there was an additive effect of AMPK’s inhibition in a low-temperature condition. Our results demonstrate the S1P$_1$R-AMPK-signaling pathway aggravates the ΔF508-CFTR’s channel defect.

Furthermore, the additive effect observed under the low-temperature condition suggests inhibition of AMPK had a different mechanism of action. Low-temperature incubation is thought to reduce the activity of proteasomes involved in CFTR degradation (Farinha et al. 2012), and this allows the mutant protein to accumulate, and it has a chance to fold correctly. The low-temperature rescued ΔF508-CFTR has a lower half-life in the plasma membrane than WT protein (Gentzsch et al. 2004), and the internalized protein is degraded rather than recycled (Sharma et al. 2004). Inhibition of AMPK may lead to increased stability in the plasma membrane for ΔF508-CFTR, or decreased degradation of the internalized protein. This could explain the enhanced channel function observed. Furthermore, the enhanced uptake of S1P-FITC and plasma membrane expression of ΔF508-CFTR observed after acute Compound C treatment (60 minutes at 37°C) suggest that the mutant protein is trapped in sub-membrane compartments. Acute inhibition of AMPK may promote trafficking of ΔF508-CFTR from these
compartments to the plasma membrane. However, trafficking assessments are required to further address this notion.

The cell-culture experiments formed the basis to test whether the S1P₁R-AMPK-signaling pathway affects the cerebrovascular function of mice that express the genetic ΔF508 mutation (i.e., FVB-129-ΔF508-CFTR). We showed that these mice have a distinct cerebral microvascular phenotype (e.g., decreased CBF, enhanced MR for PCAs), relative to WT mice, that is independent of the heart’s function. The PCAs from the mutant mice also showed enhanced vasomotor responses to exogenous S1P, and this suggests that endogenous and exogenous S1P-signaling is abnormal in ΔF508-CFTR expressing mice. Antagonizing the S1P₁R with VPC 23019 attenuated the MR, and reduced the vasomotor responses to exogenous S1P. The vascular data confirms that the S1P₁R-AMPK-signaling contributes to ΔF508-CFTR’s functional defect.

Liberman and Rodbard described the relationship between low blood pressure (i.e., hypotension), and the increased loss of sweat salt from CF patients expressing the genetic F508 deletion, almost 40 years ago (Liberman and Rodbard 1975). A later study by Super and colleagues found that older female carriers of the F508 mutation had lower systolic and diastolic blood pressure measurements relative to age-matched control patients (Super et al. 2004). Male carriers did not display lower blood pressure measurements. Furthermore, the sweat salt concentration was highest in the female carriers with the lowest blood pressure measurements. Since changes in MAP have been shown to affect CPP in humans (Biestro et al. 1998), it is possible that reductions in CBF could be related to blood pressure. However, in Chapter 4 we show that FVB-129 mice expressing the genetic F508 deletion do not have significant differences in their systolic and diastolic blood pressures, or in their MAP, relative to WT-CFTR expressing mice. The mouse studies suggest that ΔF508-CFTR does not contribute to
hypotension. Therefore, based upon the data presented in Chapter 4, changes in CBF are related to the enhanced myogenic reactivity and upregulation of S1P-signaling of the cerebral microvasculature, like the PCAs assessed.

CF patients are known to have increased pulmonary inflammation (Elizur et al. 2008), and the inflammation is in response to reduced clearance of bacteria and pathogens due to mucous buildup (Ratjen 2006). Additionally, the local inflammation eventually becomes systemic (Elizur et al. 2008). S1P and S1P,R-signaling is critical in the immune system (see review by Spiegel and Milstein 2011), and the reduced uptake of S1P could contribute to the systemic inflammatory profile, and exacerbates the CF disease phenotype further. Restoring CFTR’s ability to regulate S1P’s bioavailability may have beneficial effects on CF-associated inflammation.

6.5 CFTR in the MR

The murine VSMCs from mesenteric resistance arteries express the mature Band C form of CFTR, because antibodies detect a molecular weight band near 180 kDa (Meissner et al. 2012). Additionally, our laboratory has used membrane fractionation to demonstrate that the VSMC-WT-CFTR protein is in the plasma membrane fraction (Meissner et al. 2012). In Chapter 5, we show the VSMC-WT-CFTR is a functional channel because it secretes iodide in response to FSK, like the BHK-WT-CFTR that stably expresses the human epithelial isoform. From these data, it appears the vascular CFTR shares functional characteristics to the epithelial CFTR.

The presence of a functional vascular CFTR channel compels a discussion into the role of Cl− conductance in the MR, of resistance arteries. Secretion of Cl− by mechanosensitive Cl− channels expressed in VSMCs may contribute to the membrane depolarization associated with
pressure elevation (see review by Schubert and Brayden 2005, Schubert and Mulvany 1999). Doughty and colleagues demonstrated decreased pressure-dependent vasoconstriction in isolated rat cerebral arteries, after treatment with 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) (Doughty et al. 1998), a Cl⁻ channel blocker. However, NPPB also inhibited K⁺-dependent vasoconstriction suggesting the compound is non-specific. In a subsequent study, Doughty and Langton used self-referencing ion selective (SERIS) electrodes, and demonstrated a positive correlation between pressure-dependent vasoconstriction, and the amount of Cl⁻ secreted (Doughty and Langton 2001). Additionally, tamoxifen was shown to block both the Cl⁻ secretion and pressure-dependent vasoconstriction, but this drug also inhibits VOCCs in VSMCs (Song et al. 1996). Since VOCCs are critical to the MR, decreasing their activity could account for the decreased myogenic reactivity. Additional studies have observed non-specificity of other Cl⁻ channel blockers such as 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) (Wang et al. 1997), and glibenclamide (Nobusawa et al. 2000, Rabe et al. 1995). Thus, it has been difficult to elucidate the role of Cl⁻ conductance in the MR of resistance arteries. Cell-based experiments have demonstrated that CFTR is mechanosensitive (Gray 2010, Zhang et al. 2010). Theoretically the inhibition of Cl⁻ conductance could lead to hyperpolarization, and therefore an appropriate condition for activation of the downstream VOCCs will be absent. The end result would likely be vasodilation, and in the artery studies we would have expected to observe little-to-no pressure-dependent vasoconstriction in the ΔF508-CFTR PCAs. However, we observed S1P-dependent enhancement of myogenic reactivity in studies with the mutant mice, and this suggests that decreased anion secretion is not responsible. Additionally, the results support our previous work with CFTR_{inh}-172 treatment in hamster gracilis muscle resistance arteries (Peter et al. 2008), and PCAs from CFTR KO mice (Meissner et al. 2012). In Chapter 5, we found that *in-vitro* treatment of ΔF508-CFTR PCAs with CFTR corrector compound C18 normalized the
myogenic responsiveness in comparison to the WT PCAs. These results suggest that targeting CFTR’s expression and/or function directly can restore the regulation of endogenous S1P-signaling.

6.6 CFTR in HF

Human cardiac tissue (e.g., atrium and ventricles) expresses mRNA for both the alternatively spliced (i.e., alternative splicing at exon 5) and the non-spliced form of CFTR (Warth et al. 1996). At the protein level, Solbach and co-workers observed expression of Band C (approximately 180 kDa), and also two lower molecular weight bands (approximately 150-140 kDa), in the human left ventricular myocardium (Solbach et al. 2008). Electrophysiological experiments demonstrated that the human cardiac CFTR isoform is a functional Cl⁻ channel (Warth et al. 1996, also see reviews by Duan et al. 2005, Sorota 1999), but the importance of CFTR in the normal functioning of the heart is unclear. For example, CFTR KO mice do not have electrocardiogram parameters that are different from WT mice (Duan 2009), and other experiments in mice have suggested that CFTR protects the heart against ischemic and reperfusion injury (Xiang et al. 2011, Duan 2009, Diaz et al. 1999). In humans, Solbach and colleagues observed reduced CFTR protein expression in the myocardium of HF patients (Solbach et al. 2008), relative to non-HF patients. Therefore, it is possible that abnormal CFTR expression and/or function in the heart contribute to the tissue damage and decreased CO, observed in HF, and more experiments are required to better assess this.

The systemic effects of HF (e.g., reduced tissue and organ perfusion) are related to the increased TPR, a compensatory mechanism, and the augmentation in the MR of resistance arteries is responsible for the increase (see Section 1.5.3). Since vascular CFTR regulates the myogenic reactivity of resistance arteries, then CFTR expression and/or function contribute to
the regulation of TPR. Our previous work in mice demonstrates that TNFα is responsible for
down-regulating CFTR’s protein expression in HF, and TPR is enhanced as a result (Meissner et
al. 2012). In Chapter 5, we show that chronic TNFα treatment (24 hours) in VSMC-WT-CFTR
cells, to simulate the HF condition in-vitro, reduces CFTR’s protein expression and function
(e.g., FSK-stimulated iodide efflux and S1P-FITC uptake). However, acute TNFα treatment did
not affect the FSK-stimulated iodide efflux of BHK-WT-CFTR cells (Supplemental Figure 8B),
and this suggests that TNFα has a genomic effect on CFTR function by down-regulating mRNA
expression. TNFα has also been shown to stimulate the in-vitro phosphorylation and activation
of SphK1 (Sun et al. 2010), which proposes a direct role for TNFα in S1P-signaling. Indeed, our
laboratory recently demonstrated that in-vitro TNFα treatment enhances the MR for WT PCAs,
but not the PCAs from SphK1-KO mice (Yang et al. 2012), and this supports a link between
TNFα and the pressure-dependent production of S1P. Furthermore, PCAs from HF-SphK1-KO
mice had less myogenic responsiveness and were unresponsive to in-vitro Etanercept treatment,
relative to PCAs from HF-WT mice (Yang et al. 2012). Thus, there are two ways that TNFα
may enhance the bioavailability of S1P in HF: (i) directly by stimulating the SphK1-dependent
production of S1P, and (ii) indirectly by affecting the genomic and protein expression of CFTR.
In Chapter 5, we show C18-dependent (e.g., in-vitro, in-vivo) enhancement of CFTR protein
expression, normalization of CFTR function (e.g., channel and S1P-uptake), and normalization
of cerebral microvascular behavior (e.g., MR, CBF) in simulated and mouse models of HF. We
believe the positive effects on CFTR expression and function restores the degradation of S1P
(e.g., by SPP1), and the regulation of S1P bioavailability is re-established even though the
upstream production remains abnormal.
6.7 CFTR uptakes S1P and regulates its bioavailability

The activation of SphK1 leads to the production of S1P into intracellular and extracellular compartments. However, S1P is a zwitterion with a polar phosphate group and is unlikely to simply “flip-flop” across the plasma membrane (see reviews by Rosen et al. 2013, Hannun and Obeid 2008, Rosen and Goetzi 2005). The exact mechanism of its transport out of the cell is not known, and is enigmatic for most cell-types. A review of the published literature suggests three mechanisms for S1P-export. The first is transporter-mediated export, as demonstrated in immune cells (Mitra et al. 2002, also see review by Kim et al. 2009) and in the MCF-7 breast cancer cell line (Takabe et al. 2010). Takabe and colleagues showed the ABCC1 transporter (multidrug resistance protein 1 – Mrp 1), and the ABCG2 transporter (breast cancer resistance protein-1 – Bcrp1) export S1P (Takabe et al. 2010). Yet, mice deficient in some of the ATP-transporters thought to export S1P in-vitro do not have altered plasma S1P levels (reviewed in Lekvau 2013), and this suggests the in-vivo S1P-export mechanism is independent of the ABC-transporters that have been studied thus far. The second proposed mechanism is the export of SphK1 directly to the extracellular environment, and the subsequent production of S1P (Venkataraman et al. 2006). This mechanism is specific to the SphK1-α isoform, primarily expressed in endothelial cells, and therefore it is unknown whether this can be applied to VSMCs and the MR. Finally, Johnson and colleagues demonstrated in-vitro activation of PKC promotes SphK1’s translocation to the plasma membrane in HEK 293 cells, and increased the concentration of S1P in cell media (Johnson et al. 2002), which suggests SphK1 directly secretes S1P into the extracellular environment. While a compelling finding, once again whether this mechanism is present in VSMCs remains untested. Our studies do not address the mechanism for S1P-export, and we can only predict that endogenous S1P is exported in an autocrine manner, as others have (Kim et al. 2009).
The import of exogenous S1P is CFTR-dependent, as first demonstrated by Boujaoude and co-workers in their seminal *in-vitro* study. In Chapters 4 and 5, we show that both the human epithelial (e.g., BHK stable cell-line) and murine vascular isoforms of CFTR can uptake fluorescently labeled S1P (i.e., S1P-FITC). This supports previous findings from our laboratory (Meissner *et al.* 2012), and adds to the work of Boujaoude *et al.* CFTR is instrumental in reducing the *in-vitro* activation of S1PR-dependent signaling pathways (Meissner *et al.* 2012, Boujaoude *et al.* 2001), and uptake may divert S1P from its receptors. The inability of BHK-G551D-CFTR cells to uptake S1P-FITC, demonstrated in Chapter 4, suggests that the transport requires a functional channel or ATP-dependent gating mechanism. The absence of S1P-FITC uptake with BHK-ΔF508-CFTR cells, also shown in Chapter 4, suggests that a functional channel and proper CFTR expression in the plasma membrane is a requirement for the uptake. Our studies do not directly address the mechanism for the CFTR-dependent uptake of S1P. However, some proposed models based upon our data are discussed next.

In addition to the transport S1P and anions (e.g., Cl^−, I^−, HCO₃^−) (see Section 1.5, also Tang *et al.* 2009), CFTR also regulates the flux of GSH (see Section 4.5.3). We can then postulate that CFTR shares transport characteristics with the other ABC-transporters. Therefore examining how other ABC-transporters move substances across the plasma membrane can offer insight into the CFTR-dependent uptake of S1P. The ABCB1 transporter, also known as the Permeability-glycoprotein (P-gp), is responsible for the efflux of several types of materials including drugs (Goard *et al.* 2010, Fromm *et al.* 1999, also see reviews by Wessler *et al.* 2013, Sharom 2006), and lipids (Eckford and Sharom 2005, Elferink *et al.* 1997, also see review by Mizutani *et al.* 2008). There are three common models for the mechanism of P-gp-dependent transport (see reviews by Sharom 2011, Sharom 2008), and because P-gp and CFTR share structural similarities, the mechanistic models will be discussed (Figure 6-2). The first is the
classical pump model, where polar substances are directly transported through the hydrophilic path formed by the membrane-spanning domains (Sharom 2011, Sharom 2008). If S1P entered the cell by this mechanism, it would imply CFTR is an antiporter. In this scenario, the secretion of anions down the electrochemical gradient compensates for the uptake of S1P, and may equilibrate the cellular charge distribution. However, in Chapter 3 we show that S1P inhibits the FSK-stimulated iodide efflux in BHK-WT-CFTR cells, and this argues against the classical model. Furthermore, S1P is a hydrophobic zwitterion (i.e., has a negatively charged phosphate head, and its hydrocarbon chain is non-polar), and the structural properties suggest it cannot pass freely through the hydrophilic path formed by the pore. By itself S1P is insoluble in aqueous solutions (see review by Rosen \textit{et al.} 2013), and for our experiments S1P must first be reconstituted into a fatty-acid free BSA solution.

In the second proposed model, hydrophobic substances partition near or into the lipid bilayer and interact with P-gp in the cytoplasmic, inner leaflet (Qu and Sharom 2002). Then substances are transported into solution on the opposite end via a hydrophobic “vacuum cleaner”-like mechanism (Higgins and Gottesman 1992, also see review by Sharom 2008). As a possible mechanism for CFTR, S1P partitioning near the outer leaflet or into the lipid bilayer, would be followed by interaction with CFTR in the plasma membrane and then uptake (Figure 6-2). The vacuum model insinuates that the proximity of S1P to the plasma membrane and the ability for S1P to partition into the lipid bilayer are critical to uptake, but the zwitterionic head of S1P makes it unlikely to enter the bilayer, and this may contribute to its low content in membranes (see reviews by Kim \textit{et al.} 2009, Hannun and Obeid 2008). Experiments by Boujaoude and colleagues with labeled S1P have demonstrated an exponential relationship between the concentration of S1P and the uptake into cells, with a plateau near 80 \( \mu \text{M} \) (Boujaoude \textit{et al.} 2001). Additionally, our laboratory has previously shown that co-incubation
with unlabeled S1P decreases the uptake of S1P-FITC in murine VSMCs (Meissner et al. 2012). The dose-dependency and the competitive inhibition data suggest the S1P transport mechanism is tightly regulated, and does not solely rely on the proximity of S1P. Instead, the loss of uptake could be explained by the presence of discrete S1P binding sites in the CFTR protein. These sites may become saturated at high concentrations of labeled S1P, or when unlabeled S1P is co-incubated.

The Sharom laboratory demonstrated that purified and reconstituted P-gp, in phosphatidylcholine proteoliposomes, transports lipids (e.g., glycolipids, phospholipids) by direct binding, and then movement of the lipid from the inner to outer leaflet. This constitutes a flippase mechanism (Eckord and Sharom 2005, Sharom et al. 2005, Romsicki and Sharom 2001, also see review by Sharom 2011), and it is the third proposed mechanism for P-gp-dependent transport. For the CFTR-dependent uptake, S1P partitioning near or into the lipid bilayer may occur, and then S1P interacts with a binding pocket (or pockets) in the CFTR protein, at the outer leaflet. Then conformational changes could occur that transport S1P into the cell (Figure 6-2). The vacuum model is also thought to require lipid-binding sites (see review by Sharom 2006), and it is difficult to differentiate between the flippase and vacuum models.
Figure 6-2. Proposed mechanisms for the CFTR-dependent uptake of sphingosine-1-phosphate (S1P).

Based upon the published data on the mechanism of action for the P-glycoprotein, we propose there are three possible mechanisms by which CFTR can uptake S1P: (1) In the channel model, S1P is transported through the aqueous channel pore, and at the same time anions are secreted out. In this model, CFTR behaves as an anti-porter; (2) In the vacuum model, S1P accumulates near the plasma membrane and interacts with binding sites within the CFTR protein. The lipid is then transported from the extracellular to the intracellular environment. (3) In the flippase model, S1P interacts with a strong binding site present within the CFTR protein, at the outer leaflet. The interaction causes conformational changes, and S1P then binds to a weak binding site near the inner leaflet, and is then released into the intracellular environment.
6.8 Limitations

6.8.1 Aim 1

We assessed the S737 inhibitory residue of CFTR, which is one of two residues in the R-domain that can be phosphorylated by AMPK, and found that substituting this residue with alanine prevented the S1P-mediated reduction in FSK-stimulated iodide efflux. However, a limitation of our first study is the role of S768, the second inhibitory residue that can be phosphorylated by AMPK. Our results do not exclude the S768 residue because the two sites could act in cooperation with one another. Furthermore, the relative proximity of S737 and S768 could allow for conformational changes in the R-domain when a single site is phosphorylated.

In the first study, we also used a cell-permeable inhibitor of AMPK, Compound C, and found that it prevented the effect of S1P on iodide efflux. Compound C is an ATP-competitive inhibitor of AMPK, and is reversible (Zhou et al. 2001). Although, Compound C has no effect in AMPK KO mice, demonstrating the treatment is specific (Li et al. 2007), some in-vitro studies have demonstrated that Compound C can inhibit processes independent of AMPK (see comprehensive review by Viollet 2010). Therefore, we validated the contribution of AMPK with siRNA transfection.

Kimura and colleagues demonstrated that S1P stimulates the phosphorylation of AMPK through the S1P₁R (Kimura et al. 2010). However, the S1P₃R has also been shown to stimulate AMPK in-vitro in response to S1P (Murakami et al. 2010). To study the receptor influence we used VPC 23019, a receptor antagonist against both the S1P₁R and the S1P₃R, which binds with greater affinity to S1P₁R (Davis et al. 2005). Then we confirmed the contribution of the S1P₁R with a specific agonist (SEW 2871). A future assessment is the use of the specific S1P₁R antagonist W146 (Lim et al. 2012), and the specific S1P₃R antagonist TY-52156 (Murakami et al. 2010) and this would provide further support for the role of S1P₁R.
6.8.2 Aim 2

Our studies with the FVB-129-ΔF508-CFTR mice, suggests that the enhanced myogenic reactivity observed in ΔF508-CFTR PCAs is due to enhanced endogenous S1P-signaling. However, Ca\textsuperscript{2+} is a target of S1P and CFTR is a regulator of and regulated by Ca\textsuperscript{2+} (reviewed in Billet and Hanrahan 2013), and the arteries from the mutant mice also displayed enhanced vasoconstriction to two concentrations of PE. Therefore, abnormal Ca\textsuperscript{2+} mobilization from intracellular stores (see Divangahi et al. 2009) and/or influx via store-operated Ca\textsuperscript{2+} channels (e.g., ORAI, see Billet and Hanrahan 2013) may be enhanced in the arteries from the mutant mice, and a limitation is we did not assess changes in Ca\textsuperscript{2+}.

There are currently three established mouse models used for studying the ΔF508-CFTR mutation (reviewed in Guilbault et al. 2007), and the FVB 129 strain displays normal mRNA expression of CFTR, a low mortality rate (Wilke et al. 2011). FVB-129-ΔF508-CFTR mice display bowel obstructions (French et al. 1996, van Doornick et al. 1995), and abnormal nasal potential differences (Saussereau et al. 2013), which are classical symptoms of the CF disease in humans (Speck and Charles 2008, Delmarco et al. 1997). The mice do not display dysfunction in their respiratory system (van Doornick et al. 1995), which is the primary clinical symptom in human CF patients (reviewed in Livragi and Randell 2007). However, we used FVB-129-ΔF508-CFTR mice as a model to assess the role of vascular CFTR in the MR, and were not interested in the airway dysfunction. In Chapter 4, we showed that S1P-signaling contributes to ΔF508-CFTR’s dysfunction in the mutant’s PCAs, and FVB-129-ΔF508-CFTR mice have decreased CBF relative to WT mice. This supports the use of this mouse model to assess the cerebral effects of CF.
6.8.3  Aim 3

In Chapter 5, we found that acute treatment of HF PCAs and ΔF508-CFTR PCAs with the C18 corrector compound normalized the myogenic responsiveness, and we attributed this to a proteostatic effect by C18. In addition to C18’s effects on processing, two groups have presented preliminary evidence that suggests C18 is a potentiator of CFTR function (Eckford et al. 2014, Lui and Sheppard 2011-Abstract). The classical definition of potentiator is a drug or treatment that enhances the channel function of CFTR, and a potentiator effect by C18 is an attractive explanation for the acute treatment results. However, we did not specifically assess the possibility of potentiation. One way to address this limitation would be the use of an established potentiator compound, for example VX-770 (see Section 6.9.3).

We used ligation of the LAD coronary artery to induce myocardial infarction (MI), and this is a normotensive model of HF in mice. There are other mouse models of HF, and these represent cardiomyopathy (Ross 2002, Arber et al. 1997), or pressure overload (Rockman et al. 1994, Rockman et al. 1991). Overall the techniques to induce HF in mice cause hypertrophy of the heart as a compensatory mechanism. The strengths of the LAD model include the ability to assess a variety of modalities for cardiovascular physiology, and the ability to perform the procedure in different mouse strains or KO models (Patten and Hall-Porter 2009), and this is thought to ensure relevancy to the clinic. Indeed, the direct effect of the ligation procedure in mice is the development of a myocardial scar, which then causes dilated cardiomyopathy clinically, and this is comparable to human HF (Breckenridge 2010). Nonetheless, there are drawbacks to the LAD procedure such as the expense and expertise required (Breckenridge 2010).

There are anatomical differences in the mouse versus human cardiovascular systems, the most obvious being the heart itself. The mouse heart has a more rapid heart rate than the human
heart (e.g., 400 to 600 bpm in mice versus 60-90 bpm in humans). The difference in heart rate may be due to changes in \(\text{Ca}^{2+}\)-handling (Mayosi et al. 2006). Differences also exist in the expression pattern for the myosin isoforms between mice and humans (Swynhedauw 1986). Nevertheless, the common features are generally accepted to be similar when contrasting mice and humans (Breckenridge 2010). Furthermore, we did not assess heart function directly, and instead were interested in the microvascular effects of reduced CO.

6.9 Future Directions

6.9.1 The S1P\(_1\)R-AMPK-signaling pathway and CFTR dysfunction

In Chapter 4, we found that the S1P\(_1\)R -AMPK-signaling pathway contributes to the expression and function defects of \(\Delta\text{F508-CFTR}\). The data warrants an investigation of whether VPC 23019 treatment or AMPK siRNA transfection enhances the processing and maturation of the mutant protein. Metabolic labeling and pulse-chase experiment can be used to evaluate the conversion of Band B to Band C (Sharma et al. 2001), and would provide information on the processing and maturation. The presence of cyclohexamide would eliminate the contribution of transcription and translation. Effects on \(\Delta\text{F508-CFTR}\)’s degradation of stimulation or inhibition of S1P\(_1\)R -AMPK-signaling can be assessed with ubiquitin antibodies (Sharma et al. 2004). We also found an additive effect of AMPK siRNA transfection at 27°C, which suggests the mechanism of action for AMPK’s inhibition is different than low-temperature. Inhibition of this signaling pathway could enhance the half-life or stability of low-temperature rescued \(\Delta\text{F508-CFTR}\). The \(\Delta\text{F508-CFTR}\) protein expressed in the BHK stable cell-line has an extracellular HA tag, and therefore we can label membrane-bound protein and track the loss in signal over time (Sharma et al. 2004, Sharma et al. 2001). The increased expression of \(\Delta\text{F508-CFTR}\) in membrane fractions, after acute treatment with Compound C at 37°C suggests that inhibition of
AMPK may promote the trafficking of the mutant from sub-membrane compartments to the plasma membrane. This can be assessed with fluorescence microscopy and markers against the ER, Golgi, and endosomes.

The Ca\(^{2+}\)-signaling pathway can be assessed in the isolated PCAs from FVB-129-WT-CFTR and ΔF508-CFTR mice directly with the Fura-2 dye, as previously shown by our laboratory (Lidington et al. 2009, Peter et al. 2008, Bolz et al. 2003). Another future direction with these mice is determining the mRNA expression of S1P-signaling components (e.g., S1P\(_1\)R, SphK1, SPP1) with qPCR. It is possible that the genetic deletion of the F508 residue alters the expression pattern of these components, relative to WT mice.

Wong and colleagues at the Hospital for Sick Children in Toronto recently characterized human embryonic stem cells expressing WT-CFTR, as capable of differentiating into functional airway cells (Wong et al. 2012). It would be interesting to test whether the S1P\(_1\)R-AMPK-signaling pathway contributes to functional defects in this cell line, because it would provide information on the effect in airways. Then the effect of inhibiting the S1P\(_1\)R-AMPK-signaling pathway can be assessed in pluripotent stem cells from CF patients (Wong et al. 2012). The experiments would provide an additional translational aspect to our studies. Future vascular work would focus on human resistance arteries because our laboratory has a protocol in place for the isolation and cannulation of resistance arteries from surgical patients.

6.9.2 Correction of CFTR in HF

In Chapter 5, we found that C18 treatment partially reversed the TNF\(\alpha\)-dependent down-regulation of CFTR protein expression in cells, normalized the expression in cerebral tissue from HF mice, and C18 also normalized CFTR function. We believe that C18 enhances the stability of CFTR in the plasma membrane, or treatment indirectly decreases degradation. Stability and
membrane half-life can be assessed with the BHK-WT-CFTR cells that express an extracellular HA tag (Sharma et al. 2004). Degradation can be assessed with the previously mentioned ubiquitin antibodies.

As previously mentioned, C18 may possess dual corrector and potentiator function. Therefore acute *in-vitro* treatment with VX-770 (i.e., Kalydeco), a FDA-approved CFTR potentiator, of murine HF PCAs would answer whether potentiation of CFTR is a mechanism of the normalization of the MR. Similarly, a compound that only affects processing could be employed as a positive control for correction, for example VX-809, VRT-325, or Corr-4a. Furthermore, *in-vitro* treatment of resistance arteries from HF patients would provide a translational aspect.

Another future direction is the specificity of CFTR’s role in the myogenic responsiveness of PCAs, and the specificity of C18-dependent normalization. We have preliminary evidence that acute *in-vitro* C18 treatment does not affect the MR of PCAs from CFTR KO mice (data not shown), but these mice are difficult to breed and have a high mortality rate. Thus, we propose using a conditional VSMC KO mouse model with the Cre and flox system (Regan et al. 2000, also see review by Deng 2012). In this system, a mouse strain with the CFTR gene flanked by two loxP sites (CFTR flox/flox) are crossed with mice expressing the chimeric Cre recombinase (Cre-ER<sup>T2</sup>), under the control of the smooth muscle-specific promoter smooth muscle myosin heavy chain. The Cre-ER<sup>T2</sup> recombinase activity is controlled by a tamoxifen ligand-binding domain (ER<sup>T2</sup>), and this allows for the temporal gene deletion. Activation of Cre recombinase leads to the excision of the target gene flanked by the LoxP sites, and subsequently the targeted gene is deleted. Therefore, isolated PCAs from the VSMC targeted deletion of the CFTR would display the same enhancement of the myogenic responsiveness and constriction to S1P as the HF mice, if the enhancements were CFTR-specific. The mice would also display the reduction in
CBF. Then *in-vitro* and *in-vivo* treatments with C18 would be performed, and we would expect to see no effect of the treatment if it is indeed CFTR-specific.

6.9.3 **Assessment of the mechanism for CFTR-dependent uptake of S1P**

The structural properties of S1P suggest it does not travel through the hydrophilic path formed by the pore, but this does not rule out a binding site within the CFTR pore. The hydrocarbon chain may bind directly to hydrophobic, non-polar amino acids sites within the CFTR pore. Alternatively, the phosphate head group of S1P could bind to a CFTR polar binding site near the outer leaflet, and this would orient the hydrocarbon chain towards the lipid bilayer. Both scenarios could result in CFTR-mediated “flip-flop” of S1P. Hence, determining if there is an S1P binding site in the CFTR sequence would aid immensely in evaluating the previously discussed flippase model, and also the vacuum model. Alvarez and colleagues demonstrated the binding between the TNF receptor-associated factor 2 (TRAF2) and S1P (Alvarez *et al.* 2010). The interaction is important for the inflammatory effects of TNFα, and establishes TRAF2 as an intracellular target for S1P. Importantly, the binding cavity of TRAF2 contains a hydrophobic region (F45, L58, A59, L62, A90, F91, F92), and a positively charged region (R43, R97) that may stabilize the phosphate group of S1P (Alvarez *et al.* 2010). Remarkably, there is some alignment between the residues that compose this binding cavity and the CFTR protein, when the full amino acid sequences of TRAF2 and CFTR are analyzed (Figure 6-3). The analysis shows an identical match at residues F45 and F236 for TRAF2 and CFTR respectively. Furthermore, semi-conserved substitution matches are present at the following amino acids, in the respective TRAF2 and CFTR sequences: L58-I343, L62-M347, and R97-K447. There are also interesting categorical matches at other sites. The A59 and A90 residues of TRAF2 align with the V344 and V440 residues of CFTR respectively. These amino acids are non-polar and contain hydrophobic,
aliphatic side chains. Although the F91 and L441 residues, in TRAF2 and CFTR respectively, could match because these residues are also non-polar and contain hydrophobic side chains, the aromatic ring of phenylalanine is not an ideal structural match for the isobutyl side chain of leucine. The alignment for the majority of the residues involves CFTR’s MSD1, and the cytoplasmic region between this domain and NBD1 (see Figure 1-3 for CFTR structure). The K447 residue is present within the NBD1, and the overall sequence alignment does not involve residues within the R-domain or NBD2. However, the sequence alignment cannot predict whether the cavity is within CFTR’s pore or at a region close to the plasma membrane. Nevertheless, this preliminary evidence suggests the CFTR protein sequence possesses a S1P binding cavity. Extensive biochemical experiments are required to prove the existence of the binding site.
**Figure 6-3. Sequence alignment of TRAF2 and CFTR suggests a possible S1P binding pocket in the CFTR protein.**

The full-length FASTA amino acid sequences of human TRAF2 (accession number: NP_066961) and human CFTR (accession number: NP_000483) were acquired from the NCBI database. An alignment was performed using the T-COFFEE Multiple Sequence Alignment Server (www.tcoffee.vital-it.ch, Di Tommaso et al. 2011, Notredam et al. 2000). The result was exported to the BoxShade Server (www.ch.embnet.org), and the alignments were visualized. Identical matches are denoted as “*” and semi-conserved substitution matches (i.e., amino acids that have similar structure and properties) are denoted as “.” The red boxes outline the sequence of the S1P binding cavity identified by Alvarez et al. for TRAF2 (R43, F45, L58, A59, L62, A90, F91, F92, R97), and their alignment with CFTR residues.
6.10 Conclusion

This thesis advances the fields of microvascular physiology, and the CF and HF diseases, which are two diseases associated with S1P-signaling and the CFTR protein. We demonstrate that S1P is an S1P$_1$R-AMPK-phosphorylation dependent novel modulator of CFTR’s channel function (e.g., FSK and cAMP-stimulated iodide efflux). Furthermore, the S1P-signaling pathway contributes or aggravates the expression and function defects of a major CF disease causing CFTR mutation (e.g., ∆F508-CFTR). Mice that express the genetic ∆F508-CFTR mutation display a distinct cerebrovascular phenotype (e.g., decreased CBF), and this may explain the CNS dysfunction observed in some human CF patients. Since, the endogenous and exogenous S1P-signaling pathways through the S1P$_1$R are enhanced in the cerebral microvasculature of the mutant mice, we believe CFTR is a cerebrovascular regulator. Our data suggests that bioavailability of S1P is altered when CFTR’s expression and/or function are abnormal, and this could have implication in human CF patients (e.g., CNS dysfunction, inflammation). Finally, we demonstrated that CFTR is a new microvascular drug-target for the HF disease. Therapeutics that enhance CFTR expression and/or function represent an incredible resource for the management of cerebrovascular dysfunction observed in HF patients, and the potential exists to improve quality of life for these patients. Knowledge that arises from the thesis strongly suggest that CFTR’s vascular function can be regulated by S1P, and targeting this regulation is worth exploring for the CF and HF disease models.
Supplemental Figures
Supplemental Figure 1. Baby Hamster Kidney (BHK) cells endogenously express components of the sphingosine-1-phosphate (S1P) signaling pathway.

End point RT-PCR was performed on cDNA generated from BHK naïve cells and BHK-WT-CFTR stably transfected cells. Primer sets were against mouse isoforms of S1P-signaling components to detect endogenous expression. (A) The coloured text and lines correspond to the primer sets that were used in those particular samples. The cells express: SphK1 (244 bp RT-PCR product), S1P2R (170 bp RT-PCR product), and SPP1 (203 bp RT-PCR product). (B) The cells have little visible expression of the S1P3R (137 bp RT-PCR product), but there is expression of the S1P1R (395 bp RT-PCR product). (C) The cells also express GAPDH (111 bp). RT-PCR product.)
Supplemental Figure 2. Acute treatment of BHK-WT-CFTR cells with exogenous sphingosine-1-phosphate (S1P) does not affect the expression of total CFTR protein.

BHK-WT-CFTR cells were treated with either DMSO (30 seconds, 0.1% v/v, n=4 cell culture dishes) or S1P for various time-points (30 seconds to 1 hour, 1 µmol/L, n=4 cell culture dishes for all time-points). (A) CFTR’s total protein expression was detected with the M3A7 antibody, and the normalized signal to alpha-tubulin was not significantly different after S1P treatment. (B) The same samples were probed with the 67D4 CFTR phosphosensitive antibody, and there was a significant decrease in the normalized signaling (to total CFTR protein), and to (C) alpha-tubulin. S1P promotes phosphorylation of CFTR and does not affect CFTR’s total protein expression. Phosphorylation can be detected by normalizing the 67D4 signal to either total CFTR protein (M3A7) or to a loading control (alpha-tubulin). *denotes p<0.05 with one-way ANOVA and selected comparisons relative to DMSO control treatment.
Supplemental Figure 3. Sphingosine-1-phosphate (S1P) treatment does not affect the cell-surface expression of WT-CFTR. S1P-FITC stimulates the phosphorylation of AMP-activated protein kinase (AMPK).

(A) BHK-WT-CFTR cells were treated with either DMSO (30 seconds, 0.1% v/v, n=7 cell culture dishes) or S1P (30 seconds, 1 µmol/L, n=8 cell culture dishes), and cell-surface biotinylation was performed to detect the change in CFTR’s protein expression in the plasma membrane. Treatment with S1P did not significantly affect the membrane expression of CFTR (normalized to total CFTR protein). (B) BHK-WT-CFTR cells were treated with either BSA (30 seconds, 4% v/v, n=4 cell culture dishes) or S1P-FITC (30 seconds, 1 µmol/L, n=6 cell culture dishes), and the expression of p-AMPK and total AMPK was detected. S1P-FITC significantly increased the ratio of p-AMPK: total AMPK relative to the BSA treatment group. *denotes p<0.05 unpaired t-test.
Supplemental Figure 4. Pharmacologic inhibition of AMP-activated protein kinase (AMPK) increases the protein expression of ΔF508-CFTR in plasma membrane fractions. BHK-ΔF508-CFTR cells were treated with Compound C (20 µmol/L) for various time points up to 60 minutes, and membrane fractionation was performed to detect CFTR’s protein in the plasma membrane (PM) relative to the intracellular (IC) fractions. The 60 minutes treatment with Compound C (n=4 cell culture dishes) significantly increased the expression in the PM fraction (normalized to total protein) relative to the untreated control group (n=4 cell culture dishes). Concurrently, Compound C treatment significantly decreased the expression in the IC fraction (normalized to total protein) at the 10 minutes (n=4 cell culture dishes), 30 minutes (n=3 cell culture dishes), and 60 minutes time points. *denotes p<0.05 for the PM fraction relative to untreated. #denotes p<0.05 for the IC fraction relative to untreated one-way ANOVA with Bonferroni’s selected comparisons.
Supplemental Figure 5. Representative tracings for pressure-dependent vasoconstriction of proximal posterior cerebral arteries (PCAs) from FVB-129-WT-CFTR and FVB-129-ΔF508-CFTR

Isolated and cannulated PCAs were subjected to incremental increases (20 mmHg) of transmural pressure, and the tracings were recorded for the change in outer wall diameter. As can be seen, (A) WT-CFTR PCAs has little-to-no vasoconstriction in response to pressure, whereas (B) FVB-129-F508-CFTR PCAs displayed vasoconstriction, relative to Ca\(^{2+}\)-free.
Supplemental Figure 6. Quantitative PCR (qPCR) demonstrate greater mRNA expression of CFTR and the S1P1R in murine cerebral tissue. (A) mRNA was extracted from skeletal and cerebral tissues samples from WT C57/Bl6 mice, and cDNA was produced. Quantitative PCR (qPCR) was performed using primer sets against CFTR and glucose-6-phosphate dehydrogenase (G6PD). The mRNA expression of CFTR (normalized to G6PD) was significantly greater in the cerebral tissue samples (n=6 samples) than in the skeletal muscle tissue samples (n=6 samples). *denotes p<0.05 unpaired t-test. (B) In the cerebral tissue samples, qPCR was also performed using primer sets against the S1P1R, S1P2R, and S1P3R. The mRNA expression of S1P1R (normalized to G6PD) was significantly greater than the expression of S1P2R or S1P3R (n=7 samples for all). *denotes p<0.05 Friedman’s ANOVA and Dunn’s multiple comparison post hoc test.
Supplemental Figure 7. Representative tracings for the sphingosine-1-phosphate (S1P) dependent vasoconstriction of proximal posterior cerebral arteries (PCAs) isolated and cannulated from FVB-129-ΔF508-CFTR mice. Proximal PCAs were isolated and cannulated from C57/Bl6 WT-CFTR mice, and the concentration-dependent vasoconstriction to S1P was recorded. This was a pilot study to assess whether two S1P vasomotor response experiments could be done in the same artery. As shown in the representative tracings of the artery’s outer wall diameter, S1P promotes vasoconstriction (S1P DR 1). The arteries were washed for two hours with standard MOPS buffer, and a second dose-response assessment was performed. However, the artery remained constricted after the first assessment. Therefore, it is very difficult to wash out the highest dose of S1P (10^{-6} mol/L) in PCAs. The second assessment also showed S1P-dependent vasoconstriction (S1P DR 2). Then subsequent experiments, we excluded the highest dose of S1P, and this allowed for more reliable washout of the agonist. We also paired our treatments with contemporaneous arteries.
Supplemental Figure 8. C18 treatment partially reverses the tumor necrosis factor-alpha-(TNFα)-dependent down-regulation in CFTR's protein expression and normalizes CFTR's function in BHK-WT-CFTR cells.

(A) C18 treatment (6 µmol/L) significantly increased the expression of the CFTR protein (normalized to total protein) in the BHK-WT-CFTR stable-cell line after three to nine hours incubation (n=3-4 cell culture dishes). *denotes p<0.05 relative to time point 0 hours.

(B) Coincubation with C18 (6 µmol/L) and TNFα (10 ng/mL) for 24 hours (ratio of CFTR: total protein= 1.38±0.0577, n=4 cell culture dishes) increased CFTR’s protein expression (normalized to total protein) relative to TNFα treatment alone (0.948±0.0361n=4 cell culture dishes). However, the expression was still significantly less than the untreated control (1.88±0.0325, n=3 cell culture dishes). *denotes p<0.05 relative to untreated control. +denotes p<0.05 relative to TNFα alone.

(C) Subsequent C18 treatment after 24 hours TNFα-dependent down-regulation(n=3 cell culture dishes) significantly increased the fluorescence intensity of S1P-FITC relative to TNFα alone (mean fluorescence intensity= 233±37.8 versus 49.2±20.2 for TNF+C18
and TNF alone respectively, n=4 cell culture dishes) and normalized the value relative to the untreated control (168±16.7, n=13 cell culture dishes). *denotes p<0.05 relative to untreated control. (D) Subsequent C18 treatment after 24 hours TNFα-dependent down-regulation, significantly increased the FSK-stimulated iodide efflux (7.93±0.478 µmol/L, n=6 coverslips) relative to TNFα alone (3.15±0.530 µmol/L, n=6 coverslips), and normalized the efflux relative to the DMSO control treatment (24 hours, 0.1% v/v, 10.3±0.894 µmol/L n=6 coverslips). *denotes p<0.05 relative to DMSO control. All analysis utilized one-way ANOVA and Bonferroni’s selected comparisons.
Supplemental Figure 9. Sequestration of tumor necrosis factor-alpha (TNFα) or media replacement normalizes the TNFα-dependent down-regulation in BHK-WT-CFTR’s iodide efflux.

(A) Subsequent treatment with Etanercept (24 hours, 10 µg/mL in the presence of 10 ng/mL TNFα, 8.32±1.21 µmol/L, n=6 coverslips) after the 24 hours TNFα-dependent down-regulation significantly increased the FSK-stimulated iodide efflux relative to TNFα alone (24 hours, 10 ng/mL, 2.82±0.482 µmol/L, n=6 coverslips), and normalized the efflux of BHK-WT-CFTR cells relative to the DMSO control group (48 hours, 0.1% v/v, 9.49±0.0696 µmol/L, n=9 coverslips). Media replacement with TNFα-free media (24 hours, 11.9±0.511 µmol/L, n=6 coverslips) after the down-regulation also normalized the FSK-stimulated iodide efflux. Additional controls were
CFTRinh-172 treatment (30 minutes, 1 µmol/L, 1.54±0.321 µmol/L, n=6 coverslips) significantly decreased the iodide efflux relative to DMSO, and the absence of cAMP (n=6 coverslips) displayed no detectable efflux. *denotes p<0.05 relative to DMSO, one-way ANOVA and Bonferroni’s selected comparisons. (B) Acute treatments with TNFα (co-stimulation with cAMP cocktail, and pre-treatments for 30 minutes, 3 hours, 9 hours, 10 ng/mL, n=3 coverslips for all), did not affect the FSK-stimulated iodide efflux of BHK-WT-CFTR cells relative to co-stimulation with DMSO (n=3 coverslips). As a control, we performed an acute treatment with sphingosine-1-phosphate (S1P), and found significant reduction in the iodide efflux (3.81±0.562 µmol/L, n=3 coverslips) relative to DMSO. *denotes p<0.05 relative to DMSO, one-way ANOVA and Bonferroni’s selected comparisons.
Supplemental Figure 10. C18 treatment does not affect the sphingosine-1-phosphate (S1P)-dependent phosphorylation of BHK-WT-CFTR cells.

The phosphorylation of CFTR was detected in BHK-WT-CFTR cells with the 67D4 phosphosensitive antibody. Cells treated with S1P (30 seconds, 1 µmol/L, mean ratio of 67D4:α-tubulin= 0.510±0.0112, n=4 cell culture dishes) significantly decreased the 67D4 signal relative to the DMSO control treatment (30 minutes, 0.1% v/v, 1.64±0.176, n=4 cell culture dishes). Co-incubation with C18 did not affect the S1P-dependent decrease in the 67D4 signal (0.845±0.157, n=4 cell culture dishes). Therefore, C18 does not prevent the S1P-S1P1R-AMPK-dependent phosphorylation of WT-CFTR. *denotes p<0.05 relative to DMSO, one-way ANOVA and Bonferroni’s selected comparisons.
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