Cardiovascular Effects and Mechanism(s) of Action of Glucagon-Like Peptide-1 Metabolite, GLP-1(28-36)

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Physiology Department

University of Toronto

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Abstract

The incretin hormone, glucagon-like peptide-1 (GLP-1), is a cardioprotective peptide. GLP-1(28-36) is a C-terminal nonapeptide derived from the cleavage of GLP-1 by the endopeptidase NEP 24.11. We investigated the cardioprotective effects of GLP-1(28-36) in mouse models of myocardial ischemic injury and aimed to discover the cellular and molecular mechanisms involved.

Chronic 14d pre-treatment with GLP-1(28-36) via osmotic minipumps reduced infarct size following total left anterior descending coronary artery occlusion in mice, as compared to control scrambled peptide. Acute pre-treatment with GLP-1(28-36) prior to ischemia prevented myocardial dysfunctions by improving left ventricular developed pressure (LVDP) at the end of reperfusion, as compared to scrambled peptide, in isolated mouse hearts undergoing ischemia/reperfusion injury (IRI). Isolated hearts from Glp1r−/− mice perfused with GLP-1(28-36) showed similar LVDP recovery after IRI, suggesting GLP-1 receptor (GLP-1R)-independent actions. Pharmacological inhibition of soluble adenylyl cyclase (sAC), an intracellular enzyme responsible for catalyzing cAMP synthesis from ATP, abrogated the LVDP recovery of mouse hearts pre-treated with GLP-1(28-36). At the cellular level, GLP-1(28-36) caused concentration-
dependent increases in ATP and cAMP synthesis in mouse coronary artery smooth muscle cells (caSMCs). GLP-1(28-36) underwent cellular uptake, increased cAMP-PKA activity, and prevented cytotoxicity of caSMCs exposed to H₂O₂-mediated oxidative injury, as evidenced by decreased release of LDH. These effects were attenuated with KH7, a sAC-inhibitor, or using caSMCs isolated from sAC−/− mice. Using co-immunoprecipitation and subsequent proteomic analysis, we discovered that GLP-1(28-36) interacts with proteins involved in mitochondrial metabolism. Mitochondrial trifunctional protein subunit α (MTPα) was identified as the most significant binder of GLP-1(28-36) in mouse hearts.

Altogether, my study reveals GLP-1R-independent cardioprotective actions of GLP-1(28-36), localized to caSMCs. We identify sAC as a novel molecular pathway, although the precise mechanism by which GLP-1(28-36) increases ATP production to upregulate sAC activity remains unclear. Future investigations should focus on the role of GLP-1(28-36) in mitochondrial metabolism.
Acknowledgments

I am eternally grateful to my supervisor, Dr. Mansoor Husain, whose leadership, support and constant coaching has guided me throughout my PhD. I would also like to thank my committee members, Dr. Daniel Drucker and Dr. Anthony Gramolini, for continuously providing precious insights and guidance throughout numerous committee meetings, which has helped shape the direction and progress of my thesis. My sincere thanks to Dr. Peter Backx for providing the necessary support to perform the Langendorff experiment in his lab. And many thanks to Dr. Sanja Beca, who taught me the isolated mouse heart technique, and became a friend along the process.

Thank you to all members of the Husain lab, who made everyday a great day to be at work, particularly Dr. Talat Afroze, Dr A. Momen, Dr. Hossein Noyan-Ashraf, Dr. Sarah Steinbach, and Dr. Xuetao Sun. Also, thank you to Dr. Momen, whose surgical skills was instrumental to the success of this project. Thank you to Eric A Shikatani for help with the figures. I would also like to acknowledge Lauren Harris and Bani Bali, for their administrative support and for being always helpful and considerate.

Lastly, I dedicate this thesis to my two children, Aadi and Sarla. They taught me the true meaning of bravery in the face of hardships. Because of them, I never gave up, despite all the challenges we faced in the past five years. So, children, this is for you.
Achievements

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<td>Adenylyl cyclase</td>
</tr>
<tr>
<td>ACS</td>
<td>Acute coronary syndrome</td>
</tr>
<tr>
<td>AKAP</td>
<td>A kinase anchoring proteins</td>
</tr>
<tr>
<td>ANP</td>
<td>Atrial natriuretic peptide</td>
</tr>
<tr>
<td>ATF-1</td>
<td>AMP-dependent transcription factor-1</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>CA</td>
<td>Carbonic Anhydrase</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
</tr>
<tr>
<td>cAMP</td>
<td>3’-5’-cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>caSMC</td>
<td>Coronary artery smooth muscle cells</td>
</tr>
<tr>
<td>CCP</td>
<td>Constant coronary pressure</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary heart disease</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding protein</td>
</tr>
<tr>
<td>CV</td>
<td>Cardiovascular system</td>
</tr>
<tr>
<td>Ddox</td>
<td>2’5-dideoxyadenosine</td>
</tr>
<tr>
<td>DIO</td>
<td>Diet induced obese</td>
</tr>
<tr>
<td>DPP-4</td>
<td>Dipeptidyl peptidase-4</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cells</td>
</tr>
<tr>
<td>ECG</td>
<td>Electrocardiography</td>
</tr>
<tr>
<td>EIA</td>
<td>Enzymatic immunoassay</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>EPAC</td>
<td>Exchange proteins activated by cAMP</td>
</tr>
<tr>
<td>ES</td>
<td>Embryonic stem cells</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factors</td>
</tr>
<tr>
<td>GIP</td>
<td>Glucose-dependent insulinotropic peptide</td>
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<tr>
<td>GLP-1</td>
<td>Glucagon-like peptide-1</td>
</tr>
<tr>
<td>GLP-1R</td>
<td>Glucagon-like peptide-1 receptor</td>
</tr>
<tr>
<td>GRPP</td>
<td>Glicentin-related pancreatic polypeptide</td>
</tr>
<tr>
<td>GSIS</td>
<td>Glucose stimulated insulin secretion</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HR</td>
<td>Heart rate</td>
</tr>
<tr>
<td>i.v</td>
<td>Intravenous</td>
</tr>
<tr>
<td>IBMX</td>
<td>3-isobutyl-1-methylxanthine</td>
</tr>
<tr>
<td>icv</td>
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<td>IRI</td>
<td>Ischemia-reperfusion injury</td>
</tr>
<tr>
<td>LAD</td>
<td>Left anterior descending</td>
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<tr>
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<td>Long chain 3-hydroxyacyl-CoA dehydrogenase</td>
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LDH  Lactate dehydrogenase
LV   Left ventricle
LVDP Left ventricular developed pressure
LVEDP Left ventricular end diastolic pressure
LVESP Left ventricular end systolic pressure
MACE Major adverse cardiovascular events
MI   Myocardial infarction
MPGF  Major proglucagon fragment
MPTP Mitochondrial pore transition protein
MTPα Mitochondrial trifunctional protein alpha
NEP  Neutral endopeptidase
NO   Nitric oxide
NSTEMI Non ST-segment elevated MI
ORF  Open Reading Frame
PC   Preprohormone convertase
PDE  Phosphodiesterase
PKA  Protein kinase A
PKC  Protein Kinase C
PKG  Protein Kinase G
PPCI Primary percutaneous coronary intervention
RIC  Remote ischemic conditioning
RISK Reperfusion injury survival kinase
RLU  Relative light units
ROS  Reactive oxygen species
s.c Subcutaneous
sAC Soluble adenylyl cyclase
sAC-fl Full-length soluble adenylyl cyclase
sAC-tr Truncated soluble adenylyl cyclase
SAFE Survivor activating factor enhancement
siRNA Small interfering RNA
SMB  Streptavidin magnetic beads
SR   Sarcoplasmic reticulum
STAT-3 Signal transducer and activator of transcription 3
STEMI ST-segment elevated MI
STZ  Streptozotocin
$t$-BHP Tert-butyl hydroperoxide
T2DM Type 2 diabetes mellitus
TBS Tris-buffered saline
Chapter 1
Background

1.1 The Cardiovascular system

The cardiovascular (CV) system consists of a main pump (the heart) and a circulatory vascular system of arteries, veins and capillaries that serves to transport blood and distribute essential nutrients and oxygen to organs, tissues and cells of the body and remove metabolic by-products. The homeostatic function of the CV system is regulated in response to the demands of tissues and cells under different physiological or pathological conditions.

1.1.1 Heart

The heart is divided into 2 pairs of muscular chambers, each separated by septal walls, but connected by valves that enable unidirectional blood flow through the various chambers [1]. The right atrium receives deoxygenated blood from all tissues of the body (systemic circulation) through the superior and inferior vena cava, and pumps it into the right ventricles through the tricuspid valve. In turn, the right ventricle contracts to propel blood through the semilunar pulmonic valve into the pulmonary arteries and ultimately to the lungs (pulmonary circulation) for exchange of CO$_2$ and O$_2$. Oxygenated blood returning from the pulmonary circulation enters the left atria via the pulmonary veins and moves through to the left ventricles via the mitral valve. The thicker walls of the left ventricles then contract to pump this oxygenated blood, through the semilunar aortic valves into the aorta, and onto the entire systemic circulation.
The heart is enclosed in an outer pericardial membrane that serves to protect against infections and maintain its position inside the thoracic cavity. The pericardium is separated from the outer wall of the heart by a fluid-filled pericardial cavity that prevents frictions during heart contractions. The wall of the heart consists of 3 layers: an outer thin epicardium, a thick middle myocardium, and a thin endocardium covering the inner lumen of the heart. The epicardium represents an additional wall of protection with its layer of connective tissue and fat. The endocardium comprises a thin lining of endothelial cells that provides nutrients to contractile cells as well as regulates contractility through secretion of vasoactive substances. The myocardium is comprised mainly of contractile cells (cardiac myocytes), around a framework of collagenous fibers (fibroblasts), nerve fibers (electrical conduction system) and blood vessels (coronary circulation).

1.1.2 Cardiac myocytes

The adult myocardium is comprised of terminally differentiated cardiac myocytes (cardiomyocytes), which form 80% of the heart tissue and generate contractile force. Cardiomyocytes have a high density of mitochondria, which enable rapid production of energy in the form of adenosine triphosphate (ATP) in response to the high demands of the contracting myocardium. Due to their lack of replicative potential or regeneration, most cardiomyocytes undergo irreversible damage following an insult or injury under conditions of deprived oxygen or nutrient supply [2]. This in turn contributes to increased workload for the remaining viable cardiomyocytes, which grow larger in diameter (hypertrophy). In some cases, maladaptive hypertrophy can eventually contribute to functional decline of the myocardium and potentially lead to heart failure and death. Cardiomyocytes are supplied with oxygen and nutrients and excrete their waste products through the microvasculature of the coronary circulation.
1.1.3 Coronary circulation

Blood flow to the myocardium is supplied by left and right coronary arteries arising at the root of the aorta [3]. The right coronary artery supplies blood mostly to the right atrium and ventricle, and in some instances to the septum and inferior walls of the left ventricle. The left coronary artery branches into the left anterior descending (LAD) and circumflex arteries near its origin and supplies mostly the left atrium and ventricle. The terminal branches of the coronary arteries end in capillary beds that surround cardiomyocytes to provide the necessary supply of O$_2$ and nutrients. In turn, deoxygenated blood is sent back to the right atria mostly through the coronary sinus and anterior coronary veins.

Under normal physiological conditions, coronary blood flow through the myocardium is regulated by physical, neurohumoral and metabolic factors [4]. While physical compression of the heart chambers during systole or cardiac sympathetic nerve activation can increase coronary resistance, myocardial metabolic activity plays the most significant role in coronary blood flow. Indeed, an imbalance in the O$_2$ supply/demand ratio of cardiomyocytes alters coronary flow through the release of vasodilator substances. This ensures optimum O$_2$ supply for normal myocardial contractility. However, diminished blood flow through coronary arteries can impair cardiac function by inducing ischemia in the myocardium and resulting in pathologies such as myocardial infarction (MI).

When native coronary vessels fail to provide sufficient blood, the coronary collateral circulation can be an alternative source of blood supply [4]. Coronary collaterals, or “natural bypasses,” are anastomotic connections without an intervening capillary bed between portions of the same coronary artery and between different coronary arteries, which are formed by the process of arteriogenesis (formation of new arteries). Coronary collaterals provide an alternative pathway
of blood flow to the ischemic myocardium, which may serve to prevent MI. Native collaterals vary in size, but are usually <50 µm in most species. Large collaterals are generally restricted to the epicardium, whereas smaller collaterals run transmurally. Human hearts may have collaterals 180 µm diameter or larger.

1.1.4 Coronary Heart Disease

Coronary artery disease (CAD) is a pathology that affects the coronary arteries, usually due to atherosclerosis. The more general term CHD (Coronary heart Disease) refers to the manifestation of myocardial infarction, angina pectoris, silent myocardial ischemia, heart failure and mortality that result from CAD. The INTERHEART study identified several preventable risk factors for developing CHD in men and women, at all ages and in all regions of the world [5]. These factors include hypertension, diabetes mellitus, abnormal lipids, smoking, abdominal obesity, and alcohol consumption. While CHD is a global burden causing death and disability worldwide, the 2010 Heart Disease and Stroke Statistics update of the American Heart Association (AHA) reported that from 1990 to 2006, death rates from CHD declined about 29% in most developed countries, due in part to improvements in clinical therapy and partly to changes in lifestyle and risk factors [6]. Nevertheless, CHD remains responsible for about one-third or more of all deaths in adults over age 35, with an estimated one-half of all middle-aged men and one-third of middle-aged women in the United States will develop some manifestation of CHD [6].

The clinical manifestation of CHD is acute coronary syndrome (ACS), where patients present with ischemic discomfort, caused by an imbalance in myocardial oxygen supply-demand due to (most frequently) atherothrombotic obstruction of coronary blood flow. ACS encompasses diagnoses of unstable angina (UA), non ST-segment elevation MI (NSTEMI) and ST-segment elevation MI (STEMI), where each has different clinical outcomes[7]. Diagnostic tools that
distinguish between UA, NSTEMI and STEMI most commonly include biochemical testing for markers of myocardial necrosis (troponin and creatine kinase) or electrocardiography (ECG) [8]. Patients with UA show no elevation in troponin, with or without ECG changes indicative of ischemia, as compared to elevated troponin and ST segment and/or T wave changes persistently seen in NSTEMI cases. The most distinguishing factor between NSTEMI and STEMI is the evolution of pathologic Q waves seen with 12-lead ECGs, which reflect changes in QRS due to alterations in the sequence of depolarization as a result of an infarcted myocardium. Patients with STEMI are candidates for reperfusion therapy, while those with UA or NSTEMI are usually treated with anti-ischemic therapy.

1.1.5 Myocardial Infarction

Myocardial infarction is universally defined as myocardial death due to prolonged ischemia [9]. Experimental studies in dogs have shown that irreversible injury to the left ventricular myocardial wall begins after 20 minutes of total proximal coronary artery occlusions, in the absence of significant collateral coronary flow [7, 10]. The injury originates from the subendocardial layer and progresses as a “wave front of cardiomyocyte death” towards the epicardium, and after 1h, covers one third of the inner myocardium. After 3h occlusion, the necrosis is completely transmural, and myocardial ischemic injury is usually complete within 4 to 6 hours after coronary occlusion. Myocardial ischemia can be either global, thereby affecting the whole ventricle, or localized to a specific segment of the ventricle.

Cardiomyocytes undergo both metabolic and physiological changes within seconds of cessation of coronary blood flow. Due to the absence of O₂, energy metabolism shifts from aerobic metabolism to anaerobic glycolysis, which in turn quickly depletes energy reserves supplied by creatine phosphate, a readily available source of high-energy phosphate [10, 11]. Anaerobic
glycolysis further causes a gradual reduction in tissue ATP levels, and accumulation of by-products such as lactate and H\(^+\), which reduces intracellular pH to \(<7.0\). The increase in intracellular H\(^+\) activates the Na\(^+\)-H\(^+\) exchanger, causing entry of Na\(^+\) into the cell in exchange of removal of excess H\(^+\). Intracellular Na\(^+\) overload in turn results in the reverse activation of 2Na\(^+\)-Ca\(^{2+}\) exchangers, causing an excess entry of Ca\(^{2+}\) into the cardiomyocyte as Na\(^+\) is extruded [11]. The increase in intracellular Ca\(^{2+}\) results in cardiomyocyte hypercontracture, mitochondrial Ca\(^{2+}\) overload, and opening of the mitochondrial permeability transition pore (MPTP). These changes compromise the integrity of the mitochondria due to uncoupling of oxidative phosphorylation, and cardiomyocyte death ensues [12]. Myocardial cell death can either occur by (1) coagulation necrosis, or (2) apoptosis [7]. Coagulation necrosis is characterized by cell swelling, organelle breakdown, protein denaturation and, infiltration of neutrophils. On the other hand, cardiomyocyte undergoing apoptosis (or programmed cell death) display cell shrinkage, DNA fragmentation, MPTP opening, cytochrome c release and cleavage of a cascade of ‘caspase’ proteins leading to cell death. Cardiomyocyte death critically impairs contractile function and electrical cardiac functions and eventually leads to myocardial infarction.

### 1.1.6 Myocardial ischemia/reperfusion injury

In patients presenting with acute STEMI, the mainstay of therapeutic intervention is reperfusion, using either thrombolytic agents or primary percutaneous coronary intervention (PPCI), in order to restore coronary blood flow to the ischemic myocardium. Timely and effective reperfusion following STEMI may reestablish cardiomyocyte function, limit infarct size and preserve LV contractile function, thereby saving the patient’s life and improving outcome. Paradoxically, reperfusion itself can further induce cardiomyocyte death, a phenomenon known as ischemia/reperfusion injury (IRI) and first described by Jennings et al. in the 1960s [13]. IRI can
cause either transient reversible damages to the myocardium or result in more lethal, non-reversible injury.

Reversible injury: IRI-induced ventricular arrhythmias are frequently observed in STEMI patients undergoing PPCI, but are easily reversible with pharmacological interventions [14, 15]. Similarly, myocardial stunning is another form of transient IRI, caused by a combination of oxidative stress and calcium overload, in the absence of irreversible myocardial damage [16]. The contractile dysfunction caused by stunning can be reversed with the use of oxygen radical scavengers or calcium channel blockers [10, 17].

Non-reversible injury: Microvascular obstruction of blood flow encountered during opening of infarct-related coronary artery, and commonly referred as the “no-reflow” phenomenon, can cause irreversible damage due to inability to reperfuse the ischemic myocardium [18]. The pathogenesis of no-reflow is complex and poorly understood and there are currently no approved therapies, despite advances in diagnosis and characterization through cardiac magnetic resonance imaging. No-reflow is associated with sustained cardiac dysfunction and worse clinical outcomes through larger infarct size, reduced LV ejection fraction, and adverse LV remodelling [19, 20]. Secondly, lethal myocardial reperfusion injury is another major permanent form of IRI and accounts for up to 50% of the final infarct size [11]. Lethal myocardial reperfusion injury attenuates the clinical benefits of PPCI by inducing the death of cardiomyocytes that had originally survived the myocardial ischemic event. Lethal myocardial reperfusion injury is caused by a complex interplay between several mediators including oxidative stress, calcium overload, and inflammatory response.
1.1.7 Mediators of IRI

Experimental studies have shown that oxidative stress, calcium overload and inflammation act in concert to mediate the detrimental effects of IRI. At the onset of myocardial reperfusion, the sudden burst in oxygen supply reactivates the electron transport chain, generating reactive oxygen species (ROS). ROS is the initiator of myocardial reperfusion injury by inducing opening of the MPTP pore, acting as a neutrophil chemoattractant and mediating dysfunction of the sarcoplasmic reticulum (SR). Damage to the SR contributes to intracellular calcium overload, due to the inability to recycle calcium, generating cardiomyocyte hypercontracture. Several hours after the onset of myocardial reperfusion, neutrophils accumulate in the infarcted tissue, generating an inflammatory response that further exacerbates damage to the contractile cells.

1.1.8 Therapeutic strategies for IRI

Extensive research to understand the molecular basis of IRI has enabled the development of targeted therapies to prevent this pathology. These include both mechanical and pharmacological interventions that target innate cardioprotective pathways through myocardial ‘conditioning’ and provide protection against reperfusion injury. Such cardioprotective pathways include the reperfusion injury salvage kinase (RISK) [21] and survivor activating factor enhancement (SAFE) pathways [22, 23], which limit reperfusion injury when activated.

1.1.8.1 Cardioprotective Pathways

The RISK pathway involves the activation of pro-survival kinases such as PI3K-AKT and ERK1/2 that prevent opening of the MPTP through multiple downstream intermediate targets such as protein kinase C-ε (PKC-ε), protein kinase G (PKG), P70S6K, and GSK-3β [21]. Endothelial nitric oxide synthase (eNOS) is another component of the RISK pathway whose
activation by phosphorylation generates nitric oxide (NO) and effects cardioprotection [24]. The RISK pathway is innate to cardiomyocytes, and confers a ‘preconditioning’ state to cells prior to the ischemic event.

The SAFE pathway involves TNF-α-mediated activation of STAT-3 (Signal transducer and activator of transcription 3), which confers protection against reperfusion injury by increasing expression of anti-apoptotic Bcl-2 gene and through inactivation of pro-apoptotic factors Bad and GSK-3β [22, 23]. As opposed to RISK, the SAFE pathway is activated after the ischemic event (postconditioning) and there is not much overlap between both pathways [22]. Lastly, AMPK is another cardioprotective kinase, which is activated by preconditioning and reduces IRI by regulating glucose and fatty acid metabolism through increased glycolysis, glucose uptake and fatty acid oxidation [25, 26].

Although the above pathways are distinct in terms of their molecular constituents, they all converge towards the MPTP and the end-effector for preventing cell death.

1.1.8.2 Pre-, Post- and Remote Mechanical Conditioning

As mentioned earlier, myocardial ‘conditioning’ provides protection against IRI by targeting the RISK or SAFE pathways, although the phenomenon of ‘conditioning’ was historically discovered prior to elucidating the signaling pathways. Indeed, in 1986, Murry et al. made the important discovery that four successive cycles of 5 min coronary occlusion/5 min reperfusion preceding a 40 min coronary occlusion in dogs decreased infarct size in the area at risk by 75% [27]. This phenomenon was termed ‘ischemic preconditioning’ or IPC, and has been unanimously replicated in other canine [28], rodent [29], rabbit [30], porcine [31] and primate [32] models. Evidently, IPC is hard to implement in clinical practice, due in part to its invasive nature (except perhaps for open heart surgery) and mostly because of the timing of IPC, which
necessitates application prior to the ischemic event.

Myocardial conditioning can also be applied after ischemia at the time of reperfusion, i.e. postconditioning (IPoC). Zhao et al. first demonstrated the benefits of IPoC in anesthetized open-chest dogs by occluding the LAD coronary artery for 60 min, followed by three cycles of 30 s reperfusion/30 s coronary occlusion preceding 3h of reperfusion. Infarct size was reduced to 14% with IPoC, comparable to 15% with IPC in the same experiment vs. 25% in non-conditioned controls [33]. As opposed to IPC, IPoC is applied in the clinical setting of PPCI, through four cycles of 60 s low-pressure inflation/deflation of an angioplasty balloon placed upstream of a stent at the onset of reperfusion of the occluded vessel [34]. To-date, the benefits of IPoC as a therapeutic intervention for preventing IRI in patients undergoing PCI is not consistent, with some studies showing significant reduction in infarct size [34, 35], while others show either no difference [36] or trend towards increased infarct size [37].

Remote ischemic preconditioning (RIC) is emerging as an alternative non-invasive approach to reduce IRI, by applying a few cycles of brief ischemia/reperfusion to another organ or tissue. Examples include three cycles of 5min inflating/deflating a blood pressure cuff placed on the upper arm or lower limb of a patient [38]. The clinical benefits of RIC are still under active investigation.

1.1.8.3 Pharmacological Conditioning

Based on the invasive nature of mechanical conditioning, several pharmacological agents have been investigated in pre-clinical studies as potential therapeutics capable of modulating RISK and SAFE pathways to mimic IPC and/or IPoC and confer protection against IRI. These include surface receptor ligands on sarcolemmal membrane, such as adenosine, erythropoietin, insulin, glucagon-like peptide-1 (GLP-1) and atrial natriuretic peptide (ANP) [39]. Other agents such as
insulin, statin, nicorandil directly target intracellular components of RISK pathway, whereas cyclosporine acts directly on the MPTP pore to prevent its opening, thus reducing cell death [40].

The advantage of pharmacological conditioning is that it can be integrated in the usual therapeutic regimen of patients at risk of MI, including those with type 2 diabetes or hypertension. A plethora of agents have also been evaluated in clinical trials, but disappointingly, most of them showed conflicting results with regards to cardioprotection against IRI and/or were unable to translate the findings from basic science. A few exceptions include cyclosporin, ANP, and GLP-1, which show promise as cardioprotective agents against IRI and constitute an active area of research.

1.2 Incretin hormones

The incretin effect is defined as the increase in pancreatic insulin secretion observed with oral glucose administration as compared to the same dose of glucose given intravenously (i.v) [41]. The incretin effect underlies the existence of gut-derived factors, termed incretin hormones, that can stimulate glucose-dependent insulin secretion, following meal ingestion. The first incretin hormone to be identified in animals and humans was glucose-dependent insulinotropic peptide (GIP), mainly produced from the K-cells of the small intestine [42]. However, several lines of evidence pointed towards the existence of other incretin hormones. The most compelling in vivo finding was that patients with resection of the ileum due to Crohn’s disease or mesenteric thrombosis displayed lower incretin effects after oral glucose ingestion, as compared to healthy volunteers, despite normal GIP plasma levels [43]. Several years later, the second incretin hormone, glucagon-like peptide-1 (GLP-1), was discovered following cloning and sequencing of the mammalian proglucagon genes [44]. The incretin effects of both GIP and GLP-1 are additive,
and they both contribute nearly equally to postprandial insulin release, with more pronounced responses in the morning versus afternoon meals [45, 46]. GIP and GLP-1 are the two main incretin hormones currently known to be active in humans, and account for approximately 90% of the incretin response. Other gut hormones such as CCK, that can also mediate glucose-dependent insulin secretion, are released following meal ingestion but their plasma levels remain insignificant compared to both GIP and GLP-1. Both GIP and GLP-1 display equal potentials for development into anti-diabetic agents due to their insulinotropic effects in healthy subjects. However, the incretin effects of GLP-1, but not GIP, is preserved in patients with type 2 diabetes [47]. This has discouraged the development of GIP-based therapies for type 2 diabetes but favoured GLP-1.

1.2.1 GLP-1: synthesis, secretion and degradation

GLP-1 is synthesized within the L-cells of the intestinal epithelium located in the small intestine and colon, with highest production in the distal ileum and colon. GLP-1 is encoded by the proglucagon gene (gcg), along with other proglucagon-derived peptides (glucagon, GLP-2, oxyntomodulin and glicentin) [48]. The 160 amino acid peptide product of gcg gene, proglucagon, undergoes tissue-specific posttranslational processing, by actions of preprohormone convertase (PC) as illustrated in Figure 1.1. Indeed, in the presence of PC2, such as in the pancreas, proglucagon is cleaved into glicentin-related pancreatic polypeptide (GRPP), glucagon, intervening peptide-1 (IP1) and major proglucagon fragment (MPGF). In intestinal L cells and some brain neuronal cells, GLP-1 is the PC1/3-mediated cleavage product of proglucagon, alongside glicentin, GLP-2, intervening peptide-2 (IP2), GRPP and oxyntomodulin. GLP-1 is further processed by PC1/3 and α-amidating enzyme into a glycine-extended and a C-terminal amidated forms respectively [49, 50]. Of note, C-terminal amidation of GLP-1 has not
been reported to affect known biological activities of this peptide. The initial products of PC1/3 processing are GLP-1(1-37)amide and GLP-1(1-36)amide, which differ by a single amino acid. Further truncation results in GLP-1(7-37)amide and GLP-1(7-36)amide, both equipotent in action, with identical plasma half-life ($t_{1/2}$) and biological activities through the same receptor [51, 52]. However, GLP-1(7-36)amide is the major (80%) circulating active form [53]. Plasma levels of GLP-1(7-36)amide are low in the fasting state (7±1 pM) and rise rapidly within minutes of food ingestion to 41±5 pM [53]. GLP-1(7-36)amide secretion from the distal gut is controlled by neural and hormonal signals initiated by nutrient entry in the proximal gut, and by direct nutrient contact with L cells [54, 55].
Figure 1.1. Proglucagon and proglucagon derived peptides

Proglucagon is a 160 amino acid peptide translated from the gcg gene. Proglucagon contains cleavage sites for both PC2 and PC1/3 that generate the cleavage products shown. Processing by PC1/3 yields GLP-1, IP2 and GLP-2 peptides, whereas PC2 cleavage generates GRPP, Glucagon and IP1 as major peptides. PC2 and PC1/3, preprohormone convertases 2 and 1/3; GRPP, glycentin related polypeptide; IP1 and IP2, intervening peptide 1 and 2; GLP-1, glucagon-like peptide-1; GLP-2, glucagon-like peptide 2.
Following secretion, GLP-1(7-36)amide is rapidly metabolized by the enzyme dipeptidyl peptidase-4 (DPP-4) [56, 57], resulting in a plasma t_{1/2} of less than 3 min [58]. DPP-4 is a membrane-bound peptidase ubiquitously expressed in almost all tissues and the membranes of most cells. It can also be found in soluble form circulating in plasma. DPP-4 has a high affinity for peptide substrates with an amino-residues proline or alanine at position 2. GLP-1(7-36)amide represents an ideal substrate for DPP-4 and is rapidly cleaved at position Ala2 to generate GLP-1(9-36)amide (Figure 1.2). Therefore, it is conceivable that GLP-1(9-36) is simultaneously released with GLP-1(7-36) at the site of production.

GLP-1(7-36)amide peptides are also susceptible to cleavage by membrane-bound ectopeptidases. Neutral endopeptidase (NEP), 24.11 (neprilysin or CD10), is one of the most widely distributed ectopeptidases involved in extracellular degradation of peptide hormones and neuropeptides. In mammals, NEP expression has been reported in kidney, lung, endothelial cells, vascular smooth muscle cells, cardiac myocytes, fibroblasts, neutrophils, adipocytes, testes, and brain [59]. NEP24.11 is a 94kDa zinc metalloendopeptidase that cleaves its substrate at the N-terminal side of hydrophobic amino acids. Therefore, unlike GLP-1(9-36) which is ‘co-released’ with GLP-1(7-36) at the site of secretion, GLP-1(28-36) might only be produced extracellularly in cells and tissues specifically expressing NEP24.11. Both GLP-1(7-36)amide and GLP-1(9-36)amide have 6 potential sites for NEP24.11 cleavage, leading to the formation of many smaller metabolites. Studies examining the endoproteolytic metabolism of GLP-1(7-36)amide-related peptides by NEP24.11 are scarce. Moreover, the endogenous, in vivo, formation of smaller metabolites and/or their plasma levels have never been directly examined. An earlier study dating back to the mid-1990s characterized the in vitro endoproteolysis of GLP-1(7-36)amide by the recombinant human form of NEP24.11, using reverse-phase column liquid chromatography[60]. This study identified fourteen metabolites generated from GLP-1(7-36)amide cleavage in a time- and
concentration-dependent manner, whereas potential target bonds such as Thr\textsubscript{11}-Phe\textsubscript{12} and Leu\textsubscript{32}-Val\textsubscript{33} were not cleaved. More importantly, GLP-1(28-36)amide and GLP-1(32-36)amide were identified as the most readily generated metabolites and requiring the lowest concentration of NEP24.11 (Figure 1.2).

![Diagram of GLP-1(7-36)amide and its metabolites](image)

**Figure 1.2. GLP-1(7-36)amide and its metabolites.**

Amino acid sequences of GLP-1(7-36)amide and known biologically active metabolites in amidated forms. The cleavage sites for DPP-4 and NEP24.11 are as indicated, where DPP-4 generates GLP-1(9-36)amide and NEP24.11 produces GLP-1(28-36)amide and GLP-1(32-36)amide. DPP-4, dipeptidyl peptidase-4; NEP 24.11, neutral endopeptidase 24.11.
The metabolic stability of two GLP-1(7-36)amide metabolites, GLP-1(9-36)amide and GLP-1(28-36)amide, was recently examined using liquid chromatography-tandem mass spectrometry developed specifically for the quantitation of the intact peptides in isolated hepatocyte incubations[61]. GLP-1(9-36)amide displayed longer half-life and greater stability than GLP-1(28-36) in both mouse hepatocytes [GLP-1(9-36)amide: t1/2 = 52 minutes; GLP-1(28-36)amide: t1/2 = 13 minutes] and human hepatocytes [GLP-1(9-36)amide: t1/2 = 180 minutes; GLP-1(28-36)amide: t1/2 = 24 minutes]. However, a major limitation of this study was that the expression levels of both DPP-4 and NEP24.11 were not measured in the isolated hepatocytes, making it difficult to interpret the relative contributions of both proteolytic enzymes.

It is not known whether DPP-4- and/or NEP24.11-generated metabolites of GLP-1(7-36)amide are endogenously active with physiologically significant plasma concentrations. However, emerging evidence using recombinant forms of the metabolites GLP-1(9-36)amide, GLP-1(28-36)amide, and GLP-1(32-36)amide suggest that these peptides have pharmacological actions in various organs and tissues, as discussed further in this chapter.

Throughout the remainder of this thesis, GLP-1(7-36)amide will be referred as GLP-1, GLP-1(9-36)amide as GLP-1(9-36), GLP-1(28-36)amide as GLP-1(28-36), GLP-1(32-36)amide as GLP-1(32-36).

1.2.2 Physiological actions of GLP-1

The scope of GLP-1 actions is widespread and encompasses almost every organ and physiological systems of the body as evidenced by both animal and human studies. Besides its primary metabolic actions in the endocrine system, GLP-1 has been studied in the cardiovascular system, central nervous system, immune system, as well as specific organs including liver,
kidney, adipose tissue, and skeletal muscle. Despite such extensive pleiotropic actions, GLP-1-based therapies are only approved for their glucoregulatory effects in type 2 diabetes and as agents for weight control. The major limitations hampering the advancement of GLP-1-based treatment to other therapeutic areas include (1) lack of translation from animal to large-scale clinical trial, (2) use of pharmacological rather than physiological doses to study GLP-1 actions in pre-clinical studies, (3) lack of adequately controlled pre-clinical studies using active comparators and (4) poorly understood cellular and molecular mechanisms of actions. Indeed, GLP-1 actions are mediated by its receptor, GLP-1R, expressed in many organs and cell types. However, understanding the signaling mechanisms of GLP-1 is complicated by evidence of (A) biological actions in GLP-1-responsive tissues and cells not expressing GLP-1R, and (B) biologically active metabolites.

1.2.3 GLP-1 receptor

The actions of GLP-1 are transduced by its binding to the extracellular N-terminal domain of its seven trans-membrane Gs protein-coupled receptor (GLP-1R), originally cloned from a rat pancreatic islet cDNA library [62]. GLP-1R has also been cloned from tissues originating from human brain, heart and lungs. GLP-1R expression has been localized to multiple tissues and cell types, including the cardiovascular system, by western blot analysis or immunostaining using commercially available antisera. On the other hand, recent studies using primer pairs capable of detecting full length Glp1r mRNA have failed to validate GLP-1R expression based on antisera, raising concerns about the validity of previously reported GLP-1R-dependent actions in these tissues and cells types [63]. Following this controversy, use of newly designed, extensively validated monoclonal antisera against GLP-1R have now confirmed expression in pancreatic
beta cells, as well as vascular smooth muscle cells, atrial cardiomyocytes and sino atrial node in the cardiovascular system, amongst others[64].

GLP-1R is subject to desensitization through phosphorylation of serine residues on the intracellular carboxyterminal tail of the receptor[65]. Studies in pancreatic beta cells transfected with GLP-1R-GFP fusion proteins have shown that chronic activation of GLP-1R results in (1) internalization following cAMP-PKA dependent phosphorylation at serine 301 residues, and (2) degradation after interaction with the small ubiquitin-related receptor modifier[66]. Contrarily, chronic exposure of rodents to GLP-1R agonists has not demonstrated meaningful loss of glucoregulatory efficacy and is not associated with any significant down-regulation of GLP-1R-dependent responses, in vivo [67]. Moreover, in patients with diabetes, twice-daily treatment for 30 weeks with an approved GLP-1R agonist resulted in improved glycemic control and reduced postprandial glucose excursions, demonstrating long-term clinical efficacy of chronic GLP-1R activation [68]. The only exception appears to be the tachyphylactic effect of GLP-1R activation on gastric emptying. Intravenous infusion of native GLP-1 for 8.5 h in 9 healthy subjects was associated with delayed gastric emptying through rapid desensitization at the level of vagal nervous activation [69]. Therefore, it appears that GLP-1R receptor desensitization is not generally clinically significant in terms of glucoregulatory efficacy of its agonists. However, a different interpretation of these discrepancies could be that other pathways besides GLP-1R are involved in GLP-1 signalling, such as actions of bioactive metabolites and/or an alternate GLP-1R receptor.

The physiological importance of GLP-1R has been examined by blocking its action using either pharmacological antagonists or genetic deletion of GLP-1R in mice.
1.2.4 GLP-1R antagonist

Exendin(9-39) is a truncated peptide extracted from lizard salivary glands which is capable of blocking GLP-1R activation by acting as an antagonist at the receptor. Evidence from pre-clinical studies in mice, rats, baboons and pilot human trials have shown that exendin(9-39) could counteract the glucoregulatory effects of GLP-1R agonists through increased fasting glucose and hyperglycemic excursions accompanied by reduced levels of circulating insulin [70-73]. In the nervous system, acute intracerebroventricular (icv) injections of exendin(9-39) increased food intake in satiated rats [74], while daily icv administrations induced weight gain [75]. Exendin(9-39) is not commonly used to study GLP-1R actions partly because of some evidence of partial agonist properties for the receptor [76] and mainly due to the advent of mice with whole body or organ specific knockdown of GLP-1R.

1.2.5 GLP-1R knockout mice

The importance of GLP-1R in mediating the signal transduction pathways involved in the physiological actions of GLP-1 and/or its metabolites can be studied using GLP-1R knockout (Glp1r<sup>-/-</sup>) mice. This mutant strain was generated in 1995 by targeted disruption of Glp1r gene through germline deletion of two exons [77]. Phenotypically, Glp1r<sup>-/-</sup> mice are viable and fertile, but display abnormal glucose tolerance with diminished insulin secretion, and defective CV responses to stress, including mild cardiac hypertrophy that worsens with age [78, 79]. Glp1r<sup>-/-</sup> mice have been used extensively and have generated a large body of evidence informing the mechanisms of actions of the incretin GLP-1 in the endocrine, nervous and cardiovascular system.
To study the importance of GLP-1R in humans, a humanized GLP-1R (hGLP-1R) mouse transgenic strain has been developed, with selective expression of the human isoform of GLP-1R in islet and pancreatic ductal cells of Glp1r−/− mice under the control of pdx1 promoter[80]. The advantage of hGLP-1R is that the importance of GLP-1R expression in a specific organ or tissue can be studied (in this case pancreatic cells) without confounding effects of GLP-1R activation in other tissues, while allowing translation of the findings to humans.

On the other hand, loss-of-function studies with tissue-specific inactivation of GLP-1R provide more efficient ways of studying GLP-1R signaling. Indeed, using the Cre-LoxP strategy, nestin-Cre driver line or Phox2b have been successfully employed for targeted inactivation of GLP-1R in hypothalamus, brainstem or pancreas, whereas the mouse insulin promoter (MIP)-Cre has been used to conditionally knockdown GLP-1R in pancreatic beta cells[81, 82]. In the cardiovascular system, myosin heavy chain (MHC)-Cre mice have been used to conditionally eliminate GLP-1R expression under the control of tamoxifen in cardiomyocytes [83].

1.2.6 Drugs targeting GLP-1R

As outlined earlier, the half-life of native GLP-1 in plasma is very short (<3min), due in part to rapid degradation by DPP-4. As such, GLP-1R agonists with increased resistance to DPP-4 and capable of mimicking native GLP-1, as well as inhibitors of DPP-4 (DPP-4i) have been developed to prolong actions of GLP-1 at the GLP-1 receptor. These agents are currently approved for the treatment of type 2 diabetes.

1.2.6.1 Exenatide

Exenatide is a synthetic version of exendin-4, a 39 amino acid peptide isolated from the venom
of the Gila monster (*Heloderma suspectum*)[84, 85]. In the discovery of exenatide as a therapy for diabetes, researchers first noticed that Gila monster venom could induce insulin secretion in dispersed acini from guinea pig pancreas, with concurrent 50–60-fold increase in cellular cAMP [85]. Exenatide shares 53% homology with native GLP-1, but still binds effectively to GLP-1R on pancreatic beta-cells for its insulinotropic effects[86]. As per the amino acid sequence of exenatide shown in Figure 1.3, this analog is resistant to DPP-4 degradation due to the substitution of Ala2 by Gly2. Moreover, this peptide is not a good substrate for NEP24.11, due to lack of hydrophobic N-terminal amino acids cleavage sites [60]. Due to its stability, exenatide has a half-life of approximately 2 h following subcutaneous (s.c.) administration [87]. Exenatide is measurable in plasma for up to 10 h following s.c. injection, warranting twice-daily dosage for full glycaemic control. Unlike GLP-1, which undergoes proteolytic degradation, the elimination of exenatide from the body is thought to be through glomerular filtration [87].

1.2.6.2 Liraglutide

Liraglutide is a potent, long-acting synthetic analogue of the human GLP-1 peptide. It shares 97% homology with native GLP-1(7-37), with the addition of a γ-glutamic acid residue at position 26 to allow attachment of a C-16 fatty acid (palmitoyl) group and substitution of lysine for arginine at position 34 to ensure mono-acylation (Figure 1.3) [88]. Structure–activity studies have reported a relationship between liraglutide acylation and potency for GLP-1R as well as protraction time in vivo[89]. Indeed, the presence of a palmitoyl group on liraglutide facilitates non-covalent binding to plasma albumin, which in turn hinders DPP4 action by shielding the molecule from enzymatic access. As such, due to albumin binding, aggregation at injection site[90] and reduced susceptibility to DPP-4, liraglutide has been reported to have slow absorption ($T_{\text{max}}$: 10–14 h) and a long plasma half-life of 12–13 h following s.c. injection,
allowing for once-daily dosing[91, 92]. More importantly, the acylated modifications of liraglutide do not confer any loss in potency for GLP-1R compared with native GLP-1[88].

Pharmacokinetic studies *in vivo* revealed that liraglutide circulates in the plasma mainly as the intact molecule (≥ 89%), alongside two minor metabolites (≤ 11%), following a single s.c. injection of 0.75 mg to healthy males[93]. It is believed that NEP24.11 is involved in the degradation of liraglutide *in vivo*, with GLP-1(28-36), but not GLP-1(9-36) being the degradation products, although their respective plasma levels were not indicated in this study [93]. However, due to absence of intact liraglutide in urine and feces, and the low level of metabolites in plasma, it is speculated that liraglutide is fully degraded within the body and that its degradation products are either recycled into new proteins and lipids, or eliminated by the liver and kidney, or even excreted from the body as carbon dioxide, urea and water [93, 94].

1.2.6.3 DPP-4 inhibitors

DPP-4 inhibitors increase endogenous levels of GLP-1 by inhibiting the enzyme responsible for its degradation and thus prolonging its action. DPP-4 inhibitors currently approved by the FDA include saxagliptin, sitagliptin, alogliptin, and linagliptin. Vildagliptin is approved for use in Europe. Unlike exenatide, and to a lesser extent liraglutide, DPP-4 inhibitors do not prevent degradation by NEP24.11, thus smaller metabolites of GLP-1 are still generated with the use of these agents.
Figure 1.3. Amino acid sequences of GLP-1, and GLP-1 analogs Exenatide and Liraglutide.

Ala2 residue on GLP-1 is a site of proteolytic cleavage by DPP-4. Substitution of Ala2 by Gly2 residue in exenatide mediates resistance to DPP-4 metabolism, whereas other C-terminal substitutions prevent NEP24.11 degradation. Liraglutide is resistant to DPP-4 cleavage due to shielding of Ala2 by albumin bound to C-16 fatty acid, whereas still maintaining susceptibility to NEP24.11 cleavage to generate GLP-1(28-36).
1.2.7 GLP-1 metabolites

1.2.7.1 GLP-1(9-36)

GLP-1(9-36) was long considered an inert metabolite of GLP-1(7-36), despite the fact that it has a longer half-life than its parent peptide [61, 95]. Earlier studies examining the glucose-lowering effects of GLP-1(9-36) in healthy human subjects were contradictory. A study examining the biological activity of GLP-1(9-36) in healthy human volunteers ranging in age from 23–61 yrs old failed to demonstrate an effect of GLP-1(9-36) on glucose clearance or insulin secretion [96]. In contrast, i.v. administration of GLP-1(9-36) at 1.2 pmol/kg/min into fasting healthy human male subjects for 390 minutes, in the presence of a test meal, modestly but significantly reduced the rise in postprandial hyperglycemia, without effects on gastric emptying or on plasma levels of insulin, or glucagon, perhaps through effects on glucose disposal [97].

On the other hand, numerous pre-clinical studies in dogs, rats and mice have replicated beneficial cardiovascular actions of GLP-1(9-36). In conscious dogs with pacing-induced dilated cardiomyopathy, 48 h continuous i.v infusions with GLP-1(9-36) increased myocardial glucose uptake and improved left ventricular performance, as compared to saline control [98]. Also, in isolated rat hearts undergoing IRI, administration of GLP-1(9-36) following global ischemia significantly improved LV pressure, although the treatment failed to reduce infarct size [99]. Our group has demonstrated that isolated mouse hearts rapidly convert GLP-1(7-36) to GLP-1(9-36). In hearts isolated from both WT and Glp1r−/− mice, post- (but not pre-) ischemia treatment with GLP-1(9-36) significantly improved recovery of LV functions and decreased cellular injury as determined by infarct size measurement and LDH release [100]. Studies on cultured cardiomyocytes and endothelial cells revealed that treatment with GLP-1(9-36) increased cAMP
formation and improved cell viability after exposure to hypoxia/reoxygenation or hydrogen peroxide (H$_2$O$_2$), as determined by the MTT assay, LDH release and caspase-3 activation [100]. The cytoprotective action achieved with GLP-1(9-36) was via PI3K and ERK1/2-dependent mechanisms as shown with the use of pharmacological inhibitors. GLP-1(9-36) also exhibited significant vasodilatory effects, by increasing coronary flow in a constant-pressure perfused isolated heart model and causing dilation of pre-constricted isolated mesenteric arteries from WT mice, through a NO/cGMP-dependent mechanism. Furthermore, the cardioprotective, cytoprotective as well as vasodilatory effects of GLP-1(9-36) were maintained in Glp1r$^{-/-}$ mice, suggesting a GLP-1R-independent mechanism (Figure 1.4).

To add to these findings, a recent study has pointed towards the existence of an alternate receptor for GLP-1(9-36) [101]. Radioligand binding assays were performed using [$^{125}$I]GLP-1(9-36) in coronal and sagittal sections of the adult mouse brain containing the hypothalamus and thalamus, regions which have the highest density of mRNA encoding GLP-1 receptor. In these sections, [$^{125}$I]GLP-1(9-36) bound with similar affinity but labeled a smaller population of receptors than [$^{125}$I]GLP-1(7-36), suggesting an interaction with a receptor distinct from GLP-1R.

1.2.7.2 GLP-1(28-36)

There is emerging evidence of biological actions of GLP-1(28-36) in the endocrine system, based on findings from two separate group of investigators. The Habener group revealed that 28 d continuous subcutaneous infusion with GLP-1(28-36) prevented weight gain, development of diabetes and hepatic steatosis in diet-induced obese (DIO) mice [102]. Cellular studies showed that GLP-1(28-36) suppressed glucose production in isolated mouse hepatocytes subjected to artificially induced insulin resistance. Moreover, treatment with 100nM GLP-1(28-36) displayed cytoprotective effects by preventing decreases in ATP levels and reducing ROS production under
conditions of either tert-butyl hydroperoxide (t-BHP) or hydrogen peroxide (H2O2)-induced oxidative stress in hepatocytes isolated from DIO mice or in a rat hepatocyte cell line (H4IIe) [103]. Similarly, in INS-1 mouse β-cell lines, GLP-1(28-36) treatment prevented stress-induced apoptosis by inhibiting mitochondrial depolarization, preventing cytochrome-c release and caspase activation in response to glucolipotoxicity media, streptozotocin(STZ), and the oxidizing agents H2O2 and t-BHP [104]. Incubation with GLP-1(28-36) also protected dispersed human islets by attenuating the decreased ATP levels generated by t-BHP-induced oxidative stress. Using fluorescently-labeled GLP-1(28-36), the Habener group showed that this peptide appeared to localize subcellularly and target to the mitochondria of INS-1 cells and isolated hepatocytes, but only under conditions of glucolipotoxic stress [103, 104].

Using a different model of metabolic syndrome, the STZ-induced diabetic mice, the Jin group reported that 9 wk daily i.p injection of 18 nmol/kg GLP-1(28-36) resulted in significantly reduced fasting glucose and increased basal insulin levels, as compared to vehicle control[105]. Additionally, histomorphometric analysis revealed that this effect was accompanied by improved β-cell mass and increased β-cell proliferation as measured by BrdU incorporation. This study suggests that GLP-1(28-36) exerts these observed in vivo insulinomimetic effects through the Wnt signaling pathway, as observed by the cAMP-PKA-dependent Ser675 phosphorylation and activation of its main effector β-catenin and increased expression of cyclin D1 (a downstream target of β-cat). This group also suggests that GLP-1(28-36) induces β-cell proliferation through increased cAMP-PKA-dependent phosphorylation of transcription factors cAMP response element-binding protein (CREB) and cyclic AMP-dependent transcription factor-1 (ATF-1).

Using a high-fat fed mouse model of type 2 diabetes, the Jin group further demonstrated that 6wk daily i.p injections with GLP-1(28-36) (18.5 nmol/kg body weight) was associated with
reduced body weight and glucose output after a pyruvate challenge (the major substrate for hepatic gluconeogenesis). These effects were accompanied by reduced hepatic gene expressions of gluconeogenic enzymes \textit{Pck1}, \textit{G6pc} and gluconeogenic transcriptional activator \textit{Ppargc1a} [106]. The authors further showed that cAMP-PKA-CREB activation was involved in the repression of hepatic gluconeogenetic gene expression, contrary to previous reports of the involvement of this signaling cascade in upregulation of gluconeogenic gene expression [107].

To summarise, these studies from two independent groups complement each other and revealed important functions of GLP-1(28-36) in long-term glucoregulatory control in hepatic cells and insulinomimetic effects in pancreatic \( \beta \)-cells. Mechanistically, it appears that cAMP-PKA-dependent activation of Wnt signaling pathway and downstream activation of nuclear transcription factors involved in cell proliferation as well as suppression of hepatic gluconeogenic gene expression, are involved. Moreover, GLP-1(28-36) displays cytoprotective effects under conditions of oxidative stress by targeting to mitochondria and preventing apoptosis. However, an important caveat between the two group’s findings is that the mitochondrial-based action in hepatic and INS-1 cells observed by the Habener group does not correlate with the cAMP-PKA-dependent activation of nuclear-based transcription factors and gene expression observed by the Jin group in the same cells. Indeed, the mitochondrial membrane is impermeable to cAMP. Possible explanations include either (1) activation of distinct signaling pathways by GLP-1(28-36) based on subcellular compartmentalization to mitochondria, cytosol and nucleus, or (2) existence of a cytoplasmic-based molecule that link mitochondrial actions of GLP-1(28-36) to the activation of cytosolic cAMP/PKA leading to downstream phosphorylation and activation of nuclear transcription factors. Therefore, further investigations into the mechanisms underlying GLP-1(28-36) actions in hepatic and pancreatic \( \beta \)-cells are required.
Of note, aside from its metabolic effects, the biological actions of GLP-1(28-36) in other systems, including the cardiovascular system, had never been studied.

1.2.7.3 GLP-1(32-36)

The actions of GLP-1(32-36) on glycemic control and metabolic syndrome have been studied in dogs and mice. In dogs undergoing 180 min hyperglycemic clamps, continuous infusions with GLP-1(32-36) increased glucose utilization as compared to saline control, without any significant changes in insulin or glucagon secretion [108]. In diet-induced obese mice, GLP-1(32-36) prevented the development of obesity, insulin resistance, diabetes and hepatic steatosis. Cellular studies in brown adipose tissue and skeletal muscle from GLP-1(32-36)-treated mice revealed the involvement of this pentapeptide in fat metabolism and energy expenditure, through activation of AMP kinase and inhibition of acetyl CoA carboxylase[109].

1.2.8 Cardiovascular actions of GLP-1, analogs and related agents

1.2.8.1 Animal studies


The ability of GLP-1 and related agents to prevent myocardial IRI has been widely studied. In ex vivo models using Langendorff isolated perfused heart preparations from wild-type mice, GLP-1 and exendin-4 increased functional recovery and cardiomyocyte viability, and reduced infarct size following IRI [99, 110]. Interestingly, these beneficial effects were long-lasting, as even short treatments with exendin-4 in the newborn rat led to protection from ischemic injury in
adulthood [111]. Similarly, sitagliptin improved recovery of LVDP after ex vivo IRI when wild-type mice were pre-treated with the drug in vivo [112]. In this study, mice with genetic deletion of DPP-4 (Dpp4−/−) had significantly improved LVDP compared to wild-type mice. Studies employing rodent, canine, and porcine models have also demonstrated protective effects of GLP-1 following IRI. In the rat, GLP-1 (in the presence of valine pyrrolidide, a DPP-4i) significantly reduced infarct size at the end of a 2h reperfusion following 30 min of ischemia [113]. In conscious canines undergoing 10 min occlusion followed by 24 h reperfusion, GLP-1 improved isovolumic left ventricular relaxation, enhancing recovery from ischemia after reperfusion [114]. In porcine models, data regarding the effect of GLP-1 in IRI is less clear. An early study using recombinant GLP-1 infusions in a non-diabetic porcine model with 60-min ischemia followed by 2h reperfusion did not have an effect on infarct size [115]. However, a later study of the GLP-1R agonist exenatide in non-diabetic pigs undergoing a longer ischemic period (75 min) showed a 40% decrease in infarct size as compared to controls [116]. Although, another study employing a closed-chest non-diabetic porcine model of IRI (40 min ischemia and 2.5 h reperfusion) failed to demonstrate any infarct-sparing effect with liraglutide [117], but the dose of liraglutide used in this study was a small fraction of that used in clinic. As such, it remains to be established whether these contrasting results are due to differences in the GLP-1R agonists tested, or in the models of IRI used. In addition to cardioprotective effects in non-diabetic hearts, GLP-1R agonists have also proven to be beneficial in diabetic mice. Pre-treatment with liraglutide reduced infarct size and improved cardiac function in both normal and diabetic mice undergoing either permanent coronary artery (MI model) or ischemia (30 min)–reperfusion (40 min) injury ex vivo [118].
1.2.8.2 Clinical studies

The cardioprotective benefits observed with GLP-1 and its analog exenatide in animal studies have shown clinical translation as evidenced by small pilot studies and early phase clinical trials [119-123]. Indeed, while pre-treatment of patients with CAD with either full length GLP-1, sitagliptin or exenatide showed cardioprotective benefits, the extent and mechanism of protection differed between the agents. An unblinded, non-randomized pilot study showed that a 3d infusion of recombinant GLP-1 after successful angioplasty for STEMI significantly improved LV function in 10 patients [119]. Also, iv infusion of native GLP-1 for 30 min in CAD patients undergoing elective PCI significantly reduced the ischemic LV systolic and diastolic dysfunction that follows coronary occlusion, and that these beneficial effects were still apparent after 30d [121]. Moreover, in two separate pilot studies, infusions of both native GLP-1 and sitagliptin protected the heart from ischemic LV dysfunction induced by dobutamine-stress in patients with CAD [122, 123]. Infusion of the degradation-resistant exenatide, at the time of PPCI, in STEMI patients produced only a modest reduction in infarct size, with no difference in LV function or clinical events by 30d [120]. Another small randomized, double-blind, placebo-controlled crossover study suggested that the cardioprotective effects of exenatide may be mediated by its ability to increase myocardial blood flow and increase glucose uptake [124]. These pilot and early phase clinical studies imply that while both GLP-1 and exenatide have cardioprotective effects, the observed differences in the extent of protection may suggest different modes of action.
1.2.8.3 Cardioprotective mechanisms of GLP-1 actions

Studies using mouse cardiomyocytes and ECs have identified some of the molecular pathways involved in the cytoprotective effects of GLP-1 and GLP-1(9-36) (Figure 1.4). Using specific pharmacological inhibitors, it was shown that RISK kinases such as Akt, ERK1/2 prevented apoptosis by reducing caspase-3 activation in both cardiomyocytes and vascular ECs [100, 110]. GLP-1 and GLP-1(9-36) also increased myocardial glucose uptake through downstream targets involving P38 MAPK as well as nitric oxide production through eNOS activation. Using a pharmacological inhibitor of PKA, it was also shown that phosphorylation of Akt, ERK1/2 and eNOS were cAMP-PKA dependent. However, these effects were still apparent in the absence of the GLP-1 receptor [100, 110]. Therefore, to-date, the mechanism by which GLP-1 and related peptides mediate cardioprotection or cytoprotection is unclear.
Figure 1.4. Cardioprotective intracellular signal transduction pathways for GLP-1 and GLP-1(9-36) in cardiomyocytes and endothelial cells. Activation of GLP-1R leads to downstream activation of RISK kinases involved in mediating cytoprotection via reduced caspase activation. eNOS and MAPK kinase are involved increased myocardial glucose uptake.
1.3 cAMP signaling

1.3.1 cAMP is a second messenger

The small molecule, 3’-5’-cyclic adenosine monophosphate (cAMP) was first discovered in 1958 by E.W Sutherland, earning him the 1971 Nobel prize in Physiology and Medicine [125]. cAMP was termed a ‘second messenger’ due to its role in initiating intracellular signal transduction cascades in response to extracellular signals such as hormones or neurotransmitters (first messengers). cAMP is one of the most evolutionary conserved and intensely studied second messengers, and is involved in the regulation of diverse mammalian cellular processes including cell migration, proliferation and death, gene transcription and mitochondrial homeostasis. cAMP is synthesized from ATP in a cyclization reaction catalyzed by a family of enzymes, adenylyl cyclases (ACs), which releases a pyrophosphate from ATP [126]. The synthesis of cAMP in response to an extracellular signal is very robust and rapid. Indeed, using fluorescent imaging with cultured nerve cells, it was shown that the cAMP levels in resting cells are approximately 5 x 10^-8 M, which rise more than 20-fold within 20s of hormone stimulation [127].

However, increase in cAMP are transient, because of susceptibility to degradation by phosphodiesterases (PDEs), which rapidly hydrolyse either cAMP (or cGMP) to 5’-AMP (and 5’-GMP respectively) [126]. PDEs are a large and diverse family of enzymes, comprising eight different cAMP-degrading families (PDE1, 2, 3, 4, 7, 8, 10, 11), with multiple genes and a number of splice variants within each family, resulting in a large number of possible isoforms [128]. Different PDE isoforms have different tissue distributions, while an individual cell might express several PDE isoforms, localized to different subcellular compartments. In the
cardiovascular system, isoforms of the cAMP-specific PDE4 family are the most widely expressed and play a major role in health and disease. [129].

As a second messenger, cAMP regulates various downstream signaling pathways through activation of 2 main effector proteins: cAMP-dependent protein kinase (PKA) and guanine-nucleotide exchange proteins activated by cAMP (EPAC).

1.3.2 PKA and A kinase anchoring proteins (AKAP):

PKA is a holoenzyme, discovered in 1968 as the main downstream effector of cAMP [130]. In the resting state, PKA is a tetramer composed of two regulatory (R) subunits that bind to and suppress the activity of its two catalytic (C) subunits [131]. PKA is activated by binding of 2 cAMP molecules to each R subunits, causing a conformational change that relieves the inhibitory effects on the C subunits. The released C subunits then go on to activate and/or suppress the activity of target proteins through phosphorylation at Serine or Threonine residues. The C subunits of PKA can either phosphorylate cytosolic or membrane-anchored targets in cells, or translocate to the nucleus and phosphorylate the transcription factor CREB (CRE-binding protein), leading to the activation of cAMP-inducible genes, which play important roles in proliferation, survival, and differentiation of a wide variety of cells. However, there is now strong evidence of compartmentalization of localized cAMP-PKA signaling, through binding of PKA to AKAPs through its R subunits. Indeed, diffusion of cAMP through the cytosol is restricted, due to rapid degradation by PDEs, and AKAPs have now been identified as scaffolding platforms that ensure localized and specific cAMP signaling. AKAPs anchor several components of cAMP signaling, including receptors, ACs, cAMP, PKA, PDEs and various phosphatases and kinases, and have been identified in the nucleus [132, 133], sarcoplasmic reticulum [134] and mitochondria [135].
1.3.3 Epac

Epacs are guanine nucleotide exchange factors (GEFs) for the small GTPase Rap1. EPAC is directly activated by cAMP, which promotes its release from the guanine nucleotide GDP and its binding to GTP [136, 137]. Epac1 and Epac2 are the two main families identified in mammalian cells, with Epac1 showing wide tissue distribution including the cardiovascular system, while Epac2 has been identified in brain, pancreas, testes and other secretory cells. In the cardiovascular system, cAMP-dependent Epac1 has been identified in cardiomyocytes, vascular smooth muscle and endothelial cells and plays a major role in regulation of vascular tone, blood pressure as well as protection against oxidative stress and apoptosis [138, 139]. Epac2 is involved in the GLP-1 and/or GIP-mediated insulin secretion in pancreatic beta cells [140].

1.4 Adenylyl cyclases

Adenylyl cyclases (ACs) are enzymes that catalyze the formation of cAMP from ATP. There are six different classes of AC distributed across the phylogenetic tree from bacteria, archaea and eukaryotes, all with the sole function of cAMP synthesis [141]. In mammals, AC class III is the most predominant, with 10 isoforms (AC1-10) identified based on sequence homologies in the catalytic domain [142]. Furthermore, based on cellular location, these isoforms can be further subdivided into two groups: AC1-9 (tmAC) and AC10 (sAC).

AC1-9 are located at the plasmalemma and form the transmembrane ACs (tmAC), which are mainly regulated by the α-subunit of the stimulatory Gs or inhibitory Gi proteins through activation of GPCRs by extracellular ligands. AC1-9 isoforms are differentially expressed in tissues, but their expression is mostly conserved between rodents, canines and humans, with AC5 and AC6 being the most highly expressed isoforms in the heart [143]. TmACs can be
pharmacologically activated (both in vitro and in vivo) by the diterpene, Forskolin, isolated from the Indian Coleus plant [144]. Forskolin binds directly to a hydrophobic pocket near the catalytic sites of AC1-8, but not AC9 and sAC, which lack this binding site[145]. On the other hand, the activity of all tmACs can be selectively inhibited by the cell permeable 2′5-dideoxyadenosine (Ddox), which binds to a distinct purine domain (P-site) found on tmACs but not sAC [146].

AC10, also known as soluble adenylyl cyclase (sAC), is localized intracellularly and is insensitive to G-protein or Forskolin activation. sAC is structurally, molecularly and biochemically distinct from tmACs. Unlike tmACs, the mammalian sAC protein does not have any membrane spanning domains, and its catalytic domains C1 C2 are more closely related to cyanobacterial ACs than other eukaryotic ACs [147]. The structure, activity and physiological functions of sAC are reviewed in detail in the next section.

1.4.1 Soluble adenylyl cyclase

1.4.1.1 Discovery of sAC

For a very long time, tmACs, were believed to be the only source of cAMP in mammalian cells. An earlier study in the mid 1970s identified the presence of a distinctive Mn-2+-sensitive adenylyl cyclase, insensitive to forskolin, in the cytosolic fractions of rat testis tissue homogenates, and named it “soluble” AC [148]. However, it was not until the new millennium that evidence about an intracellular, receptor-independent source of cAMP started to emerge. Buck et al. were the first to provide molecular evidence that sAC was distinct from tmACs, after cloning the sAC gene from rat testis cDNA library and purifying a N-terminal 50-kDa sAC protein containing two distinct catalytic domains different from those of tmACs [147].
1.4.1.2 Structure of sAC

In rodents, RT-PCR analysis of testis extracts identified two alternative splice variants of a single sAC gene, encoding a full-length (sAC-fl) and truncated (sAC-tr) protein [149]. sAC-fl (187 kDa) is an isoform coded from the open reading frame (ORF) of the entire sAC mRNA, containing catalytic domains C1 & C2 and a non-catalytic C-terminal region (Figure 1.5). sAC-tr (≈50 kDa) is translated from a truncated sAC transcript lacking exon 11 with premature termination of the ORF after the catalytic domain, resulting in a smaller protein lacking the non-catalytic C terminal region (Figure 1.5). In humans, both sAC-fl and sAC-tr splice variants have been cloned and characterized in the cytosol of multiple tissues and cell lines including brain, heart, testis, small intestine, liver and kidney [150]. Heterologous expression of sAC cDNAs in HEK293 cells indicate that sAC-tr is 10 times more active than sAC-fl, based on cAMP generating activity [149]. This is because of an autoinhibitory region of 9 amino acids located immediately C-terminal to catalytic domain C2 in sAC-fl, but absent in sAC-tr, which was identified by site-directed mutagenesis of both sAC-fl and sAC-tr and subsequent heterologous expression and activity of the deleted mutants in HEK293 cells [151]. The mechanism of autoinhibition is unclear, as well as its physiological importance in the regulation of sAC activity, and studies investigating this specific aspect of sAC biology are lacking.
**Figure 1.5. Linear structure of soluble adenylyl cyclase protein, depicting the catalytic domains (C1 & C2) and the non-catalytic domain.** Full-length sAC protein (sAC-fl) contains both catalytic and non-catalytic C-terminal region, whereas truncated sAC protein (sAC-tr) contains only catalytic domains CI and C2 regions.
1.4.1.3 Regulation of sAC

sAC is directly activated by bicarbonate ions (HCO$_3^-$) and requires two divalent metal cations (Mg$^{2+}$ and calmodulin-independent Ca$^{2+}$) in the catalytic site in order to coordinate binding and cyclization of ATP. The role of bicarbonate in the activation of sAC was first demonstrated in stable HEK293 cell lines expressing either sAC-fl or sAC-tr, where addition of NaHCO$_3$, within physiologically relevant mammalian serum concentration, caused immediate increases in cAMP levels[152]. Moreover, purified recombinant sAC-tr protein were stimulated more than seven-fold in the presence of NaHCO$_3$, independent of pH changes. These findings have been validated by structure-kinetics modelling studies using crystal structures of sAC enzyme in complex with ATP analogs, calcium and bicarbonate ions [153]. It has been proposed that Ca$^{2+}$ bound to the γ-phosphate of ATP interacts with specific residues in the sAC catalytic site, resulting in an ‘open state’. This facilitates binding of the second divalent cation Mg$^{2+}$ to the α-phosphate of ATP, inducing a ‘closed state’, followed by release of both β and γ-phosphate and cyclization of the α-phosphate with adenosine to produce cAMP. HCO$_3^-$ acts allosterically at the second catalytic site to increase Vmax of sAC by causing closure of the active site and releasing the ATP-Mg$^{2+}$ inhibition [154]. sAC has been termed a ‘physiological sensor’ of HCO$_3^-$ ions, because the EC50 of sAC for HCO$_3^-$ is close to the normal serum concentration of HCO$_3^-$ in mammals at approx. 20 mM[152]. As such, small changes in physiological HCO$_3^-$ concentrations leads to increased sAC activity. Bicarbonates are in close equilibrium with CO$_2$, through reversible catalysis by the zinc metalloenzyme carbonic anhydrases (CA). It has been shown that sAC can be activated by bicarbonate generated from both extracellular and cytosolic intracellular CAs in a number of epithelial and endothelial cells [155]. Moreover, using isolated mitochondria from skeletal
muscle cells, it was shown that sAC can also be activated by metabolically generated CO2-/HCO3- inside the mitochondria [156].

1.4.1.4 Inhibition of sAC

1.4.1.4.1 Pharmacological inhibitors:

Specific pharmacological inhibitors against either sAC or tmACs have been exploited to distinguish whether a cAMP signaling pathway is initiated by sAC or tmACs. Bitterman et al. determined the specificity and potency of commercially available AC inhibitors, using stably transfected cells overexpressing either sAC or tmACs [157]. Of these, they found that the compound, KH7, inhibited the cAMP-forming activity of physiologically stimulated sAC, with an IC50 of ~8.0 µM. In contrast, KH7 had negligible inhibitory effect on cAMP-generating capacity of cells overexpressing tmACs, displaying an IC50 of ~138 µM. KH7 was first identified as an inhibitor of sAC through a library screen of 15,312 small lipophilic molecules, and was shown to inhibit the activity of both recombinant purified human sAC-tr protein and heterologously expressed sAC-tr in cellular assays [158]. While KH7 displays noncompetitive inhibition with either the substrate (Mg2+-ATP) or sAC activators Ca2+ or HCO3-, its exact mechanism of action is currently unknown. However, based on its specificity for sAC but not tmACs, KH7 is commonly used for studying the actions of sAC in a variety of cell-types and tissues [159-161].

1.4.1.4.2 Genetic inhibitors

Besides pharmacological inhibition, genetic knockdown of sAC through small interfering RNA (siRNA) has been successfully employed as a complementary tool for studying cellular actions
of sAC in various species ranging from bovine and rodents to humans. Using a single siRNA targeting the sAC gene in bovine corneal endothelial cells, Li et al. demonstrated that sAC was important in protecting these cells against staurosporine-induced apoptosis [162]. Similarly, transient transfection of a mouse osteoclast cell line with a custom-designed 21-nucleotide sAC-siRNA revealed the importance of sAC in preventing bone loss [163]. Corredor et al. studied effect of bicarbonate-activated sAC on axon growth in rat retinal ganglion cell survival using a pool of 2 siRNAs against sAC. After 48h transfection, they detected qualitative reductions in sAC PCR products in siRNA-treated retinal ganglion cells, as compared to scrambled siRNA [164]. In a human prostate carcinoma cell line, use of a commercially available pool of 4 siRNAs targeting the human sAC gene revealed the importance of sAC in controlling survival and proliferation of cancer cells [160]. To summarise, these above studies have commonly reported between 70-80% reduction in sAC expression following transient siRNA transfection, as measured by either mRNA expression through RT-PCR or protein expression through western blot. While partial reductions in sAC activity with pharmacological inhibitors and siRNAs represent the only alternatives for studying sAC in human cells, the use of transgenic mice with total sAC knockout offer more reliable alternatives for animal studies.

1.4.1.5 sAC knockout mice

The sAC knockout mice (sAC-KO) was first generated by targeted modification of exons 2, 3, and 4 of the murine sAC locus encoding the C1 catalytic domain in embryonic stem cells (ES) [165]. Blastocysts were injected in mice and chimeras were selected and mated with C57BL/6 females for germline transmission of the selected allele. Heterozygous mice were then interbred to produce homozygous sAC-KO mice (named as Sacy^{tm1Lex}/Sacy^{tm1Lex}), which lack both germline and somatic isoforms of sAC-tr and sAC-fl. sAC-KO mice are viable, and
behavioural/gross anatomical examinations did not detect any abnormalities in 10-week old, male and female homozygous (sAC<sup>−/−</sup>), heterozygous (sAC<sup>+/-</sup>) and wild-type littermate controls (sAC<sup>+/+</sup>) [165]. This group also observed these animals up to 8 months of age and reported no health issues. However, male homozygous, sAC<sup>−/−</sup> mice are infertile, due to severe sperm motility defects resulting in impaired fertilization [158]. Fertility is not affected in male heterozygous mutants, as shown by normal-sized litters produced by mating of sAC<sup>+/−</sup>, similar to sAC<sup>+/+</sup> male mice.

Although the cardiovascular phenotype of sAC-KO has not been studied, the mouse genome database report an extensive phenotypic characterization, whereby female sAC-KO display increased circulating cholesterol and triglyceride levels and both male and female homozygous knockout mutants have slightly elevated heart rates [166]. Surprisingly, the numerous somatic functions of sAC predicted by both pharmacological inhibitors and genetic deletion were inconsistent with just a prominently infertile phenotype coupled with minor cardiovascular dysfunctions in the sAC-KO mouse. It was suggested that either compensatory mechanisms or the presence of yet unidentified sAC locus were responsible for the lack of somatic phenotypes. Indeed, Farell et al. identified a promoter upstream of coding exon 5 in brain tissues that gave rise to a new somatic isoform of sAC containing only the catalytic domain C2, but with no adenylyl cyclase activity [167]. These findings have since been contradicted with the advent of monoclonal antisera against sAC, which have confirmed the lack of sAC-tr and sAC-fl expression in somatic tissues from sAC-KO mice as compared to wild-type littermate controls.

Recently, Zippin et al. generated a new strain of sAC-KO mice by backcrossing the Sacy<sup>tm1Lex</sup> allele into the C57BL/6 background and analyzing mutant mice following the 10th generation of backcrossing, named as sAC-C1 KO mice [168]. This group reported defective glucoregulation after i.p glucose tolerance test, accompanied by reduced glucose stimulate insulin release (GSIS),
despite histologically normal pancreatic morphology, islet structure and size in sAC-C1 KO mice. To-date, the advent of tissue-specific sAC knockout is awaited in order to assess the importance of sAC in various somatic functions [169].

1.4.1.6 Cellular localization of sAC

sAC is distributed intracellularly to discrete subcellular microdomains in the cytoplasm, nucleus, mitochondria, centrioles, mitotic spindles and mid-bodies, as evidenced by immunocytochemistry or western blotting using both monoclonal and polyclonal antisera against sAC [133]. Each of these locations contains known cAMP targets/effectors and has been functionally characterized. sAC has been identified inside the nucleus of skin keratinocytes, where it stimulates the activity of nuclear PKA to phosphorylate CREB, leading to modulation of genes involved in hyperproliferative skin disease [170]. Moreover, live cell imaging using a recombinant sAC protein attached to a nuclear localization domain and fluorescent probe mCherry revealed that sAC associates to a microdomain involving AKAP, PKA and PDEs inside the nucleus of HEK293 cells [132]. As opposed to the nucleus, Acin-Perez et al. discovered a different role for sAC inside the mitochondria, where the inner mitochondrial membrane is known to be impermeable to cAMP [156]. Using isolated mitochondria from mouse liver, this group showed that cAMP was generated inside the mitochondria through activation of a mitochondrial pool of sAC by metabolically generated CO$_2$/HCO$_3^-$.

In turn, activation of mitochondrial PKA regulates oxidative phosphorylation and increases ATP production by inducing phosphorylation of mitochondrial proteins. Lastly, activation of cytosolic pool of sAC has been shown to play a role in both cell death and cell growth. In prostate carcinoma cell lines, sAC is abundantly expressed and diffusely located throughout the cytoplasm, where it controls cell proliferation through activation of cAMP-dependent Epac2/Rap1/B-Raf signaling pathway.
Conversely, in rat cardiomyocyte undergoing acidosis/ischemia, cytoplasmic sAC translocate to the mitochondria upon activation, where it promotes the apoptotic pathway involving cytochrome c release [171].

1.4.1.7 Physiological actions of sAC

The role of sAC relative to health and disease has been studied in multiple tissues and organs, including reproductive organs, lungs, kidney, and brain. Its importance in the cardiovascular system, which is still a developing area of research, will be discussed here, specifically the dual role of sAC in the regulation of cell death and cell survival. For example, in isolated rat cardiomyocytes and coronary endothelial cells underdoing simulated ischemia/reperfusion injury in vitro, sAC was reported to activate mitochondrial-dependent apoptosis [171, 172]. Indeed, after induction of ischemia, sAC was found to co-translocate from the cytosol to the mitochondria, alongside the pro-apoptotic protein Bax, followed by PKA-dependent Bax phosphorylation, cytochrome c release, and formation of cleaved caspase-9 and ROS. Both pharmacological and genetic inhibition of sAC abrogated the mitochondrial Bax translocation and induction of apoptosis. Similarly, the mitochondrial translocation of sAC/Bax was also observed in rat aortic smooth muscle cells subjected to oxysterol-induced apoptosis [173]. However, based on the absence of any significant increases in cAMP observed in the apoptotic cardiomyocytes and aortic smooth muscle cells, its was concluded that mitochondrial sAC-translocation, rather than sAC-activation per se, was responsible for triggering sAC-dependent apoptosis[174].

In contrast, the same group of investigators report unpublished pilot experiments whereby sAC plays a protective role in adult rat cardiomyocytes subjected to IRI. They indicate that inhibition of sAC in reperfused ischemic cardiomyocytes blocked the recovery of mitochondrial function
and Ca\textsuperscript{2+} homeostasis and lead to cell death [174]. Moreover, another independent group observed an anti-apoptotic role for sAC in bovine corneal endothelial cells exposed to staurosporine-induced apoptosis, as confirmed through the use of both pharmacological and siRNA-mediated inhibition of sAC [162]. On a separate note, activation of isolated mitochondrial pool of sAC under normal physiological conditions increased oxidative phosphorylation and ATP generation and preserved mitochondrial integrity, thereby attributing a potentially protective role to sAC in every cells of the body, especially mitochondria-rich cardiac cells [156]. It appears that the discrepancies observed in the pro- and anti-apoptotic roles of sAC may be ascribed to differences between the models employed to induce apoptosis, and further studies are required to establish the protective role of sAC in the cardiovascular system.
Chapter 2
Rationale, Hypothesis and Research Aims

2.1 Rationale

To-date, *in vitro* and *ex vivo* models of IRI and *in vivo* models of MI have supported the beneficial cardiovascular actions of GLP-1 and GLP-1(9-36), mediated through a GLP-1R-independent mechanism. However, this cardioprotective, GLP-1R-independent signaling mechanism remains poorly understood. Given the fact that both GLP-1 and GLP-1(9-36) are believed to be further degraded into GLP-1(28-36), studies that further our understanding of the cardiovascular mechanism(s) of action of this smaller metabolite is of potential interest and could specifically support its use as a cardiovascular therapeutic against myocardial ischemic injury.

Drugs such as GLP-1R agonists and DPP-4i, which target the incretin systems, are an important class of therapeutics currently in clinical use for managing T2DM and in a few select cases for obesity. Manufacturers of these agents are mandated by the FDA to evaluate their long-term cardiovascular safety in large, randomized, double-blinded, and placebo-controlled clinical trials. This regulation was implemented because patients with T2D are at higher risk for major adverse cardiovascular events (MACE) such as MI, stroke and death, and (with a few exceptions) many agents approved for T2DM have failed to clinically reduce MACE or in some cases were associated with worsen cardiovascular outcomes. Findings from these large-scale cardiovascular safety trials, such as SAVOR-TIMI and TECOS, have recently been announced, most of them showing non-inferiority to usual T2DM care. A limitation to interpreting these studies is that the
CV mechanisms of actions of these drugs are not clear. Since GLP-1R agonists and DPP-4i differ in the relative levels of metabolites, including GLP-1(28-36), that they generate, a clear understanding of the CV role and mechanisms of action of GLP-1(28-36) may progress our interpretation of CV outcomes trial and expand the current knowledge of GLP-1 biology.

To summarize, a study of the cardiovascular effects and mechanisms of actions of GLP-1(28-36) has two important implications: (1) potential for drug development as a cardiovascular therapeutic, and (2) likely to interpret the difference in cardiovascular outcomes between the different classes of degradable and non-degradable GLP-1 drugs.

2.2 Hypothesis and Research Aims

2.2.1 Central Hypothesis

GLP-1(28-36) exerts direct cardioprotective effects against myocardial ischemic injury, through a novel molecular pathway independent of the GLP-1R receptor.
2.2.2 Aim 1

To assess the cardioprotective effects of GLP-1(28-36) in two independent models of myocardial ischemic injury in mice. (Presented in Chapter 4)

2.2.3 Aim 2

To investigate the GLP-1R-independent, cellular and molecular signaling mechanisms involved in the cardioprotective actions of GLP-1(28-36), in vitro and ex vivo. (Presented in Chapter 4)

2.2.4 Aim 3

To identify the binding partner(s) of GLP-1(28-36) in mouse heart. (Presented in Chapter 5)
3.1 Mice

Protocols were approved by the University Health Network Animal Use and Care Committee, in accordance with Guidelines for the Canadian Council for Animal Care. C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME) and housed for at least 2 weeks before experimentation. sAC+/− mice, B6;129S5-Adcy10tm1Lex/Mmnc, identification number 11659-UNC, were obtained from the Mutant Mouse Regional Resource Center 8U42OD010924-13 (Chapel Hill, NC), a NIH funded strain repository, donated to the MMRRC by Lexicon Genetics Incorporated. The mice were bred in our facility with C57BL/6J strain to generate sAC-null (sAC−/−) and genotyped according to the supplier’s protocol. Breeding and genotyping of Glp1r−/− mice have been described [77]. All experiments were performed on 10-12 weeks old male mice.

3.2 Drugs and Chemical Reagents

GLP-1(7-36) and GLP-1(28-36) peptides, with C-terminal amidated modifications, were synthesized by Bachem (Torrance, USA) and supplied in trifluoroacetate salt with >98% purity (confirmed by high performance liquid chromatography). KH7 and 2,5-dideoxyadenosine (Ddox) were a generous gift from Dr. D.H. Maurice (Queen’s University, Kingston, Ontario, Canada). Ketamine was purchased from MTC Pharmaceuticals (Ontario, Canada) and xylazine from Bayer Inc. (Ontario, Canada). All other reagents were from Sigma-Aldrich (Ontario, Canada).
3.3 Scrambled Peptide

To evaluate the specificity of GLP-1(28-36), a nonapeptide with the same 9 amino acids arranged in a completely different order (AGKFWRLIV), named SCRAM(28-36), was used as control. SCRAM(28-36) was designed using the online “Peptide Scrambled Library Design Tool” from Genscript (NJ, USA) and searched against UNIPROT/SWISSPROT protein databases, using BLAST (NCBI), for sequence similarity with known proteins. Out of 50 scrambled sequences, SCRAM(28-36) was selected as the peptide with least predicted biological activity, based on sequence alignment score <40% and non-significant E-value >=1.0.

3.4 Mouse model of myocardial infarction

Two different experimental protocols were employed to study the functional importance of the GLP-1(28-36) in cardioprotection against ischemic injury. Live animals were used to assess the protective effect of GLP-1(28-36) on myocyte death after coronary occlusion, in vivo whereas an isolated heart Langendorff preparation was used to study the protective effect of GLP-1(28-36) on cardiac contractile function against IRI injury, ex vivo.

3.4.1 Experimental MI model, in vivo

3.4.1.1 Peptide Infusions

A micro-osmotic pump system with flow moderator (Alzet®, Model #1002, California, USA) was used for continuous, subcutaneous infusions in mice. The model of micro-osmotic pump used has a reservoir volume of 90±10 µl with a nominal pumping rate of 0.25±0.05 µl/hr for 14 days at 37°C. Filling, handling and surgical implantation of micro-osmotic pumps were
performed as per manufacturer’s protocol. Prior to filling, peptides were diluted to desired dosage with 0.9% saline in order to maintain stability at 37°C for the duration of the experiment. A small 1.0 ml syringe with a 27 gauge blunt-tipped filling tube was used for filling the pumps, under sterile conditions. Prior to implantation, the pumps were primed by overnight incubation in sterile saline at 37°C. The next day, mice were anesthetized via intraperitoneal injection with a cocktail of ketamine (100 mg/kg) and xylazine (10 mg/kg), a small incision was made in the skin between the dorsal scapulae, the pump was implanted between the subcutaneous connective tissues, and the skin incision was closed with sutures. Mice were allowed to recover under normal housing conditions and monitored closely for infections or open sutures.

3.4.1.2 LAD Ligation

Fourteen days after micro-osmotic pump implantations, mice were re-anesthetized by intraperitoneal injection with ketamine:xylazine (ratio 100:10 mg/kg), intubated and mechanically ventilated with room air. An incision was made in the ventral side to open the thorax and pericardium and expose the heart. Global ischemia was generated by permanent ligation of the left anterior descending coronary artery using a 7.0 silk suture. The chest was closed with sutures, antiseptics were applied locally, and the animals were allowed to recover. For consistency and accuracy, the LAD ligation procedure was performed by a single surgeon, who was blinded to treatment conditions. At day 4 post-MI, animals were sacrificed and hearts were surgically excised for measurement of infarct size.

3.4.1.3 Infarct Size Measurement

Freshly excised hearts were washed twice in cold PBS to remove excess blood, and cut into ≈ 2mm sections perpendicular to the long axis. The sections were incubated at 37°C in 2% 2,3,5-
triphenyltetrazolium chloride (TTC) for 15 min, after which they were washed with PBS and fixed in 4% paraformaldehyde for 1h prior to image acquisition using a digital scanner. Image J software (NIH, USA) was used to measure infarct area (TTC unstained white area) and total left ventricle (LV) area. Infarct size was expressed as a percentage of total ventricular area, which, in global ischemia, is equal to the area at risk.

3.4.1.4 Weight & glucose measurements

Body weight (g) and fresh heart weight (mg) were measured on the day of sacrifice, at 4d post-MI. Blood samples were collected from the tail vein on day1 (beginning) and day14 (end) of peptide infusions and non-fasting glucose was measured using a glucometer (Roche Accu-Chek).

3.4.2 Mouse model of ischemia/reperfusion injury

A second experimental protocol was employed to study the direct cardioprotective effects of GLP-1(28-36) against IRI injury. A Langendorff setup was used to measure cardiac contractile function of isolated mouse hearts undergoing IRI injury [110]. The in vivo mouse model provides a reliable model that mimics clinical situations of MI. However, the contributions of other organs (such as pancreas or nervous system) to the observed cardiovascular effects cannot be ascertained. Therefore, the ex vivo isolated heart model of retrograde perfusion provides an alternative method for determining the direct cardiac actions of compounds. However, the disadvantage of the isolated heart model is that it lacks the neural and hormonal regulation of in vivo settings.
3.4.2.1 Buffer preparations

Isolated hearts were perfused with Kreb-Hensleit buffer, prepared as follows: NaCl (118mM), KCl (4.7mM), NaHCO₃ (25mM), KH₂PO₄ (1.2mM), MgSO₄ (1.2mM), Sodium pyruvate (2mM), and glucose (11mM). Stock solutions (10X) were prepared and stored at 4°C. On the day of experiment, fresh 1X Kreb-Hensleit solution was prepared from stock and filtered through a 0.45µm membrane filtration system. CaCl₂ solution (final concentration of 2.5mM) was added and the buffer oxygenated with 95% O₂ and 5% CO₂ throughout the experiment to maintain pH 7.4. The temperature was kept constant at 37°C with a thermo-circulating system (Harvard Apparatus).

3.4.2.2 Isolated Heart Preparations

Mice were anesthesized with intraperitoneal injections of ketamine (100 mg/kg)/xylazine (10 mg/kg). Heparin(1000 IU/kg) was added to the injections to prevent formation of blood clots during surgery. After thoracotomy, the heart was quickly excised by cutting the aorta ≈ 4-5mm above the aortic valve. The excised heart was immediately placed in warm Kreb-Hensleit buffer for a few seconds to empty residual blood through spontaneous contractions. The heart was then transferred to a dish containing cold Kreb-Hensleit buffer, in view of preventing contractions and preserving energy expenditure. Within seconds, a grooved 20-gauge stainless steel cannula, connected to a Langendorff apparatus via a short intravenous (IV) line, was inserted and tied to the aorta with surgical sutures. Perfusion was immediately started at a constant perfusion pressure (CCP) of 80 mmHg, and the aortic cannula was then attached directly to the Langendorff apparatus by removing the connecting IV line. A CCP of 80mmHg was monitored throughout the experiment via a transducer connected to the cannula/heart combination. The
isolated heart was surrounded by a water jacket to maintain constant temperature at 37°C.

In order to maintain optimal contractile function of the heart, cannulation time was restricted to less than 1min, and the heart was allowed to perfuse for 3min prior to any other intervention. Once the heart was observed to be beating regularly, a fluid-filled plastic balloon (1cm diameter), connected to a pressure transducer, was inserted into the left ventricle via a left atrial incision. The balloon was then inflated to measure heart rate (HR), left ventricular end systolic pressure (LVESP) and end diastolic pressure (LVEDP), obtained from computer analysis software (Acknowledge 3.7.1. Biopack System; CA, USA). Isovolumetric LVEDP was maintained between 8-10 mmHg by adjusting the volume of the balloon inside the ventricles. The heart was allowed to equilibrate for 20min in order to allow adjustment to the retrograde perfusion conditions. At the end of equilibration phase, only hearts displaying HR greater than 300 beats/min underwent ischemia/reperfusion protocol.

### 3.4.2.3 Ischemia/Reperfusion Protocol

After 20 min of equilibration, hearts were perfused with either Kreb-Hensleit buffer or specific inhibitors for 20 min, followed by 20 min perfusion with peptide of interest. All inhibitors and peptides (diluted at 10X the desired concentrations) were administered through a syringe pump (Harvard Apparatus) at one-tenth of the coronary flow rate. The drugs were thoroughly mixed with perfusion buffer to achieve the desired concentration at 37°C, prior to entering the aorta. Global ischemia was then generated by clamping inflow to the heart for 30 min, after which reperfusion was reinstated for 40 min. In the untreated control group, hearts were perfused with Kreb-Hensleit buffer for a total of 40 min prior to ischemia. Our lab has previously observed that the isolated heart undergoes hypothermia during the ischemia phase. Hypothermia has been linked to “hibernation induction triggers” known to confer myocardial protection after IRI [175].
To prevent this, hearts were immersed in Kreb-Hensleit buffer without CaCl$_2$ and glucose and maintained at 37°C for the entire duration of the global ischemic phase. At the end of IRI protocol, hearts were dismounted, wiped dry, weighed, and quickly frozen in liquid nitrogen and stored at -80°C. Data acquisition of HR, LVEDP, and LVESP were continuously monitored throughout the entire protocol.

### 3.4.2.4 Measurement of mechanical function

In isolated heart IRI models, left ventricular developed pressure (LVDP) is a commonly defined index of contractile function, measured as the difference between LVESP and isovolumetric LVEDP [176]. Functional recovery was defined as the measure of cardiac performance and expressed as a percentage of LVDP at the end of 40min reperfusion over LVDP just prior to global ischemia. Functional recovery represents the improvement in myocardial mechanical functions following prolonged global ischemia.

### 3.4.2.5 Coronary flow

Coronary venous effluents emerging from the perfused heart per minute was collected at time-intervals throughout the IRI protocol, and expressed as flow rate ml/min. Coronary flow rate was used to calculate pumping rate to achieve the desired final perfusion concentration of drugs. Coronary venous effluents were assayed for extracellular release of lactate dehydrogenase (LDH) or cAMP.

### 3.4.2.6 Lactate dehydrogenase release

In addition to cardiac function, the extent of myocardial tissue injury were assessed by measuring LDH release in coronary effluents. LDH is an enzyme released extracellularly when
damaged or necrosed cells lose membrane integrity. An enzymatic assay kit, TOX7 (Sigma-Aldrich, Ontario, Canada) was used to measure LDH levels. This assay is based on the reduction of NAD (substrate) by LDH to produce reduced NADH, which stoichiometrically converts tetrazolium (dye) to a colored substance that can be measured spectrophotometrically. More specifically, the coronary effluents were centrifuged at 250 x g for 4min to pellet cells. An aliquot of the supernatant was mixed 2X the volume of LDH assay mixture consisting of 1:1:1 ratios substrate:dye:cofactor, in 96-well flat bottom plates. The plates were incubated in the dark for 20 min at room temperature, at the end of which the reaction was terminated by addition of 1/10 volume 1N HCl to each well. Absorbance at 490 nm (Abs$_{490}$) was measured in a Thermo Max microplate reader (Molecular Devices, Union City, CA). Background absorbance was measured at 690 nm (Abs$_{690}$) and LDH activity was expressed as Abs$_{490}$ - Abs$_{690}$ per coronary flow rate and standardized to wet heart weight (Abs$_{490}$ - Abs$_{690}$ · ml$^{-1}$ · min$^{-1}$ · g$^{-1}$).

### 3.4.2.7 cAMP assay

In a first attempt to unravel the mechanistic action of GLP-1(28-36), levels of second messenger cAMP were measured in coronary effluents, using a separate protocol. The necessity for a different protocol arose from the fact that cAMP is susceptible to rapid degradation by phosphodiesterases and can only be quantified in the presence of a phosphodiesterase inhibitor. However, since cAMP plays a major role in cardiac muscle contractility, inhibiting its degradation would largely affect the LVDP readouts from the IRI protocol. Therefore, a separate group of isolated hearts was perfused for 10min with Kreb-Heinsleit buffer containing the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX,100mol/L), after the 20min equilibration phase. The perfusion was then switched to buffer with or without peptides for another 10min. Coronary effluent was collected at timed intervals and cAMP levels quantified
using an enzymatic assay kit as per the manufacturer’s protocol. cAMP was expressed as a function of coronary flow rate and heart weight (pmol · ml⁻¹ · min⁻¹ · g⁻¹).

3.5 Cell culture and drug treatment

Whole left coronary arteries were dissected from C57BL/6J mice, sAC-KO and wild-type littermate controls and coronary artery smooth muscle cells (caSMC) were isolated as previously described[177]. caSMCs were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS: Wisent Inc., Quebec, Canada), 1% Platelet-Derived Growth Factor-BB (PDGF-BB: Sigma-Aldrich, Ontario, Canada) and 1% penicillin-streptomycin (Wisent Inc., Quebec, Canada) and passages 3-5 were used for experiments.

Primary human caSMCs were purchased from Cascade Biologics (Gibco, C-017-5C) and maintained in M231 media (Gibco, M-231) supplemented with Smooth Muscle Differentiation Supplement (Gibco, S-007-25).

Mouse atrial cardiomyocyte cell line (HL-1) was cultured on plates coated with 0.02% gelatin and 12.5 µg/ml fibronectin and maintained in Claycomb Media (Sigma-Aldrich, Ontario, Canada) supplemented with 10% fetal bovine serum, 100 µM norepinephrine and 2mM L-glutamine.

Ventricular cardiac myocytes were freshly isolated from 1-3 days-old C57BL6/J mice as per method described [178], with some minor modifications. More specifically, hearts were excised from neonatal pups, and washed in ice-cold Hank’s buffer (Life Technologies) [containing 136 mM NaCl, 4.2 mM KCl, 5.6 mM dextrose, 0.44 mM KH₂PO₄, 0.34 mM NaH₂PO₄, 4.2 mM NaHCO₃] and supplemented with 5 mM HEPES (pH 7.4) and 100 U/ml penicillin-streptomycin
(Wisent Inc., Quebec, Canada). The atria were removed and only the ventricles were minced into 2-4 pieces and digested with 1mg/ml collagenase type II (Worthington Biochemicals) for 2 h with gentle rocking at room temperature (RT). Following digestion, cells were pelleted by centrifugation at 800 g for 5 min at RT, and the pelleted cells were re-suspended in DMEM supplemented with 10% FBS and pre-plated for 1 h at 37°C in a humidified incubator to separate fibroblasts. After pre-plating, neonatal cardiomyocytes were cultured on plates coated with 0.02% gelatin and 12.5 µg/ml fibronectin and maintained in DMEM/F-12 medium (Life Technologies) supplemented with 10% FBS, 1% penicillin/streptomycin, 3 mM sodium pyruvate, 2 mM L-glutamine, 0.2% (v/v) BSA, 0.1 mM ascorbic acid, and 0.5% (v/v) insulin-transferrin-selenium. Cells were incubated for at least 24 h to inhibit noncardiomyocyte growth.

All cell types, including cardiomyoctyes, HL-1 and caSMCs, were serum-deprived for 24h prior to treatment with the phosphodiesterase inhibitor IBMX (450 µM) for 30 min at 37°C in a humidified incubator, after which the drug of interest and/or vehicle PBS was added to the media to achieve the desired final concentration. After 10 min incubation, cells were lysed for measurement of cAMP, PKA or ATP. All experiments were performed 3 times and within each experiment every condition was tested with three replicates.

3.6 Cellular injury in vitro

A cellular injury model of oxidative stress generated by treating either neonatal cardiomyocytes [100] or human caSMCs [179] with hydrogen peroxide (H₂O₂: 100 µM) has been previously used in our lab. Human or mouse caSMCs were grown to 70% confluency in fully supplemented media. After 24h serum-deprivation, cells were pre-treated for 20 min with vehicle PBS or 100nM of GLP-1(28-36) or SCRAM(28-36), followed by 48h incubation with 100 µM H₂O₂.
Cell culture media was replenished with fresh vehicle, GLP-1(28-36) or SCRAM(28-36) after first 24h incubation with H₂O₂. For human caSMCs, specific inhibitors KH7 (20 µM) and Ddox (50 µM) were added for 3h prior to peptide treatments. At the end of 48h H₂O₂ incubation, cellular necrosis was determined from LDH release assayed in duplicates from aliquots of cell culture media using a commercially available cytotoxicity kit (TOX7: Sigma-Aldrich, Ontario, Canada). For determination of early apoptosis, mouse caSMCs were treated for 20 min with peptides of interest followed by 7h incubation with 100 µM H₂O₂. Cells were then lysed for protein determination of the apoptotic marker by western blot.

### 3.7 siRNA Transfection

Human caSMCs were transiently transfected with siRNA to knockdown soluble adenylyl cyclase (sAC) as previously described, with some minor modifications[160]. siRNA duplexes consisted of a pool of four different pre-designed sequences targeting the human sAC mRNA sequence (L-006353-00: Thermo Fisher Scientific Biosciences Corp., Ontario, Canada), with a scrambled, non-targeting siRNA pool used as control (D-001810-10; Thermo Fisher Scientific Biosciences Corp.). Briefly, the transfection reagent was prepared by incubating anti-sAC siRNA or scrambled siRNA with Lipofectamine RNAiMax™ (Life Technologies, Ontario, Canada) in Opti-MEM medium (Life Technologies) for 20 min at room temperature. This mixture was then added to human caSMCs (cultured for 24h at 50% confluency in antibiotics-free supplemented media) in order to achieve a final siRNA concentration of 50 nM. Transfection was carried out at 37°C for 24h, after which the medium was switched to normal supplemented medium and the cells were allowed to grow for another 48h. Knockdown of sAC was determined by RT-PCR using primers against human isoform of sAC as previously described[180], which revealed approximately 50% decrease in sAC gene expression.
3.8 Western Blot

Protein extraction and quantification were as described[118, 179]. Whole heart protein extracts from C57Bl6/J mice and cell lysates from caSMCs were resolved on SDS-PAGE (Novex®: Life Technologies), blotted on PVDF membrane, then probed overnight at 4°C for sAC (R21, 1:1000, CEP Biotech Inc.), cleaved caspase-3 (#9661, 1:1500, Cell Signaling Technology), hadha (#54477, 1:1000, Abcam) and GAPDH (sc-25778, 1:10000, Santa Cruz Biotechnology) as loading control. Protein bands were detected using corresponding horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibodies (1:10000, Biorad), imaged by chemiluminescence with MicroChemi 4.2 imaging system (DNR Bio-Imaging Systems Ltd., Jerusalem, Israel), and quantified using the analysis software Quantity One version 4.6.

3.9 cAMP assay

For in vitro measurements of cAMP production, cardiomyocytes and caSMCs (~2 x10^5 cells) were lysed with 0.1N HCL for 20 min at RT, followed by centrifugation at 1000 g for 10 min. A sample of the supernantant was assayed for total protein concentration using a bicinchoninic acid (BCA) assay kit (Sigma-Aldrich). Supernatant with protein concentration >1 mg/ml was diluted 10X with enzymatic immunoassay (EIA), and acetylated with a mixture of KOH and acetic anhydride in order to increase the sensitivity of cAMP detection, as per the manufacturer’s protocol. cAMP concentrations in total cellular lysates were determined using a cAMP enzymatic immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI), against a standard curve.
3.10 PKA activity assay

PKA activity in mouse and human caSMCs cellular lysates were measured using the PepTag® non-radioactive cAMP-dependent protein kinase kit (Promega, Madison, WI), as previously described[105]. Human or mouse caSMCs were plated in 6-well plates at a density of 1 x10⁶ cells/well and treated with peptides or controls as per drug treatment protocol above. Cells were then washed 2X with ice-cold PBS, and suspended in ice-cold PKA extraction buffer (25mM Tris-HCl (pH 7.4), 0.5mM EDTA, 0.5mM EGTA, 10mM β-mercaptoethanol, 0.5mM PMSF, and protease inhibitor cocktail) and homogenized in a Dounce homogenizer with 25 strokes on ice. Lysates were centrifuged for 5 min at 4°C at 14,000 g and the supernatant was assayed the same day. Lysates with a total protein concentration of 2µg/ul were incubated at RT for 30 min with 2µg/ul of the PKA-specific peptide substrate PepTag® A1 Peptide (a.a sequence L-R-R-A-S-L-G). The 30 min reaction time is expected to produce a linear relationship between phosphorylation and PKA activity for 0-16ng of PKA, as per the manufacturer’s protocol. The cAMP-dependent Protein Kinase, Catalytic subunit was supplied as control enzyme by the manufacturer and was used at a concentration of 2ug/ul in our assay. Following enzymatic reaction, non-phosphorylated (+1 charge, *moves towards anode*) and phosphorylated (-1 charge, *moves towards cathode*) PepTag® A1 Peptide were separated by 0.8% agarose gel electrophoresis and visualized under UV light.
3.11 ATP assay

Following drug treatment, mouse caSMCs (~2 x 10^5 cells) were washed twice with ice-cold PBS, and lysed by addition of 2.5% (w/v) trichloroacetic acid to extract ATP. Cells were scraped from the dish and centrifuged at 10,000 x g for 5 min at 4°C, after which the supernatant was immediately diluted 10X and neutralized with TRIS-acetate buffer, pH 7.75. ATP was measured using the ENLITEN® ATP assay system bioluminescence detection kit (Promega, Madison, WI). Briefly 10µl of sample was added to 100µl of luciferin/luciferase (rL/L) reagent, and relative light units (RLU) signals were measured with a luminometer. A delay time of 2s after rL/L addition and a 9s integration time were used as per manufacturer’s recommendations. All samples were assayed in duplicates and ATP levels expressed as average RLU.

3.12 Statistical analyses

Data are expressed as mean ± SEM. One-way ANOVA was used to analyze differences between ≥ 3 treatment groups using Prism v4.0 (GraphPad Software Inc., San Diego, CA). Two-way ANOVA was used to compare differences between GLP-1(28-36) and Scram(28-36) across multiple doses or different mouse strain. The Bonferroni post-hoc test was used for multiple comparisons. P<0.05 was considered to be statistically significant.
Chapter 4
GLP-1(28-36) protects against myocardial ischemic injury by activating soluble adenylyl cyclase in coronary artery smooth muscle cells

Running title: Mundil et al.; Cardioprotective actions of GLP-1(28-36)

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This chapter is presented verbatim as a manuscript submitted to Circulation by Mundil et al.

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4.1 Abstract

The molecular and cellular mechanisms of action underlying the ability of the incretin glucagon-like peptide-1 (GLP-1) to prevent myocardial ischemic injury are not known. The ability of GLP-1 and its primary metabolite GLP-1(9-36) to protect mouse hearts lacking a functional GLP-1 receptor suggested a receptor-independent mechanism.

Here we show that the cardioprotective effects of a small secondary metabolite of GLP-1, namely GLP-1(28-36), are mediated by the soluble adenylyl cyclase Adcy10 (sAC) expressed in mouse coronary artery smooth muscle cells (caSMC). In both \textit{ex vivo} and \textit{in vivo} mouse models of ischemia-reperfusion injury and chronic left anterior descending coronary artery ligation respectively, treatment with GLP-1(28-36) was as cardioprotective as with the parent peptide GLP-1, and abolished by scrambling its nine amino-acid sequence. Using pharmacological inhibitors, siRNA and sAC-null mice, we demonstrate that GLP-1(28-36) acts directly on both human and mouse caSMC, resulting in sAC-dependent increases in cAMP, activation of PKA, and cytoprotection from oxidative stress injury. Furthermore, we show that GLP-1(28-36) modulates sAC-generated cAMP activity by increasing intracellular levels of ATP.

GLP-1(28-36) is a small peptide that targets a novel molecular (sAC) and cellular (caSMC) pathway for the treatment of myocardial ischemic injury.
4.2 Introduction

In acute myocardial infarction (MI), the size of the resulting infarct is the most determinant cause of mortality and morbidity from coronary artery disease (CAD)[181]. While early reperfusion with percutaneous coronary intervention (PCI) or thrombolysis is of unequivocal benefit in this condition, both treatments are inherently complicated by acute reperfusion injury[182]. Indeed, consensus reviews of animal[11] and human[183] studies suggest that as much as 50% of the tissue injury post-MI is attributable to ischemia-reperfusion injury (IRI). As such, therapies aimed at limiting IRI may have high potential for clinical impact. Although many treatments shown to be effective in animal models of IRI have failed to translate into clinical usage, glucagon-like peptide-1 (GLP-1) and the degradation-resistant GLP-1 analog exenatide have shown benefits against IRI in both animal models[99, 110, 116, 184] and small early phase clinical trials[120-122, 185].

The predominant active form of GLP-1 is the 30-amino acid peptide GLP-1(7-36)amide [abbreviated hereafter as GLP-1], which acts through a Gs-coupled receptor known as GLP-1R. However GLP-1 is short-lived, being rapidly degraded to the non-insulinotropic 28-amino acid metabolite GLP-1(9-36), by action of the ubiquitously expressed enzyme dipeptidyl peptidase-4 (DPP-4)[57, 186]. GLP-1 and GLP-1(9-36) are also known to be cleaved by action of neutral endopeptidase (NEP24.11) into many smaller carboxy-terminal metabolites, of which the nine-amino acid, C-terminal cleavage product GLP-1(28-36) has biological functions[102-105]. At present, several DPP-4 degradation-resistant GLP-1R agonists, and numerous DPP-4 inhibitors have been approved for the treatment of type 2 diabetes and are widely used in clinic.
With regards to the potential utility of these agents in clinical settings of myocardial IRI, the first report by Nikolaidis et al[119] was a non-randomized, un-blinded study of 10 treated (vs. 11 untreated) patients, in whom a 72 h continuous intravenous infusion of human recombinant GLP-1 following PCI was shown to improve cardiac function in patients with acute MI and left ventricular (LV) dysfunction[119]. Similar effects of native GLP-1 were reported in another group of 20 non-diabetic patients with normal LV function undergoing elective PCI[121]. Subsequently, a randomized, placebo-controlled double-blinded study in 74 patients undergoing primary PCI for acute MI showed that a 6 h intravenous infusion of exenatide reduced infarct size as a function of area at risk when initiated early after the onset of chest pain[185]. In addition to these small clinical studies, the cardioprotective benefits of GLP-1, DPP-4 inhibitors (DPP-4i) and GLP-1 analogs have been demonstrated in multiple pre-clinical models of cardiac injury including mice[110, 187], rats[99], dogs[184], and pigs[116].

However, the mechanisms underlying these effects have not been fully elucidated, with some pre-clinical studies revealing inconsistent results (Reviewed in[188]). For example, we observed that both GLP-1 and its presumed ‘inert’ metabolite GLP-1(9-36) showed coronary and mesenteric artery vasodilation, as well as unequivocal cardioprotection from IRI ex vivo, in isolated tissue preparations from both wild-type (WT) and GLP-1R knockout (Glp1r−/−) mice, while the non-degradable GLP-1R agonist exenatide did not manifest these effects in the absence of a functional GLP-1R[100, 110]. To us, this suggested either the existence of an alternate receptor for GLP-1 and/or GLP-1(9-36), or receptor-independent mechanisms of action. More recently, mice with cardiomyocyte-specific deletion of GLP-1R continued to manifest cardioprotective effects of the GLP-1 agonist liraglutide in an in vivo model of MI[83], suggesting either that the cardioprotective effect of this agent are derived from non-cardiomyocyte or GLP-1R-independent actions.
To explore this issue, we wondered whether the secondary metabolite, GLP-1(28-36), might mediate cardioprotection. Indeed, Habener and colleagues have suggested that this 9-amino acid metabolite of GLP-1 appears to traffic to the mitochondria of isolated hepatocytes in vitro [103], and exhibits anti-apoptotic effects in INS-1 beta cells exposed to gluco-lipotoxic oxidative stress in association with inhibitory effects on mitochondrial depolarization, cytochrome-c release and caspase activation [104]. Moreover, continuous infusions of the nona-peptide prevented weight gain and improved glycemic control in diet-induced obese mice [102].

Here we show, for the first time, direct cardiovascular effects of this small peptide agent. In both in vivo and ex vivo models of myocardial ischemic injury, GLP-1(28-36) prevented LV dysfunction, reduced infarct size, and protected specific cell types from oxidative stress injury. We show that the cardioprotective actions of GLP-1(28-36) do not depend on a functional transmembrane GLP-1R, but are mediated intracellularly through Type 10 soluble adenylyl cyclase (sAC) accompanied by increased cAMP levels and PKA activation.

While the production of cAMP has traditionally been attributed to the activation of Type 1-9 transmembrane adenylyl cyclase (tmAC) associated with heterotrimeric G-protein-coupled receptors in the plasmalemma, there is strong evidence of intracellular, compartmentalized sources of cAMP generated by sAC [152]. This enzyme has been localized to the cytosol, nucleus, mitochondria, and centrioles of mammalian cells, and is activated by bicarbonate and divalent cations (e.g., Ca$^{2+}$, Mg$^{2+}$, Mn$^{2+}$), while serving as a physiological ATP sensor to catalyze the production of cAMP [133, 168]. Although the therapeutic potential of sAC is being studied in several organs (Reviewed in [189]), we are the first to report a role in incretin signaling in the heart. More specifically, we demonstrate that GLP-1(28-36) activates sAC and stimulates
cAMP release by increasing intracellular levels of ATP from mouse coronary artery smooth muscle cells (caSMC).

4.3 Methods

See Chapter 3, Sections 3.1 to 3.12

4.4 Results

4.4.1 Pretreatment with GLP-1(28-36) reduces myocardial infarct size in mice.

We began our study by directly examining the therapeutic relevance of GLP-1(28-36) in vivo in 10-12 week old male WT C57BL/6J mice subjected to myocardial ischemic injury by permanent ligation of the left anterior descending (LAD) coronary artery following 14d subcutaneous infusions of GLP-1(28-36) [18.5 pmol/kg/min][102], saline, Scram(28-36) [18.5 pmol/kg/min; negative control], or GLP-1 [3.5 pmol/kg/min; positive control][190] (Figure 4.1A). TTC-stained heart sections harvested 4d post-MI revealed obvious reductions in the unstained infarct areas of GLP-1(28-36)-treated hearts as compared to Scram(28-36)- or saline-treated controls (Figure 4.1B). Blinded histomorphometric analysis confirmed that pre-treatment with GLP-1(28-36) significantly decreased LV infarct size at 4d post-MI as compared to Scram(28-36)- and saline-treated controls (24.9±2.4%, n=7, vs. Scram: 32.5±1.8%, n=7; saline: 34.3±2.8% n=9; p<0.05 for both) (Figure 4.1C), with the effect of GLP-1(28-36) treatment comparable to that of GLP-1 (23.0±1.9%, n=13; p=ns).
As infusions of GLP-1(28-36) and GLP-1 have been shown to influence body weight and blood glucose levels in high-fat fed mice[102], and we ourselves have documented an effect of calorie restriction and weight loss on cardioprotection[118], we wanted to examine the consequence of our various 14d infusions on these parameters. Under the ad libitum food conditions used, heart weights, body weights, heart/body weight ratios and non-fasting tail blood glucose measurements were not affected by treatment assignment (Figure 4.2A,B). These results suggest that the reductions in infarct size observed in mice treated with GLP-1(28-36) are unrelated to changes in body weight or glycemic control.
Figure 4.1

A

Osmopump implantation

LAD ligation

Infarct measurement

-14d
0d
4d

B

Saline
Scram
28-36
GLP-1

C

Infarct size (%LV)

Saline Scram 28-36 GLP-1

*
Figure 4.1. Pre-treatment with GLP-1(28-36) reduced infarct size in mice. A, Schematic of the *in vivo* animal protocol employed. B, Representative photomicrographs of TTC-stained heart sections depict infarct (white) vs. viable (red) tissue at 4d post-MI. Significantly less infarcted area was visible in GLP-1(28-36) and GLP-1 (positive control)-treated hearts as compared to saline or Scram(28-36) controls. C, Grouped data: 14d pre-treatment with GLP-1(28-36) in WT mice significantly reduced %LV infarct size at 4d post-MI. Effects of saline only (n=9), GLP-1 (3.5 pmol/kg/min; n=13), GLP-1(28-36) and Scram(28-36) (both 18.5 pmol/kg/min; n=7). Data shown are mean±SE. *P<0.05 vs. the corresponding control by 1-way ANOVA with Bonferroni post hoc test.
Figure 4.2. Pre-treatment with GLP-1(28-36) in a mouse permanent LAD-ligation model of experimental MI. C57BL/6J mice were pre-treated for 14d by continuous infusion with peptides shown, followed by LAD coronary artery ligation and were sacrificed on d4 post-MI. Non-significant changes in heart/body weight ratios were observed amongst treatment groups measured at d4 post-MI. B, Non-significant changes were observed in non-fasting blood glucose levels between d0 and d14, measured using glucose-strips from tail vein blood samples. n=7-13/group.
4.4.2 Direct cardioprotective effects of GLP-1(28-36) do not depend on the GLP-1R.

To assess whether GLP-1(28-36) can exert direct effects on the heart, we next employed an ex vivo isolated heart global IRI model under constant coronary perfusion pressure[110] (Figure 4.3A). Representative left ventricular developed pressure (LVDP) tracings show that a 20 min perfusion with GLP-1(28-36) [6nM] prevented loss of LVDP following IRI as compared to buffer- and Scram(28-36) [6nM]-treated controls (Figure 4.3B). Of note, the dose of GLP-1(28-36) employed for these experiments was selected on the basis of its dose-response effect on LVDP post-IRI (Figure 4.3C). Indeed, recovery of LVDP was greater in GLP-1(28-36) vs. buffer- or Scram(28-36)-treated hearts (57.6±6.6%, n=12 vs. buffer: 22.7±3.6%, n=13; Scram: 35.9±5.6% n=4; p<0.05 for both comparisons), and comparable to GLP-1 (67.3±8.6%, n=13; p=ns) (Figure 4.3D; black bars). To further quantify the effects of GLP-1(28-36) on tissue IRI, we measured release of LDH in coronary effluents from the isolated perfused hearts. Pretreatment with GLP-1(28-36) significantly reduced LDH release during reperfusion as compared to buffer- or Scram-treated controls (Figure 4.3E). Again, the effect of GLP-1(28-36) on cardiac enzyme release was similar to that of the parent GLP-1.

To address the question of whether the cardioprotective actions of GLP-1(28-36) require a functional transmembrane GLP-1R, we repeated our ex vivo experiments using hearts isolated from mice with genetic deletion of two exons of the GLP-1R gene[77, 78]. These studies revealed that the ability of GLP-1(28-36) to prevent loss of LVDP following IRI was preserved in hearts from Glp1r/- mice. As observed previously[110], the parent peptide GLP-1 also retained its ability to protect the heart from IRI in the absence of a functional GLP-1R (Figure 4.3D; white bars).
Figure 4.3

A

<table>
<thead>
<tr>
<th>Equilibrium</th>
<th>Perfusion</th>
<th>Ischemia</th>
<th>Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 min</td>
<td>40 min</td>
<td>30 min</td>
<td>40 min</td>
</tr>
</tbody>
</table>

B

Buffer control

Scram

LV press. (mmHg)

28-36

GLP-1

LV press. (mmHg)

110 min

110 min

C

% LVDP Recovery

GLP-1(28-36) (nM)

0  0.3  3  6  30

****

****
Figure 4.3
Figure 4.3. GLP-1(28-36) exerts direct GLP-1R-independent cardioprotective effects in isolated mouse hearts. A, IRI protocol of retrograde, non-recirculating, perfusion of isolated hearts from male 10-12 wk old WT or Glp1r−/− mice showing sequence and duration of perfusion, ischemia and reperfusion. B, Representative tracings show LVDP recordings from isolated, perfused WT hearts. GLP-1(28-36) and GLP-1 perfused hearts displayed higher LVDP during reperfusion as compared to buffer-only or Scram(28-36) controls. C. Concentration-dependent effect of GLP-1(28-36) on LVDP recovery in isolated hearts undergoing IRI. LVDP recovery, expressed as % of LVDP recorded at end of reperfusion divided by LVDP prior to ischemia, was significantly increased at a minimum concentration of 6 nM, n=3-12/group. D, Perfusion with 6nM GLP-1(28-36) caused higher %LVDP recovery as compared to 6nM Scram control in both WT (n=4-13/group; black bars) and Glp1r−/− (n=3-5/group; white bars) hearts. The effect of GLP-1(28-36) was comparable to that of 0.3nM of the parent peptide, GLP-1. E, LDH release into coronary effluents from perfused WT mouse hearts, measured at timed intervals by ELISA and normalized to coronary flow(ml)/min/weight of heart (g). Decreased LDH release was observed with GLP-1(28-36) (n=3) at 10-40 min of reperfusion, as compared to Scram control (n=3). Data shown are mean±SE. *P<0.05 at 50min reperfusion vs. Cntl and Scram by One-way ANOVA, ***P<0.001, ****P<0.0001 vs. corresponding control by One-way ANOVA with Bonferroni post hoc test.
4.4.3 Cardioprotective actions of GLP-1(28-36) require soluble adenylyl cyclase

To investigate GLP-1R-independent cardioprotective actions of GLP-1(28-36), we next examined the involvement of the second messenger, cAMP. Previous studies in pancreatic beta-cells had demonstrated that GLP-1(28-36) stimulates cAMP-PKA[105], a key upstream signaling pathway in pharmacological- and ischemic cardiac pre-conditioning[191]. Based on increasing evidence in the literature of alternative, ‘receptor-independent’ sources of cAMP, through the activation of intracellular sAC in mammalian cells[152, 192], we hypothesized that GLP-1(28-36) might be mediating its GLP-1R-independent effects through a mechanism involving sAC. We first probed for sAC expression via immunoblot analysis and found abundant protein expression in mouse heart as well as specific heart chambers (Figure 4.4A).

In testing whether GLP-1(28-36) had any effect on cAMP release, we found that coronary effluents of isolated mouse hearts perfused with GLP-1(28-36) showed only a modest increase in extracellular cAMP levels which was not statistically different from controls (Figure 4.4B). However, using isolated WT mouse hearts perfused with the sAC inhibitor, KH7, we observed loss of cardioprotection with GLP-1(28-36) pre-treatment, but not with GLP-1 as measured by LVDP at the end of reperfusion (25.2±1.7 mmHg, n=3, vs. 73.1±5.3mmHg, n=3; p<0.0001) (Figure 4.4C,D). On the other hand, perfusing isolated hearts with 2’5’-Dideoxyadenosine (Ddox), to inhibit membrane-bound AC (tmAC), did not affect the cardioprotective actions of GLP-1(28-36), but did reduce the cardioprotective effect of GLP-1 (67.6±5.8 mmHg, n=3, vs. 27.1±5.3mmHg, n=3; p<0.0001). The scrambled peptide Scram(28-36), did not produce any recovery of LV function in the presence of either the sAC (36.4±5.2 mmHg, n=3, p=ns) or tmAC inhibitors (37.6±5.3 mmHg, n=3, p=ns). These data suggest that while the
cardioprotective actions of GLP-1 in the isolated mouse heart require tmAC, those of GLP-1(28-36) depend solely on sAC.

As GLP-1 biology is complex and may involve several parallel mechanisms with or without transmembrane receptors and distinct metabolites, we chose next to focus on the direct actions of the single metabolite GLP-1(28-36) using in-depth *in vitro* studies into its cellular and molecular actions in cardiovascular-specific cell types.
Figure 4.4
**Figure 4.4. Cardioprotective effects of GLP-1(28-36) are sAC-dependent.** A, Protein expression of sAC isoform in tissue lysates from different cardiac chambers of WT mice, with mouse brain as negative control. Monoclonal R21 antibody (CEP Biotech) was used to detect the 50 KDa isoform of sAC, known to be the most active form of sAC in mammalian cells. GAPDH was used as loading control (Representative blot of n=3 replicates). B, Levels of cAMP release in coronary effluents were measured at timed intervals during initial 10 min perfusion of WT hearts with either 6nM GLP-1(28-36) or Scram, buffer-only control, or 0.3nM GLP-1. Hearts were pre-perfused with buffer containing phosphodiesterase inhibitor IBMX (100 µmol/L). cAMP levels were quantified using an enzymatic immune-assay kit and normalized to coronary flow(ml)/min/weight of heart (g). At the end of 10 min perfusion, higher (but non-significant) increases in cAMP levels were observed in GLP-1(28-36) and GLP-1-perfused hearts as compared to Scram and buffer-only controls. C, Schematic of IRI protocol showing perfusion of isolated WT mouse hearts with sAC inhibitor (KH7, 20 µmol/L) or tmAC inhibitor (Ddox, 50 µmol/L) prior to peptide perfusion. D, % LVDP recovery from IRI injury in WT mice perfused with GLP-1(28-36) is blocked by KH7, but not by Ddox (n=3/group). Data shown are mean±SE. ****P<0.0001 vs. corresponding control by Two-way ANOVA with Bonferroni post hoc test. Br, brain; Hrt, heart; LV, left ventricle; RV, right ventricle; Atr, atria; Sep, septum.
4.4.4 GLP-1(28-36) enters caSMC, increases cAMP-PKA activity and prevents cytotoxicity.

In surveying each specific cardiovascular cell types, we found that the effect of GLP-1(28-36) on intracellular cAMP levels in both atrial and ventricular cardiomyocytes was no different than that of Scram(28-36) (Figure 4.5A). Of note, the adrenergic agonist isoproterenol did not stimulate cAMP response from neonatal ventricular cardiomyocytes, contrary to expectations. This data can be interpreted as a lack of beta-adrenergic receptor expression under the current conditions of neonatal ventricular cardiomyocyte isolation and culture. By contrast, even a brief (10 min) incubation with GLP-1(28-36) caused a 2.3±0.4 fold increase in intracellular cAMP release in mouse caSMC, as compared to Scram(28-36) (p<0.0001). In this cell type, the effect of GLP-1(28-36) on cAMP levels was as robust as that of the adrenergic agonist isoproterenol (Figure 4.5B). To visualize whether GLP-1(28-36) undergoes cellular uptake, we synthesized a biotinylated GLP-1(28-36), placing biotin at the C-terminus of the peptide via a lysine link. Consistent with rapid generation of cAMP in response to this peptide, biotinylated GLP-1(28-36) entered caSMC with the strongest intracellular signals observed within 15 min (Figure 4.6). However, we also observed intracellular localization of the scrambled control under similar experimental conditions (Appendix 3). As such, it appears that while the scrambled control might carry the same signal as GLP-1(28-36) for entering cells, it is functionally not active, based on the lack of cAMP response observed earlier (Figure 4.5 A, B).

We next assayed PKA[105, 193], ERK[194] or eNOS[195] activations as putative downstream targets of GLP-1(28-36)-induced cAMP or molecules known to be involved in GLP-1-mediated cytoprotection[100]. Of these, we found the enzymatic activity of PKA to be increased 1.8±0.3 fold in caSMC exposed to GLP-1(28-36) as compared to Scram(28-36) (p<0.01) (Figure 4.7A).
Again, this effect of GLP-1(28-36) was quantitatively similar to that produced by isoproterenol (Figure 4.7A).

To establish the functional significance of these findings, we next tested the ability of GLP-1(28-36) to prevent cytotoxicity in an in vitro model of oxidative stress[196]. In this experiment, GLP-1(28-36) prevented caSMC from cell damage as evidenced by decreased release of LDH (Figure 4.7B). Finally, the ability of GLP-1(28-36) to decrease the cleaved caspase activation observed in this model, suggests that the agent prevents apoptosis (Figure 4.7C).
Figure 4.5. GLP-1(28-36) stimulates cAMP production in mouse caSMC, but not cardiomyocytes. Cells were incubated with 450 µM IBMX for 30 min to inhibit cAMP degradation by phosphodiesterases, then treated for 10 min with 100 nM each of GLP-1(28-36), Scram, IPE, Forsk or PBS control (n=3/treatment, each in triplicate), and lysed for measurement of cAMP levels by enzymatic immunoassay. GLP-1(28-36) did not stimulate cAMP release from A, neonatal mouse primary ventricular cardiomyocytes (black bars) or HL-1 mouse atrial cardiomyocyte cell-line (white bars), but increased cAMP levels in B, mouse caSMC, compared to Scram control. Data shown are mean±SE. ****P<0.0001 by One-way ANOVA vs. corresponding control with Bonferroni post hoc test.
Figure 4.6. Cellular uptake of GLP-1(28-36). Mouse caSMC were stimulated with either (A) 50µg/ml unlabelled GLP-1(28-36) or 50µg/ml biotinylated GLP-1 for (B) 0 min, (C) 5 min, (D) 15 min. Cells were washed, fixed, permeabilized and stained with Streptavidin-Alexa Fluor 488 conjugate to detect biotinylated GLP-1(28-36) (green). Hoechst dye (1mg/ml) was used to counterstain nuclei (blue). Images acquired by an Olympus FluoView confocal microscope, 40X objective. Scale bar 10µm.
Figure 4.7
Figure 4.7. GLP-1(28-36) activates PKA and prevents cytotoxicity in mouse caSMC. A, Cells were incubated with 450 µM IBMX for 30 min to inhibit cAMP degradation by phosphodiesterases, then treated for 10 min with 100 nM each of GLP-1(28-36), Scram, IPE, Forsk or PBS control (n=3/treatment, each in triplicate), and lysed with PKA lysis buffer. PKA extracts (2 µg protein) were incubated with coloured Peptag®-A1 substrate and PKA activity determined by separation of phosphorylated (+p-Peptag®) and non-phosphorylated (-p-Peptag®) substrate by agarose gel electrophoresis. Densitometric analysis of phosphorylated bands showed increased PKA activity with GLP-1(28-36) as compared to Scram. Image is representative of three independent experiments. B, Mouse caSMC were pre-treated with 100 nM of GLP-1(28-36) or Scram for 20 min followed by incubation with H<sub>2</sub>O<sub>2</sub> (100 µM) for 48h to induce oxidative stress (n=3.each in triplicate). Cell culture media was replenished once with fresh peptide after 24h incubation with H<sub>2</sub>O<sub>2</sub>. At the end of 48h, LDH release was assayed in duplicates from aliquots of cell culture media by ELISA. Pre-treatment with GLP-1(28-36) reduced LDH release vs. Scram or PBS. C, Whole cell lysates were extracted from mouse caSMC undergoing similar 20 min peptide pre-treatment schedule and apoptosis was assessed by cleaved caspase-3 activation at end of 7h incubation with or without H<sub>2</sub>O<sub>2</sub> (100 µM). Representative Western blot and normalized densitometric analysis (n=3/group) show that GLP-1(28-36) prevented activation of cleaved caspase-3 in the presence of H<sub>2</sub>O<sub>2</sub> vs. controls. Data shown are mean±SE. **P<0.01, *** P<0.001, ****P<0.0001 by One-way ANOVA (except for C, by Two-way ANOVA), N.S. (non-significant) vs. corresponding control with Bonferroni post hoc test.
4.4.5 Cytoprotective effect of GLP-1(28-36) is attenuated in the absence of sAC

We next investigated the importance of sAC to the molecular actions of GLP-1(28-36) in caSMC. The ability of GLP-1(28-36) to stimulate cAMP release was lost in caSMC isolated from sAC-null mice (GLP-1(28-36): 94.8±6.0 vs. Scram(28-36): 77.7±6.8, n=3, p=ns) but not WT littermate controls (GLP-1(28-36): 173.7±15.6 vs. Scram(28-36): 87.4±9.1, n=3, p<0.01) (Figure 4.8A). Moreover, GLP-1(28-36) failed to protect sAC−/− caSMC from oxidative stress injury, as evidenced by non-significant decreases in LDH release vs. Scram (0.74±0.03 vs. 0.83±0.03, n=3, p=ns) and compared to littermate controls (0.51±0.02 vs. 0.78±0.04, n=3, p<0.01) (Figure 4.8B). Taken together, these data show that GLP-1(28-36) acts through a molecular pathway involving catalytic generation of cAMP from sAC, in order to prevent cytotoxicity in caSMC undergoing oxidative injury.
Figure 4.8. GLP-1(28-36) actions on cAMP and cytoprotection in caSMC are sAC-dependent. A, cAMP response after treatment with GLP-1(28-36) is lost in caSMC isolated from sAC<sup>-/-</sup> mice as compared to sAC<sup>+/+</sup> littermates (n=3/treatment group, each in triplicate). B, Pre-treatment with GLP-1(28-36) prior to oxidative stress injury with H<sub>2</sub>O<sub>2</sub> did not reduce cytotoxicity as measured by LDH release in sAC<sup>-/-</sup>caSMC, vs. sAC<sup>+/+</sup> controls (n=3/treatment group). Data are mean±SE. *P<0.05, **P<0.01 by Two-way ANOVA with Bonferroni post hoc test.
4.4.6 GLP-(28-36) increases levels of sAC substrate ATP

To investigate the mechanism by which GLP-1(28-36) activates sAC, we assessed whether the peptide increases production of ATP, the substrate for sAC. Indeed, previous studies have reported that sAC acts as a physiological ATP sensor in glucose-responsive cells, with sAC-generated cAMP reflecting intracellular ATP levels [168]. Using WT mouse caSMC, brief 10 min incubations with GLP-1(28-36) caused concentration-dependent increases in ATP levels, at a minimum effective concentration of 100 nM, as compared to Scram(28-36) (Figure 4.9A). This effect was paralleled with increases in sAC activity, as measured by cAMP accumulation in the same cell lysates (Figure 4.9B). In contrast, we did not observe significant GLP-1(28-36)-driven cAMP accumulation in caSMC isolated from sAC−/− mice, although the ATP response was still apparent (Figure 4.9C,D). Under physiological conditions, intracellular ATP concentrations range between 1-10 mM in mammalian cells [168]. Therefore, ATP levels are almost never limiting in cells and any increase in intracellular ATP levels should not change cAMP generations in cells. However, the K_m of tmAC for ATP-Mg^{2+} is approximately 100µM while the K_m of sAC for ATP-Mg^{2+} is approximately 1mM [168]. As such, due to the lower affinity of sAC for ATP, any minor change in ATP levels would affect cAMP production from sAC. We therefore conclude that these data support a mechanism whereby GLP-1(28-36) modulates sAC activity to generate cAMP in caSMC by increasing levels of the substrate ATP.
Figure 4.9
Figure 4.9. GLP-1(28-36) stimulates sAC-dependent cAMP release in mouse caSMC by increasing levels of sAC substrate, ATP. Cells were pre-treated with IBMX for 30 min to inhibit phosphodiesterases, followed by 10-min incubation with indicated concentrations of GLP-1(28-36) or Scram. ATP was extracted with ice-cold 2.5%(w/v) trichloroacetic acid, neutralized with Tris-Acetate buffer (pH7.75), and added to luciferin-luciferase reagent (Promega) at a ratio of 1:10 for measurement of RLU in a luminometer. cAMP was measured from the same cells lysates, using an enzymatic immunoassay kit. A, GLP-1(28-36) caused a significant increase in intracellular levels of ATP at a minimum effective concentration of 0.1 µM, in caSMC isolated from both $sAC^{-/-}$ and B, $sAC^{+/+}$ littermate controls. This effect was accompanied by parallel accumulation of intracellular cAMP in C, $sAC^{+/+}$ caSMC, but lost in D, $sAC^{-/-}$ caSMC. Data represent mean ±SE, n=3/concentration/treatment group (each in triplicate). ***$P<0.001$, analysed by Two-way ANOVA with Bonferroni post hoc test. RLU, relative light units.
4.4.7 GLP-1(28-36) stimulates cAMP release and prevents cytotoxicity in human coronary artery smooth muscle cells via sAC.

To further elucidate the mechanism of cAMP-dependent, GLP-1(28-36)-mediated cytoprotection, and potential translation of our findings to a clinical context, we next assessed the actions of the peptide in commercially available human caSMC. These cells also manifest a 2-fold increase in cAMP release in response to GLP-1(28-36), which was abolished in the presence of the sAC-inhibitor KH7, but not the tmAC-inhibitor Ddox (Figure 4.10A). Mirroring our data from sAC knockout mice, genetic deletion of sAC in human caSMC through targeted siRNA also abrogated the cAMP response to GLP-1(28-36) (Figure 4.10B,C), providing further evidence of a non-receptor, sAC-dependent mechanism of action. Again, similar to results observed in mouse caSMC, GLP-1(28-36) activated PKA and conferred cytoprotection against oxidative stress injury in human caSMC (Figure 4.10D, E).
Figure 4.10

A

![Graph showing cAMP levels with various treatments.]

B

![Western blot images showing sAC and GAPDH.]

C

![Another graph showing cAMP levels with different treatments.]
Figure 4.10

D

Figure 4.10 continued

E

Figure 4.10 continued
Figure 4.10. sAC-dependent actions of GLP-1(28-36) are conserved in human caSMC. A, Human caSMC were treated with sAC inhibitor KH7 (25 µM), tmAC inhibitor Ddox (50 µM) or PBS for 3h. IBMX (250 µM) was added during the last 30 min to inhibit cAMP degradation. After 3 h, cells were treated with GLP-1(28-36) or controls, as previously described, and cAMP levels determined (n=3/group, each in triplicate). The increase in cAMP stimulated by GLP-1(28-36) was lost by inhibiting sAC, but not tmAC. B, RT-PCR showing sAC mRNA levels decreased by ~50% after transient transfection of human caSMC with 50 nM siRNA against sAC (vs. scrambled siRNA control) using Lipofectamine RNAimax (Upper panel). Intracellular cAMP levels in cell lysates determined following 10 min pre-treatment with 100 nM each of GLP-1(28-36), Scram and Forskolin, at 72h post-transfection with siRNA, show significant decrease vs. scrambled siRNA control (Lower panel). C, GLP-1(28-36) increased cAMP-dependent PKA activity in human caSMC vs. Scram control, as shown by increased amount of phosphorylated Peptag® substrate with densitometric analysis (lower panel, n=3/group) of agarose gel electrophoresis bands (upper panel, representative image of 3 independent experiments). D, Human caSMC underwent similar inhibitor and peptide treatment as in A above, followed by 48h incubation with H2O2 (100 µM) to induce oxidative stress (n=3/group, triplicate wells each). Cellular toxicity was measured by LDH release, assayed in duplicates from aliquots of cell culture media, as previously described. Inhibiting sAC with KH7 caused significant increases in LDH release vs. tmAC inhibitor Ddox or PBS controls, indicating loss of cytoprotective effects of GLP-1(28-36) without functional sAC. Data represent mean ± SE, *P<0.05, **P<0.01 by One-way ANOVA, (except for B, by Two-way ANOVA), with Bonferroni post hoc test.
4.5 DISCUSSION

The current study demonstrates that GLP-1(28-36) functions as a cardioprotective agent in two distinct mouse models of ischemic injury. This effect of GLP-1(28-36) is independent of the known GLP-1R, and appears to be mediated by a mechanism involving intracellular sAC in caSMC. We show that GLP-1(28-36) enters mouse caSMC and localizes intracellularly. GLP-1(28-36) activates sAC as evidenced by increased production of cAMP in caSMC from WT but not sAC-knockout mice. Furthermore, GLP-1(28-36) protects mouse caSMC from oxidative stress injury through a sAC-dependent mechanism. We also reveal that GLP-1(28-36) activates sAC by increasing production of its substrate ATP. Finally, we show with pharmacological inhibitors (KH7) and gene silencing (siRNA) that the sAC-mediated actions of GLP-1(28-36) are conserved in human caSMC.

While our study represents the first to examine the effects of GLP-1(28-36) in the cardiovascular system, and the first to implicate sAC in its biology and in IRI overall, there is a small emerging body of evidence supporting a biological role for GLP-1(28-36) in preventing obesity, insulin resistance, diabetes mellitus, and hepatic steatosis and gluconeogenesis in mice[102-106]. This C-terminal metabolite of GLP-1 has displayed cytoprotective effects in isolated mouse hepatocytes and pancreatic β-cells exposed to oxidative stress injury, and was suggested to do so by trafficking to the mitochondria and limiting stress-induced apoptosis by inhibiting mitochondrial depolarization, cytochrome-c release and caspase activation[103, 104]. Others have shown in both clonal INS-1 cells and primary hepatocytes that GLP-1(28–36) increase cellular cAMP levels, PKA enzymatic activity, and phosphorylation of cAMP response element-binding protein (CREB), cyclic AMP-dependent transcription factor-1 (ATF-1), and β-catenin (Ser675) [105, 106]. Just as putative roles for CREB, ATF-1 and β-catenin remain to be
examined in the cardiovascular actions of GLP-1(28-36), future studies are required to elucidate the potential role of sAC in the biological actions of GLP-1(28-36) reported in pancreatic β-cells and hepatocytes. Indeed, recent reports that sAC-null mice display impairments in both pancreatic insulin secretion and intraperitoneal glucose tolerance[168, 169] support our own findings implicating sAC in GLP-1(28-36) and/or overall GLP-1 biology.

Our data also reveal an anti-apoptotic role for GLP-1(28-36) through activation of sAC and its downstream cAMP-PKA effectors. However, previous findings have reported dual roles for sAC in both cell death and cell growth[197]. More specifically, it has been reported that following ischemia/oxidative stress or acidosis injury in rat coronary endothelial cells, cardiomyocytes and aortic smooth muscle cells, sAC-dependent apoptosis is triggered by translocation of sAC from cytosol to mitochondria with sub-mitochondrial PKA activation, Bax phosphorylation and cytochrome c release[159, 171-173]. In contrast, sAC induces cell proliferation through a PKA-independent but EPAC/Rap1/B-Raf–dependent signaling pathway in prostate carcinoma cells[160]. While the pro- vs. anti-apoptotic mechanisms of sAC is still under investigation, our data support an anti-apoptotic role for sAC in caSMC. Unlike the above models, where activation of sAC was studied after injury, we demonstrate that GLP-1(28-36) acts as a preconditioning agent by activating sAC through production of ATP, leading to downstream cAMP-PKA-activation. Importantly, the role of PKA in activating multiple downstream targets involved in pro-survival signaling is well established[100, 110, 113, 198-200].

We previously showed that both GLP-1 and GLP-1(9-36) cause release of cGMP and cAMP in coronary effluents of isolated perfused hearts from WT and Glp1r-/− mice[110], directly implicating GLP-1 and its DPP-4-generated metabolite in GLP-1R-independent production of cAMP[100, 110]. Others showed that both GLP-1 and DPP-4i limit infarct size in animal models
of MI by increasing intracellular cAMP levels and activating PKA[113, 199]. Indeed, preventing
degradation of cAMP by phosphodiesterase inhibition increased the infarct-sparing effect of a
DPP-4i in mice[198]. Importantly, unlike β-adrenergic stimulation-induced increases in
myocardial cAMP, which enhance contractility, GLP-1 showed negative inotropic effects in
rodent cardiac myocytes, implicating a compartmentalized mechanism of cAMP production
away from the contractile apparatus[200]. These findings provide evidence that the generation of
an intracellular source of cAMP is involved in GLP-1-mediated cardioprotection.

Meanwhile, some uncertainty exists as to why we did not observe significant increases in
extracellular cAMP release in coronary effluent from GLP-1(28-36)-perfused mouse heart
(Figure 4.4B), as compared to the robust response in caSMC in vitro (Figure 4.5B). We can
only speculate that the molecular actions of GLP-1(28-36) are localized intracellularly to specific
cardioprotective agents against MI and/or IRI. As such, we were somewhat
surprised by our in vitro data revealing that caSMC was the primary site of action of the
protective effects of GLP-1(28-36). Having said that, studies examining the specific role(s)
of coronary vascular cells in IRI are scarce. To our knowledge, the strategy of selectively
preventing cytotoxicity of coronary vascular cells following MI and/or IRI to affect whole organ
cardioprotection has never been directly studied. While our data suggest that such an approach
may be feasible, further work is required on the relative importance of coronary vascular cells vs.
cardiac myocytes in the pathophysiology of IRI. For example, tissue-specific knockouts of sAC
(e.g. SMC vs. endothelial cell vs. cardiomyocytes) may be required to parse the cell-specific role
of this cAMP-generating pathway in IRI, with the lack of GLP-1(28-36)-mediated responses in
sAC-null caSMC providing a strong rationale.
Lastly, it is not yet known whether GLP-1(28-36) is a physiologically relevant, endogenously active metabolite. Indeed, assays for the *in vivo* measurement of plasma levels and tissue activity of GLP-1(28-36) have yet to be developed. Moreover, it is not yet known whether manipulating NEP24.11 to prevent the degradation of GLP-1 into GLP-1(28-36) will affect the clinically observed cardioprotective actions of GLP-1. Despite these limitations, we believe that the potential promise of GLP-1(28-36) as a synthetic peptide for use as a therapy to prevent IRI following MI is worthy of further exploration.

In conclusion, we have identified novel molecular and cellular targets for GLP-1(28-36) in the cardiovascular system. Moreover, we believe that the mechanistic pathways uncovered have potential for impact in the important clinical problem of IRI where no approved therapies currently exist.
Chapter 5

Identification of novel interacting proteins of GLP-1(28-36) in the heart

STATEMENT OF CONTRIBUTIONS

Protein digestion, mass spectrometry analysis and database search were performed by Dr. Jonathan Krieger, SPARC BioCenter, Hospital for Sick Children, Toronto Ontario.
5.1 Abstract

We previously showed that soluble adenylyl cyclase (sAC) is an important component of the molecular mechanism underlying the cardioprotective and cytoprotective actions of GLP-1(28-36) against myocardial ischemic injury. However, how GLP-1(28-36) activates sAC is not known.

We used a discovery approach to determine the binding partners of GLP-1(28-36) in the mouse heart. Affinity pull down with streptavidin magnetic beads and biotin-labeled GLP-1(28-36) versus scrambled negative control and subsequent mass spectrometry analysis identified 12 potential interactors of GLP-1(28-36). Surprisingly, sAC was not identified as a binding partner. The mitochondrial trifunctional protein subunit alpha (MTPα) was found to be most significantly bound to GLP-1(28-36) with 10.5±2.2 average fold increase in total spectral count versus scrambled peptide.

We concluded that MTPα may play an important role in sAC activation through the generation of ATP from fatty acid metabolism. However, based on the limitations of our experimental approach, we propose further extensive studies to firmly conclude on the present findings.

5.2 Introduction

From the previous chapter, we identified a molecular signaling mechanism of GLP-1(28-36) in the cardiovascular system, involving the catalytic generation of cAMP through soluble adenylyl cyclase (sAC), possibly by stimulating ATP production, the substrate for sAC. Using confocal microscopy, we also showed that GLP-1(28-36) localizes intracellularly in mouse coronary
artery smooth muscle cells (caSMCs), thereby excluding the possibility that it might be acting as a ligand for membrane-bound surface receptors. Tomas et al. have shown using fluorescent labelled GLP-1(28-36) that the peptide is capable of entering isolated hepatocytes and concentrating in mitochondria [103]. Incidentally, mitochondria are the site for metabolic generation of ATP by oxidative phosphorylation, either from glucose or fatty acid metabolism. Moreover, sAC also plays a role in the mitochondria, where it drives mitochondrial protein phosphorylation and regulates oxidative phosphorylation through the activation of intra-mitochondrial cAMP-PKA [156, 201]. But sAC is also present in the cytoplasm and nucleus, where it generates cAMP and plays important functions in cell survival and cell death [159, 170, 172, 173]. Therefore, while we strongly suspect that GLP-1(28-36) is activating sAC intracellularly, there are many unanswered questions that limit the strength with which we can make this assertion. The key question that remains is that it is not known how GLP-1(28-36) actually activates sAC. Based on our previous data, it may activate sAC through a yet unidentified pathway by indirectly increasing the local availability of its substrate ATP, which is a known physiological sensor for sAC activation [168]. Alternatively, it is conceivable that GLP-1(28-36) could directly bind sAC or cofactors in its enzyme complex as an allosteric modulator.

Determining the molecular/biochemical basis for how GLP-1(28-36) activates sAC has important implications. First, it will entail a new discovery in the molecular pathway underlying the cardioprotective actions of an incretin metabolite in the field of IRI. Second, this may even inform how GLP-1(28-36) effects glucoregulation in hepatocytes and pancreatic β-islet cells, where an emerging role for sAC has recently been proposed[168, 169]. Finally, this could form the basis for manipulating sAC as a therapeutic target against IRI and/or type 2 diabetes.
We therefore conducted an affinity pulldown experiment followed by an unbiased proteomic analysis to identify binding partners for GLP-1(28-36) in the mouse heart. We here show that GLP-1(28-36) does not physically interact with sAC in the heart. Instead, we discovered that GLP-1(28-36) binds a group of proteins localized to the inner mitochondrial membrane which regulate transport, protein binding and oxidative phosphorylation. More specifically, we identify mitochondrial trifunctional protein alpha (MTPα), an enzyme involved in fatty acid metabolism, as the main interacting protein of GLP-1(28-36).

5.3 Materials and Methods

5.3.1 Preparation of heart extracts

Hearts were dissected from 8 wk old, male, C57BL/6J mice, washed twice with cold PBS, snap-frozen and stored at -80°C for subsequent analysis. The frozen hearts were pulverized in a mortar and pestle bathed in liquid nitrogen. The frozen powder was dissolved and homogenized with 100 strokes on ice in 1 ml RIPA lysis buffer (Tris pH 7.4 50 mM; EDTA pH 8.0 1 mM; NP-40 1%; NaCl 150 mM; Na orthovanadate 2.5 mM; PMSF 1 mM; Protease inhibitor cocktail (Complete Mini Roche, Roch, Madison WI); Phosphatase Inhibitor cocktail (Sigma P5726)). The cells were allowed to swell on ice for 30 min, and centrifuged at 14,000 rpm for 30 min at 4°C. Lysate proteins were quantified using BCA reagent (Sigma-Aldrich, ON, Canada).

5.3.2 Affinity pulldown with magnetic beads

The affinity pulldown was performed at 4°C with 1000 µg pre-cleared heart extracts in the presence of 50 µg/ul biotin-labeled peptides in a total volume of 300 µl binding/wash buffer (Tris–buffered saline (TBS, Pierce Product No. 28379) containing 0.1% Tween™-20 detergent,
(TBST)). First, to limit non-specific binding, heart protein extracts (1000 µg) were pre-cleared for 1 h with 50 µl of Pierce™ Streptavidin Magnetic Beads (Pierce Biotechnology, IL) that were prepared and washed according to manufacturers instructions. The pre-cleared heart extracts were then collected using a magnetic stand, and the magnetic beads were discarded. Affinity capture was done by end –to-end mixing of pre-cleared heart extracts with either BIOT(28-36) or control BIOT(scram) for 4 h. The captured complexes were then immobilized on 50 µl Pierce™ Streptavidin Magnetic Beads with further end-to-end mixing for 2 h. After immobilization, the beads were collected with a magnetic stand and the supernatant (unbound fraction) was saved at 4°C for further analysis. The beads were washed 3 times with 300 µl TBST. Recovery of the bound proteins was achieved by adding 100 µl of IgG Elution Buffer, pH 2.0 (Pierce Product No. 21028) to the beads. Elution was performed at room temperature with mixing for 5 min. The beads were magnetically separated, and the supernatant containing the target proteins (eluents) were saved at 4°C for further analysis.

5.3.3 Mass spectrometry analysis

**Digestion:** Eluents underwent tryptic digestion by either (1) in-solution digestion after elution from beads, or (2) resolving on 10% gels with SDS-PAGE, staining with Coomassie blue and visible bands excised and digested. Peptides were extracted with 25 mM ammonium bicarbonate, evaporated to dryness in a speedvac and reconstituted with 5 µl 0.1% TFA in water. LC MS/MS was performed with no further clean up, using a linear ion-trap instrument (Thermofisher LTQ).

**Database Searching:** Tandem mass spectra were extracted and charge state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using Sequest (Thermo Fisher Scientific, San Jose, CA, USA; version 1.4.0.288) and X! Tandem (The GPM, thegpm.org; version CYCLONE (2010.12.01.1)). Sequest was set up to search Uniprot_mouse-
Sep92014.fasta (unknown version, 16678 entries) assuming the digestion enzyme trypsin. X! Tandem was set up to search the Uniprot_mouse-Sep92014 database (unknown version, 16683 entries) also assuming trypsin. Sequest and X! Tandem were searched with a fragment ion mass tolerance of 0.60 Da and a parent ion tolerance of 2.0 Da. Deamidation of asparagine and glutamine, oxidation of methionine, carbamidomethyl of cysteine, and biotinylation of lysine of the N-terminus were specified in Sequest as variable modifications. Glutamic acid to pyro-glutamic acid of the N-terminus, ammonia-loss of the N-terminus, glutamine to pyro-glutamine of the N-terminus, deamidation of asparagine and glutamine, oxidation of methionine, carbamidomethyl of cysteine, and biotinylation of lysine of the N-terminus were specified in X! Tandem as variable modifications.

**Criteria for protein identification:** Scaffold (version Scaffold_4.3.4, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 5.0% probability to achieve an FDR less than 0.1%. Peptide Probabilities from X! Tandem were assigned by the Peptide Prophet algorithm (Keller, A et al Anal. Chem. 2002;74(20):5383-92) with Scaffold delta-mass correction. Peptide Probabilities from Sequest were assigned by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 76.0% probability to achieve an FDR less than 1.0% and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii, Al et al Anal. Chem. 2003;75(17):4646-58). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters.
5.3.4 Western blot

See Chapter 3 (General Material & Methods), section 3.8

5.3.5 cAMP assay

See Chapter 3 (General Material & Methods), section 3.9

5.4 Results

5.4.1 Characterization of biotinylated GLP-1(28-36)

We first designed a biotin-labeled GLP-1(28-36) (a.k.a BIOT(28-36)) capable of strong immobilization on streptavidin magnetic beads while maintaining efficiency in pulldown of interacting partners. BIOT(28-36) was synthesized by attaching a biotin group to the C-terminal end of GLP-1(28-36) via a Lysine amino acid residue linker, as per manufacturer’s protocol and specifications (Genscript, NJ, USA). A scrambled version of BIOT(28-36) (a.k.a BIOT(scram)) was synthesized by similar methods using SCRAM(28-36), for use as a negative control (Figure 5.1A). The amino acid sequence of both BIOT(28-36) and BIOT(scram) were verified by HPLC and estimated to have >95% purity.

The functional potency of both BIOT(28-36) and BIOT(scram) were tested by quantifying cAMP formation in mouse caSMCs, which is a highly reproducible in vitro assay previously used to characterize the actions of GLP-1(28-36) under various experimental conditions (See Chapter 4). Brief 10 min incubation of mouse caSMCs with BIOT(28-36) showed comparable cAMP accumulation as GLP-1(28-36), and this effect was lost with the negative controls BIOT(scram), SCRAM(28-36) and biotin only (Figure 5.1B). As a positive control, the beta-
adrenergic receptor agonist, isoproterenol, also stimulated cAMP increase in caSMCs. Thus, we deduced that addition of Lys-Biotin group to GLP-1(28-36) had no major effect on its functional activity, and that BIOT(28-36) may be suitable for pulldown of interacting partners, with BIOT(scram) acting as an effective negative control.
Figure 5.1

A

BIOT(28-36)

\[ \text{NH}_2 \rightarrow \text{GLP-1(28-36)} \rightarrow \text{Lys} \rightarrow \text{Biotin} \]

BIOT(scram)

\[ \text{NH}_2 \rightarrow \text{SCRAM(28-36)} \rightarrow \text{Lys} \rightarrow \text{Biotin} \]

B

![Bar chart showing cAMP levels for different treatments with statistical significance](image)

- Red: Biotinylated
- Blue: Unlabelled
- Black: Positive Cntl

Statistical significance:
- Biotin: \( P = 0.002 \)
- Biot(SCRAM): \( P = 0.002 \)
- Biot(28-36): \( P = 0.006 \)

Figure 5.1
Figure 5.1. Structure of biotin-labeled GLP-1(28-36) and its potency for cAMP production.

A. BIOT(28-36) was designed by attaching a biotin group to the C-terminal end of GLP-1(28-36) via a lysine amino acid residue. BIOT(scram) was designed similarly using the previously described scrambled sequence of GLP-1(28-36). B. Mouse caSMCs were incubated with 450 µM IBMX for 30 min to inhibit cAMP degradation by phosphodiesterases, then treated for 10 min with 100 nM each of BIOT(28-36), GLP-1(28-36) and negative controls BIOT(scram), SCRAM(28-36), biotin only and positive control Isoproterenol (n=3/treatment, each in triplicate wells). Cells were lysed for measurement of cAMP by enzymatic immunoassay. BIOT(28-36) was as potent as GLP-1(28-36) in stimulating cAMP accumulation as compared to their respective scrambled controls. Data represent mean ± SE, analysed by Student t-test. P<0.05 is considered significant.
5.4.2 Optimization of BIOT(28-36) pulldown from mouse heart

Having established that BIOT(28-36) is a functional peptide and that BIOT(scram) is an effective negative control, we next investigated the interacting partners of GLP-1(28-36) in a pulldown experiment with streptavidin magnetic beads, as per the protocol outlined in Figure 5.2. We chose to conduct this investigation in heart lysates from C57Bl/6J mice due to the abundant availability of protein extracts, as opposed to primary cultured mouse caSMCs, where extraction of large amounts of protein is a technical challenge. We began our study by first optimizing the affinity pulldown conditions in order to get a high recovery of interacting proteins and maintain low background. We incubated 25, 50 and 100 µg/µl BIOT(28-36) and/or BIOT(scram) with 1000 µg mouse whole heart extracts (WHE), followed by immobilization on 0.5 mg streptavidin magnetic beads (as recommended by the manufacturer). A low pH elution buffer (IgG elution buffer pH 2.0) was selected for elution (instead of commonly used SDS-PAGE reducing sample buffer) to prevent streptavidin leaching and ensure efficient release of proteins, while also minimizing the presence of detergent that could interfere with mass spectrometry analysis. Following elution, protein samples were resolved on a gel via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by coumassie blue staining. Based on visual inspection, we selected a concentration of 50 µg/µl of BIOT(28-36) and BIOT(scram) for further experimentation, since higher concentrations showed increasingly more non-specific bands (Figure 5.3).
Figure 5.2. Pulldown protocol of GLP-1(28-36). Whole heart protein extracts (WHE) from wild-type mice were pre-cleared with streptavidin (SA) magnetic beads for 1h at 4°C. Biotin labeled GLP-1(28-36) (BIOT(28-36)) or scrambled control (BIOT(scram)) were added to the WHE and mixed end-over-end for 4h at 4°C. Streptavidin magnetic beads were added and mixed for 2h at 4°C to immobilize biotin-labeled peptides, after which the SA beads were isolated using a magnetic stand, washed and the proteins associated with BIOT(28-36) and/or BIOT(scram) were eluted from the beads using a low pH IgG elution buffer. In one set of experiments, the eluents were separated by SDS-PAGE, stained with Coumassie blue and significantly visible bands were cut, trypsinized and subjected to mass spectrometry analysis. In another group, the eluents underwent in-solution trypsin digestion, and proteins were identified by mass spectrometry analysis.
Figure 5.3. Optimization of BIOT(28-36) pulldown from mouse heart. Protein extracts prepared from mouse whole heart were incubated with increasing concentrations of BIOT(28-36) and BIOT(scram) and isolated with streptavidin magnetic beads. The captured biotinylated peptide complex was eluted with IgG elution buffer pH 2.0. Eluents, inputs and unbound fractions were resolved on 10% gel SDS-PAGE and stained with coumassie blue. Image was captured using a GS-800 calibrated imaging densitometer (Biorad, CA). 50 µg/µl of biotinylated peptides were selected as the optimal concentrations showing highest recovery of interacting proteins with BIOT(28-36) and fewer non-specific bands in the BIOT(scram) lane.
5.4.3 GLP-1(28-36) interacts with proteins involved in mitochondrial metabolism

Since we detected multiple faint bands in our initial optimization with BIOT(28-36), we next chose an unbiased approach for identifying the binding proteins of GLP-1(28-36). Indeed, after elution from the streptavidin beads, an in-solution tryptic digestion of the whole eluent was carried out, prior to mass spectrometry analysis. Following LC-MS/MS and protein identification from 3 independent pulldown experiments, 44 vs. 72 (replicate 1), 79 vs. 61 (replicate 2), and 105 vs. 29 (replicate 3) proteins were identified from BIOT(28-36) vs. BIOT(scram) eluents respectively (Figure 5.4). However, proteins belonging to families of common contaminants of purification procedures, and deemed not to be of biological relevance, were also identified and included ribosome, spliceosome, histone, heat shock and keratin associated proteins. Based on the high number of interacting proteins with BIOT(scram), it was evident that a significant amount of non-specific binding was present in our pulldown assay. In order to identify BIOT(28-36) interactors with more confidence, a semi-quantitative analysis of fold change increase in total spectral counts of proteins eluted from BIOT(28-36) vs. BIOT(scram), was performed using Scaffold version 4.3.4 (Figure 5.5). Based on total spectral fold-change increase, 14 proteins were identified as being significantly bound to BIOT(28-36), out of which 12 were replicated in all 3 independent experiments (Table 5.1).
Figure 5.4. Venn diagram depicting number of proteins bound to BIOT(28-36) and BIOT(scram). Following affinity pull down and LC-MS/MS, samples were analysed using Sequest and X! Tandem and proteins were identified using Scaffold_4.3.4 to compare interacting partners of BIOT(28-36) vs. BIOT(scram). Data represent three independent pulldown experiments labeled as Replicate 1, 2, and 3. 28-36 denotes BIOT(28-36); Scram denotes BIOT(scram).
Figure 5.5. Scatterplot denoting semi-quantitative analysis of fold-change increase in total spectral count bound to BIOT(28-36) vs. BIOT(scram). Affinity pull-down, LC-MS/MS and protein identification were as previously described. Semi-Quantitative analysis was performed in Scaffold_4.3.4. Each graph represents an independent biological replicate. Each data point in red towards the lower right portion of the graph denotes a protein with significant fold-change increase in total spectral count vs. BIOT(scram). 28-36 denotes BIOT(28-36); Scram denotes BIOT(scram).
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<td>RYR2_MOUSE</td>
<td>13 0 2 0 3 0 3</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>11</td>
<td>ATP-dependent 6-phosphofructokinase,</td>
<td>85</td>
<td>PFKAM_MOUSE</td>
<td>12 0 5 0 6 0 3</td>
<td></td>
<td></td>
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<td>No.</td>
<td>Description</td>
<td>Accession</td>
<td>Score</td>
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<tr>
<td>12</td>
<td>Coiled-coil-helix-coiled-coil-helix domain-containing protein 3, mitochondrial GN=Chchd3</td>
<td>CHCH3_MOUSE</td>
<td>26</td>
<td>10</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>3</td>
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<tr>
<td>13</td>
<td>Calsequestrin-2 GN=Casq2</td>
<td>CASQ2_MOUSE</td>
<td>48</td>
<td>8</td>
<td>0</td>
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<td>14</td>
<td>Voltage-dependent anion-selective channel protein 3 GN=Vdac3</td>
<td>VDAC3_MOUSE</td>
<td>31</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td></td>
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</tbody>
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However, a major limitation of this study was the inability to identify the bait, i.e., BIOT(28-26) and BIOT(scram), in the LC-MS/MS analysis, even after manually inputting the biotinylated peptide sequences in the ‘Uniprot_mouse-Sep92014’ database. This could be explained by the extremely strong interaction between streptavidin and biotin, which might prevent elution of the baits from the beads, under the elution conditions used in this experiment. As such, it is difficult to conclude whether the 12 identified proteins are specifically bound to BIOT(28-36) or are non-specific binders. Having said this, we believe that BIOT(scram) is a strong negative control, and the list of 12 protein interactors identified with BIOT(28-36) but not BIOT(scram) is worthy of further investigation.

5.4.4 MTPα is associated with GLP-1(28-36) in mouse heart

Despite the limitation of our experimental approach, we attempted to further validate the interacting partners of BIOT(28-36). From the short-list of 12 interactors of BIOT(28-36), mitochondrial trifunctional protein subunit alpha (MTPα, gene name hadha) topped the list with the highest average fold-change increase of 10.5±2.2 in total spectral count vs. BIOT(scram) (Table 5.2). To further explore this finding, an alternate approach to the pulldown experiment was employed. Instead of ‘in-solution’ digestion, the eluents were resolved on 10% gel by SDS-PAGE, stained with Coomassie blue and visible bands were cut, digested and analysed by mass spectrometry. Based on this ‘biased’ approach, MTPα was also identified as the most significant interacting protein of BIOT(28-36) with average total spectrum count of 238±61 vs. BIOT(scram) 20.5±5.5, representing an average fold-change increase of 11.6±0.2 (Table 5.2).

To validate the interaction between BIOT(28-36) and MTPα, the pulldown assay was repeated, followed by immunoblot detection of MTPα using polyclonal Hadha antibody (Figure 5.6). While there was a visibly stronger Hadha band associated with BIOT(28-36) as compared to
BIOT(scram), we also detected significant presence of Hadha in the unbound and input fractions. This can be explained by the fact that the mouse heart lysates were oversaturated with Hadha, exceeding the binding capacity for the concentration of BIOT(28-36) used in this assay. However, this data adds to the previous mass spectrometry analysis to confirm a visible difference between BIOT(28-36) and BIOT(scram) in their binding affinity for Hadha.

<table>
<thead>
<tr>
<th>Sample Digestion for Mass Spec</th>
<th>Biological replicate</th>
<th>Total Spectrum Count</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>BIOT (28-36)</td>
<td>BIOT (scram)</td>
</tr>
<tr>
<td>In Solution</td>
<td>N = 1</td>
<td>200</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>N = 2</td>
<td>166</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>N = 3</td>
<td>94</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Mean ± SE</td>
<td>153.3 ± 31.25</td>
<td>15.7 ± 4.7</td>
</tr>
<tr>
<td>Gel Bands</td>
<td>N = 1</td>
<td>299</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>N = 2</td>
<td>177</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Mean ± SE</td>
<td>238 ± 61</td>
<td>20.5 ± 5.5</td>
</tr>
</tbody>
</table>
**Figure 5.6. GLP-1(28-36) binds MTPα (gene name Hadha) in mouse heart.** Western blot analysis of eluents (bound fraction) and unbound proteins from pull down assays using streptavidin magnetic beads coated with either biotin labeled GLP-1(28-36) or scrambled peptide control in pre-cleared mouse whole heart lysates. Levels of MTPα (gene name hadha) were higher with BIOT(28-36) (Lanes 1,2) as compared to those eluted from BIOT(scram) (Lanes 3,4).
5.4.5 GLP-1(28-36) does not interact directly with sAC in mouse heart

We previously demonstrated that GLP-1(28-36) exerts direct cardioprotective effects on isolated mouse hearts via sAC-dependent mechanisms (Chapter 4). While this could mean that GLP-1(28-36) interacts physically with sAC for its actions, we did not detect the presence of sAC in any of the 5 mass spectrometry runs performed so far. To confirm this, we repeated the pull down assay, followed by immunoblot analysis with sAC antibodies (Figure 5.7). As expected, no bands corresponding to sAC protein was detected in the eluents from BIOT(28-36) and BIOT(scram), suggesting that sAC might not interact directly with GLP-1(28-36). Again, this finding is in no way conclusive, based on the limitation of our experimental approach as outlined previously.
Figure 5.7. GLP-1(28-36) does not bind soluble adenylyl cyclase. Western blot analysis of sAC from pulldown of BIOT(28-36) vs. BIOT(scram) in mouse whole heart lysates indicates lack of sAC expression (Lanes 2,3). sAC was however detected from the input samples (Lane 4) as well as in the unbound fractions (Lanes 5,6). sAC-tr denotes truncated active isoform of sAC protein; sAC-fl denotes full-length isoform; sAC, soluble adenylyl cyclase.
5.5 Discussion

We previously demonstrated that GLP-1(28-36) protects isolated mouse hearts against IRI and prevented oxidative stress injury of cultured caSMCs through a sAC-dependent mechanism. We also showed that, in caSMCs, GLP-1(28-36) stimulates sAC-dependent cAMP production through dose-dependent increases in ATP levels, the substrate for sAC. While this suggest a role of GLP-1(28-36) in the metabolic generation of ATP, the exact mechanism by which GLP-1(28-36) generates ATP and/or activates sAC remains unknown. We performed an unbiased affinity capture experiment with subsequent proteomic analysis in order to identify potential interactors of GLP-1(28-36) in the heart.

A list of 14 protein interactors of GLP-1(28-36) were identified in the mouse heart, as compared to scrambled negative control, out of which 12 were replicated in 3 pulldown experiments. Our proteomic analysis revealed that sAC was not a direct interacting partner of GLP-1(28-36). On the other hand, MTPα was identified as the main interacting protein associated with GLP-1(28-36) under our experimental conditions.

Mitochondrial trifunctional protein (MTP) is a hetero-octamer enzyme consisting of four alpha (MTPα) and four beta subunits (MTPβ) that catalyzes the final three steps of mitochondrial long chain fatty acid beta-oxidation[202]. MTPα subunit contains the long chain 3-enoyl-CoA hydratase activity and long chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) activity while the MTPβ subunit has the long chain 3-ketoacyl-CoA thiolase activity. MTP is associated with the inner mitochondrial membrane, where it promotes metabolic substrate channeling from fatty acid oxidation to oxidative phosphorylation to drive the electron transport chain of Complex I and III [203]. Human defects in the MTP complex are recessively inherited and patients with
MTP deficiency is present predominantly with cardiomyopathy, neuromyopathy, or sudden death in infancy [204]. Moreover, mice with genetic deletion of the MTPα subunit allele display neonatal hypoglycemia, significant necrosis of cardiac myocytes and survive only 6-36 hours after birth [205]. As such, our findings of an interaction between GLP-1(28-36) and MTPα implicates a role in mitochondrial long-chain fatty acid beta-oxidation and has significant clinical implications.

In line with our current finding, there is emerging evidence of a role of GLP-1 in hepatocyte handling of free fatty acids by improving hepatocyte transport and β-oxidation, thereby preventing lipotoxicity associated with nonalcoholic liver disease [206, 207]. It was shown that the liver from mice fed a high-fat diet treated with the GLP-1 analog liraglutide had significantly higher levels of fatty acid binding protein, acyl-CoA oxidase II and LCHAD (a component of MTPα), as compared to standard chow fed mice [206]. Recently, it was shown that a smaller metabolite of GLP-1, GLP-1(32-36), was associated with increased fatty acid β-oxidation in skeletal muscle of diet-induced obese mice through increased inhibition of acetyl CoA carboxylase, an inhibitor of fatty acid oxidation [109]. Whether GLP-1(28-36) similarly affects fatty acid β-oxidation in the cardiovascular system is unknown. Nonetheless, the actions of GLP-1(28-36) in fatty acid metabolism through MTPα requires further investigation, as this could explain the demonstrated ability of this peptide to increase ATP production.

The role of GLP-1 in mitochondrial-based metabolism requires further investigation. Nonetheless, there are many lines of evidence to support that GLP-1(28-36) might interact with and modulate MTPα inside the mitochondria. Several studies have shown that a consensus motif for mitochondrial localization signal (MLS) is alternating neutral and basic amino acid residues, which are present in the C-terminal end of GLP-1(28-36) as VKGR [208-210]. Additionally,
another peptide known to localize to the mitochondria and bind MTPα is pentagatin (AWMDFamide) [211] while the growth hormone-releasing peptide hexarelin (HWAWFKamide) modulates beta-oxidation in adipocytes [212]. Both pentagastrin and hexarelin have somewhat similar structures as GLP-1(28-36) (FIAWLVKGRamide), suggesting that GLP-1(28-36) might modulate fatty acid oxidation inside the mitochondria.

Despite the important implications of our discovery, we also acknowledge several limitations of the experimental approach. As such, further experiments are proposed to conclusively test the findings presented in this chapter. Firstly, a reverse pulldown assay using biotinylated MTPα immobilized on streptavidin beads is warranted in order to confirm binding affinity with GLP-1(28-36). Secondly, as we previously showed that GLP-(28-36) increases ATP production in caSMCs, an investigation into the importance of MTPα and fatty acid metabolism on GLP-1(28-36) actions in this cell type is also necessary. Lastly, since the heart is very rich in mitochondria, our finding of mitochondrial-based interacting proteins should be taken with caution. However, since all comparisons were made against BIOT(scram) which is a strong negative control, we believe our findings are worthy of further exploration and validation.

To conclude, this study provides preliminary data for a putative role of GLP-1(28-36) in mitochondrial fatty acid β-oxidation in mouse heart. Further extensive validations are required to confirm the present findings in specific cardiovascular cell types.
5.6 Future Directions

5.6.1 Reverse affinity capture for MTPα in mouse heart

Based on our previous affinity capture of biotin-labeled GLP-1(28-36) with streptavidin magnetic beads (SMB), we will validate our findings with a reverse pulldown experiment using MTPα as the bait to capture GLP-1(28-36) in mouse heart lysates. More specifically, a cleavable biotin-labelled MTPα will be synthesized using EZ-Link™ Sulfo-NHS-SS-Biotin (ThermoFisher, IL, #21331). Advantages of this biotin reagent includes a 24.3 angstroms spacer arm to reduce steric hindrances associated with streptavidin binding, as well as a disulfide bond that can be cleaved with DTT to release the bait (i.e MTPα) from the streptavidin column. The biotin-labeled MTPα will be incubated with heart lysates obtained from 10-12 week old male wild-type mice, and spiked with GLP-1(28-36) peptides. Any physical interactions that occur between the peptide and MTPα will thus be allowed to occur in vitro. To account for non-specific binding, biotin-labeled MTPα will be incubated with either (1) heart lysates only or (2) lysates spiked with SCRAM(28-36). Then, the biotin-labeled MTPα will be pulled down using SMB, after which any and all specific and non-specific binding partners of the MTPα/SMB will be eluted. Eluents will then be subjected to mass spectrometry analysis. Presence of GLP-1(28-36) in the eluent from GLP-1(28-36)-spiked lysates but not from SCRAM(28-36)-spiked or unspiked lysates will suggest a specific interaction between GLP-1(28-36) and MTPα.

5.6.2 Affinity pulldown of MTPα in mouse caSMCs

We previously showed that GLP-1(28-36) exerts biological actions in mouse caSMCs, more specifically causing a dose-dependent increase in ATP production. We will therefore test
whether GLP-1(28-36) interacts with MTPα in this cell type, by repeating the BIOT(28-36) affinity pulldown and MTPα reverse affinity pull down in caSMCs isolated from wild-type mice.

5.6.3 Functional studies with GLP-1(28-36) and MTPα

While the reverse affinity capture experiment using MTPα as the bait to pull down GLP-1(28-36) is an important validation step to confirm the binding interaction between the two partners, this approach has a few limitations. First, we lack an activity assay to determine whether biotinylation of MTPα affects the biological function of the enzyme. Secondly, we could not identify any positive control in the scientific literature that could possibly be used to test the binding capacity of MTPα in a pulldown experiment. Thirdly, physical interaction between MTPα and GLP-1(28-36) might not translate into biological functions. Therefore, functional studies using pharmacological inhibitors and/or genetic deletion of MTPα should inform us of the importance of MTPα in GLP-1(28-36) signaling mechanism in the cardiovascular system.
Chapter 6

General Discussion and Future Directions

6.1 GLP-1(28-36): A cardioprotective synthetic peptide of pharmacological relevance

There is extensive supporting evidence that GLP-1, GLP-1(9-36), and related drugs, exert cardioprotective effects in distinct animal models of cardiovascular disease under both normoglycemic [100, 110] and hyperglycemic conditions [112, 118] (Reviewed in [213, 214]). The cardioprotective benefits observed with GLP-1, its analog exenatide, and DPP-4i in animal studies have shown clinical translation as evidenced by small pilot studies [119-123]. However, the mechanisms by which GLP-1 and its related metabolites exert their cardioprotective actions are still poorly understood, due to the complexity of GLP-1 biology.

We previously showed that GLP-1 and its metabolite GLP-1(9-36) deliver cardioprotection by acting directly on the heart, but through GLP-1R-independent mechanisms [100]. Moreover, GLP-1, GLP-1(9-36) and exenatide had direct cytoprotective actions on cultured cardiomyocytes and endothelial cells, confirming that these effects do not depend on their pancreatic glucoregulatory actions in vivo [100, 110]. Moreover, using tissue-specific knockout mice, the Drucker lab revealed that the cardioprotective effects of GLP-1R agonists do not require cardiomyocyte-specific expression of GLP-1R [83]. As such, these findings lead towards two plausible hypotheses that could explain the cardioprotective mechanism of action of GLP-1: (1) the existence of an ‘alternate’ receptor, or (2) the involvement of metabolites.
I now show independently that the metabolite GLP-1(28-36) also displays GLP-1R independent actions in two mouse models of myocardial ischemic injury. Infusions of GLP-1(28-36) via osmotic minipumps reduced infarct size following chronic LAD coronary artery occlusion in normoglycemic mice as compared to infusions of scrambled peptide, *in vivo*. Also, pre-treatment of isolated mouse hearts *ex vivo* with GLP-1(28-36) improved LV functions following IRI as compared to hearts pre-treated with scrambled peptide whereas identical cardioprotective actions of the metabolite were observed in mice with genetic absence of a functional GLP-1R.

However, the fact that the endogenous levels of circulating GLP-1(28-36) is currently unknown limits the physiological interpretation of my findings. In this thesis, I have employed pharmacological doses of GLP-1(28-36) in order to elicit biological responses. A minimum effective concentration of 6nM was required for significant improvement in LVDP functions in isolated mouse hearts undergoing IRI (*Figure 4.2*), whereas the minimum concentration required in cellular assays was 100 nM. Other groups have demonstrated biological significance with a minimum concentration of 100 nM [103] in isolated mouse hepatocytes and ranging from 50 nM [105] to 10 µM [104] in pancreatic beta cells. Using ELISA-based assays, the physiological human plasma levels of the parent peptide GLP-1 has been measured as 7±1 pM in the fasting state, rising to 41±5 pM within minutes of food ingestion [53]. While assays for measuring *in vivo* plasma levels of GLP-1(28-36) has not yet been developed, we can only speculate that its levels will not be above that of its parent peptide. In the meantime, head-to-head studies comparing GLP-1 and/or GLP-1(9-36) with GLP-1(28-36), while manipulating its endogenous degradation with the use of NEP24.11 inhibitors, should inform us on the relative importance of the formation of GLP-1(28-36) in the cardioprotective actions of GLP-1.
Having said this, my discovery that GLP-1(28-36) is a cardioprotective peptide adds clinical value to the field of pre-conditioning therapeutics. To-date, a large variety of pharmacological agents have been investigated as pre-conditioning therapeutics, but have demonstrated either conflicting results or lack of translation of any beneficial cardioprotection from IRI in multi-center clinical trials, despite their stipulated pre-clinical benefits[39]. Although a few agents such as atrial natriuretic peptide (ANP)[215], cyclosporine[216], glucose-insulin-potassium (GIK)[217, 218] and even the GLP-1R agonist exenatide[120] have been investigated in Phase II/III trials in STEMI patients, to date, there remains no approved clinical therapy targeting IRI. GLP-1(28-36) represents a novel cardioprotective agent with potential for translation to the clinic, based on the stipulated clinical benefits of its parent peptide. Being a small nine-amino acid peptide, GLP-1(28-36) is easier to synthesize and is predicted to have better pharmacokinetics by being less susceptible to proteolytic degradation. Furthermore, by not activating GLP-1R, GLP-1(28-36) is predicted to exhibit none of the undesirable increases in heart rate[219], sympathetic activation[220] and effects on blood pressure[219-221] that accompany exenatide and other GLP-1R agonists. This distinction is physiologically important and of medicinal relevance.

My thesis concludes that GLP-1(28-36) is pharmacologically relevant as a synthetic cardioprotective peptide.

### 6.2 Coronary arteries vs. cardiac myocyte in cardioprotection

Having demonstrated that GLP-1(28-36) was cardioprotective in the mouse heart both *in vivo* and *ex vivo*, we were somewhat surprised to discover that the cellular actions were localized to coronary artery smooth muscle cells, but not cardiomyocytes, *in vitro*. This is in contradiction to
published findings from our lab, where it was shown that both GLP-1(9-36) and exenatide stimulated cAMP release, activated pro-survival kinases PI3K and ERK1/2 and protected cultured neonatal mouse cardiomyocytes against simulated IRI [100]. The effect of GLP-1(9-36) was still evident in cardiomyocytes isolated from Glp1r<sup>-/-</sup> pups. However, in my hands, GLP-1(28-36) did not stimulate cAMP increase from neonatal cardiomyocytes prepared under the same conditions, despite opposite expectations. While I fully acknowledge that some uncertainty exists as to how GLP-1(9-36) have effects on isolated cardiomyocytes that appear distinct from those of GLP-1(28-36), I can only speculate that, at least in cardiomyocytes, GLP-1(9-36) acts through an alternate mechanism to effect cAMP increase. Having said this, the possibility that GLP-1(28-36) might stimulate cAMP formation and exert direct cytoprotective effects in adult mouse cardiomyocytes cannot be excluded. Based on the putative sAC-dependent mechanism of action of GLP-1(28-36), preliminary co-immunofluorescence microscopy of the adult mouse heart using a sAC- and cardiomyocyte-specific antibody suggests that sAC may be present in ventricular cardiomyocyte at this later development stage (Appendix 1, white triangles). However, these immunofluorescence data also support the premise that abundant expression of sAC in the adult mouse heart exists within coronary VSMCs (Appendix 1, white arrows).

There is some evidence to imply that cardiac cells outside of the myocardial compartment might be involved in the cardioprotective signaling pathway of GLP-1. Recent findings suggest that mouse ventricular cardiomyocytes do not express a GLP-1R, based on localization of full-length GLP-1R mRNA predominantly to atrial instead of ventricular mouse cardiomyocytes [222]. Using cardiomyocyte-specific knockout mice, it was shown that the cardioprotective effects of GLP-1R agonists do not require cardiomyocyte-specific expression of its receptor [83]. These two findings strongly support a paradigm shift whereby the cardioprotective mechanisms of
action of GLP-1 and its metabolites are, at least in part, dependent on biological activity in cells/tissue outside of the cardiomyocyte compartment.

The specific role played by cardiac vascular cells, namely caSMCs and endothelial cells (ECs), as opposed to cardiomyocytes, in mediating IRI, has not been well studied. Although it is well recognized that both macro- (epicardial) and micro- (myocardial) vessel dysfunction, including the activation of inflammation, thrombosis and vasospasm occurs following IRI [223, 224], whether preventing cytotoxicity of caSMCs (and ECs) following IRI is “cardioprotective” has not been directly examined. In my study, pre-treatment with GLP-1(28-36) was associated with cytoprotective effects on mouse caSMCs undergoing oxidative stress through exposure to hydrogen peroxide, as evidenced by decreased release of LDH and reduced expression of cleaved caspase-3. This effect was also replicated in a commercially available source of caSMCs from human tissues, indicating conservation of GLP-1(28-36) actions across species. While the \textit{in vitro} model of oxidative stress employed in my study served as a starting point to investigate the cellular protective effects of GLP-1(28-36), this model is nonetheless limited by the fact that it does not totally replicate the molecular changes occurring during IRI \textit{in vivo}.

Further work using either \textit{in vivo} MI mouse models or isolated mouse heart undergoing IRI is clearly required to examine the relative importance of coronary vascular cells \textit{vs.} cardiac myocytes in the pathophysiology of IRI and GLP-1(28-36) actions. Based on my data showing lack of GLP-1(28-36)-mediated cytoprotective responses in sAC-null caSMCs, tissue-specific knockouts of sAC (e.g. SMC \textit{vs.} endothelial cell \textit{vs.} cardiomyocytes) would clearly be informative to parse the cell-specific role of GLP-1(28-36) in IRI.
My thesis concludes that, while the cytoprotective actions of GLP-1(28-36) was localized to caSMCs, this is by no means the only cell type involved in the cardioprotective actions observed in vivo. The role of cardiomyocytes cannot be excluded and require further investigations.

6.3 Is sAC the molecular target for GLP-1(28-36)?

In this thesis, sAC was identified as a potential molecular target for GLP-1(28-36). In isolated wild-type mouse hearts subject to IRI, pharmacological inhibition of sAC with KH7 (20 µmol/liter) blocked the cardioprotective actions of GLP-1(28-36) but not those of full-length GLP-1, as evidenced by LVDP recovery (25.2±1.7 mmHg for GLP-1(28-36) vs. 73.1±5.3 mmHg for GLP-1; P<0.0001) (Figure 4.3D). On the other hand, perfusing isolated hearts with the tmAC-inhibitor Ddox (50 µmol/liter) blocked the effect of full-length GLP-1, but did not inhibit the cardioprotective action of GLP-1(28-36) (27.1±5.3 mmHg for GLP-1 vs. 67.6±5.8 mmHg for GLP-1(28-36); P<0.0001). In cellular assays, GLP-1(28-36) did not increase cAMP formation from neonatal ventricular cardiomyocytes, perhaps because at this developmental stage there appears to be no significant expression of sAC in this cell type, as opposed to caSMCs (Appendix 2). Using the same concentration of inhibitors in vitro, we found that blocking sAC activity with KH7 abolished cAMP response in cultured human caSMCs (Figure 4.10A) and also failed to protect from oxidative stress injury (Figure 4.10D). These data suggest that the cardioprotective actions of GLP-1 in the isolated mouse heart require tmAC, while both cardioprotective and cytoprotective actions of GLP-1(28-36) depend on sAC and not tmAC.

At this stage, it is difficult to reconcile how blocking tmAC (a downstream effector of GLP-1R) abolished the cardioprotective effects of GLP-1, when we have previously published that this response is independent of GLP-1R [110]. This discrepancy can be attributed to some non-
specific inhibitory effects of Ddox, the inhibitor for tmAC. Indeed, it has been previously shown that Ddox is specific for tmACs at concentrations ranging from 30 to 50 µmol/liter, while higher concentrations (>500 µmol/liter) may also inhibit sAC. It is plausible that continuous perfusion of isolated hearts with GLP-1 in the presence of 50 µmol/liter of Ddox resulted in intracellular accumulation of the inhibitor, thereby exerting non-specific inhibitory actions on sAC. Another possible interpretation of this data could be that GLP-1 acts through an unidentified Gs-coupled receptor, distinct from the signalling pathway activated by GLP-1(28-36). While the present project was solely focused on the mechanisms of actions of GLP-1(28-36), we believe that other ‘alternate’ pathways of GLP-1 actions should be further explored in the future.

Studies employing pharmacological inhibitors should always be interpreted with caution due to potential for non-specificity. Having said this, pharmacological inhibition of sAC by KH7, in doses ranging from 12 to 25 µmol/liter, has been successfully employed in multiple studies to examine the signaling pathway of sAC [159, 160, 168, 171, 172, 225]. Furthermore, the specificity and potency of KH7 was directly validated in a study using cellular systems whose cAMP is derived either exclusively through sAC or through tmACs, and it was concluded that KH7 is a specific inhibitor of sAC without affecting tmAC activity [157]. Therefore, it can be reasonably argued that the dose of KH7 (20 µmol/liter) used in my study was effective to specifically block sAC and reveal the mechanism of action of GLP-1(28-36).

Nonetheless, based on the limitations of pharmacological inhibitors in terms of non-specificity, we validated the importance of sAC through genetic deletion using siRNAs. Although we achieved approximately 50% gene silencing with anti-sAC siRNA in human caSMCs, the cAMP response to GLP-1(28-36) was completely abrogated as opposed to scrambled siRNAs (Figure 4.10B). Further use of caSMCS derived from sAC−/− mice vs. littermate controls further
confirmed the importance of sAC in the cAMP forming activity and cytoprotective effects of GLP-1(28-36), as compared to sAC-insensitive Forskolin as positive control (Figure 4.8A, B).

While my findings imply that sAC is an important target in the signaling pathway of GLP-1(28-36), there are two limiting factors that prevent a firm conclusion. First, the role of GLP-1(28-36) needs further validation in sAC−/− mouse models of IRI and MI (Proposed in section 6.4.1). Second, the ability of GLP-1(28-36) to regain biological functions by rescuing the sAC phenotype in caSMCs from sAC−/− mice should be tested (Proposed in section 6.4.2).

The next question is how does GLP-1(28-36) activate sAC? As previously detailed in section 1.4.1.3, direct regulators of sAC include HCO₃⁻ and Ca²⁺ ions, which catalyze and coordinate binding and cyclization of ATP [153]. Therefore, possible hypotheses for sAC activation include either generation of intracellular HCO₃⁻ and/or Ca²⁺ or increase in substrate levels of ATP. We also considered the hypothesis that GLP-1(28-36) could be acting as an allosteric modulator of sAC to coordinate ATP binding. However, if GLP-1(28-36) was causing intracellular release of Ca²⁺, we would have expected a change in ionotropic effects in isolated perfused mouse hearts, which was not the case. Similarly, any increases in intracellular HCO₃⁻ would have been reflected by pH indicator changes in cell culture media, after incubation with GLP-1(28-36). The role of GLP-1(28-36) as an allosteric modulator of sAC was also a weak hypothesis, as no such functions have been identified in the literature since the crystal structure of human sAC was characterized [154, 226].

Based on previous report by the Habener group stating that incubation with GLP-1(28-36) raised cellular ATP levels in INS-1 beta cells, we hypothesized that our peptide could be modulating sAC activity through ATP production. Indeed, Zippin et al. demonstrated that sAC-generated cAMP in INS-1 cells reflect alterations in intracellular ATP that do not affect tmAC-generated
cAMP [168]. In other words, sAC, but not tmACs, is a metabolic sensor of intracellular ATP. Using the same assay as Zippin et al. for measuring cellular ATP, we showed that GLP-1(28-36) caused concentration-dependent increases in intracellular ATP levels in caSMCs isolated from both wild type and sAC−/− mice (Figure 4.9A,C). In the absence of sAC, ATP production was not accompanied by similar increases in cAMP (Figure 4.9B,D). This suggests that GLP-1(28-36) might modulate sAC-cAMP activity by increasing levels of its substrate, ATP. As additional support of this evidence, our proteonomic analysis of binding partners for GLP-1(28-36) revealed a list of potential interactors involved in mitochondrial ATP production (Chapter 5).

Having said this, it is unclear at this stage whether GLP-1(28-36) generates ATP production through cytosolic glycolytic pathway or mitochondrial oxidative phosphorylation. Further studies are required to determine the role of GLP-1(28-36) in metabolism (Proposed in section 6.4.3).

To conclude, my thesis provides compelling evidence that the cardiovascular actions of GLP-1(28-36) are dependent on sAC. It is conceivable that GLP-1(28-36) might indirectly activate sAC through the metabolic production of ATP. Elucidating the exact mechanism by which GLP-1(28-36) activates sAC is important, since sAC is slowly emerging as a therapeutic target in multiple pathophysiologica conditions, ranging from impaired fertility to cystic fibrosis, glucose regulation and cancer.
6.4 Future Directions

To further promote GLP-1(28-36) as a pharmacologically relevant synthetic cardioprotective compound, future investigations should first be directed towards confirmation of the role of sAC in GLP-1(28-36) biology using sAC-null mice, through both loss of function and gain of function studies. Elucidation of the role of GLP-1(28-36) in metabolism and how it activates sAC should also be the main focus of future experimental plans.

6.4.1 Loss of Function studies in isolated hearts from sAC-null mice undergoing IRI

To confirm that the cardioprotective effects of GLP-1(28-36) require sAC, acute ex vivo experiments in sAC-/- mice vs. wild-type littermate controls will be conducted using the Langendorff model of isolated perfused hearts undergoing IRI. We chose the ex vivo model of IRI as opposed to in vivo MI model to minimize any compensatory effects of sAC gene deletion that might occur in vivo. Moreover, these experiments will be in normoglycemic conditions to minimize any confounding effects, as others have suggested insulin-like, glucose-suppressant actions of GLP-1(28-36)[102, 103].

In this experiment, hearts will be isolated from male sAC-/- mice and wild-type littermate controls (N=15/group) and perfused under constant pressure (80 mmHg) on a Langendorff apparatus with 6nM each of (i) Ex-4 (non-degradable Glp1r-agonist that should work via tmAC), (ii) buffer-only and (iii) SCRAM(28-36) (negative controls), as well as (iv) GLP-1(28-36), prior to IRI as described in Chapter 3, section 3.4.2. Cardiac performance will be assessed as functional recovery of LVDP at end of reperfusion as a percentage of LVDP prior to ischemia. Infarct size as a percentage of total heart tissue will used to quantify IRI through TTC staining.
LDH release in coronary effluent will be used as another measure of tissue injury and will be quantified as previously detailed in Chapter 3, section 3.4.2.6.

Loss of functional recovery in sAC−/− mice perfused with GLP-1(28-36) will conclusively implicate sAC in the mechanism of action of the metabolite. If GLP-1(28-36) achieves some protection in the absence of sAC, it would imply that it also works through alternate pathways. These might include PKA-independent activation of PI3k, Akt, ERK1/2 and/or eNOS (see Chapter 1, section 1.18), in which case inhibitors of these survival kinases (and other) mediators of protection from IRI will be used with the same experimental setup to unravel alternative molecular targets of GLP-1(28-36).

6.4.2 Gain of function studies in sAC-null caSMCs

We will next test whether GLP-1(28-36) recovers biological functions in caSMCs harvested from sAC−/− mice (caSMC-KO), in which the sAC phenotype has been rescued (caSMC-rescued), as compared to wild-type littermate controls (caSMC-WT). To this end, we will generate a stable cell line of caSMC-rescued expressing sAC as per a method successfully employed in our lab to rescue KO phenotypes in vascular smooth muscle cells [227]. Mouse cDNA vectors for sAC-fl and sAC-tr, carrying an antibiotic resistance cassette will be obtained from commercial vendors (Life Technologies or Origene). The expression constructs will be electroporated in caSMC-KO, and the transfected cells allowed to recover for 24 h and then subjected to 14 days of antibiotic selection to generate caSMC-rescued. The success of transfection and antibiotic selection will be validated by western blot, using rabbit polyclonal antibodies against sAC.

Finally, all assays previously performed to test GLP-1(28-36) actions in caSMC-KO will be repeated in caSMC-rescued, against caSMC-WT as positive control and caSMC-KO as negative
control. Given the weight of evidence supporting a role of sAC in GLP-(28-36) actions, we expect all biological functions to be restored in caSMC-rescued. Contrariwise, this would imply that other downstream mechanisms such as RISK or SAFE pathways are involved in the cytoprotective actions of GLP-1(28-36) in caSMCs and should be further explored.

6.4.3 Mitochondrial studies to examine a role of GLP-1(28-36) in metabolism

We tested whether GLP-1(28-36) localizes to the mitochondria of mouse caSMCs by co-immunostaining biotinylated GLP-1(28-36) with Mitotracker Red (Appendix 4). Although we observed some co-localization, the results were not conclusive. This can be explained by the loss of mitochondrial integrity following staining, thereby disrupting the interaction with the bioactive GLP-1(28-36) peptide prior to cell fixation and imaging. But, based on the premise that GLP-1(28-36) binds mitochondrial proteins and also stimulates ATP production, we will study the function of GLP-1(28-36) on metabolism using freshly isolated intact and functional mitochondria from mouse hearts. Briefly, hearts will be harvested from 8-10 weeks old male C57Bl6/J mice, homogenized in ice-cold isolation buffer using a Teflon-glass homogenizer and subjected to differential centrifugation and density gradient purification with 18/30/60 % Percoll™ gradient solutions to isolate intact mitochondria as per protocol described by Schulz et al. [228]. Metabolic activity will be determined by the extent of oxidative phosphorylation, measured as the rate of oxygen consumption and ATP synthesis in intact mitochondria [156, 201, 229]. Isolated mouse heart mitochondria will be incubated with or without the addition of varying concentrations (10 nM – 10 µM) of GLP-1(28-36), negative control (SCRAM(28-36)) or positive control (8Br-cAMP) and the rate of oxygen consumption will be measured, using an
oxygraph equipped with a Clark electrode, as described [156, 230]. In the same assay, ATP content will also be measured using the Enliten ATP Determination kit (Promega) as described in Chapter 3, Section 3.11.

If GLP-1(28-36) effects mitochondrial metabolism, we expect an increase the rate of oxygen consumption and ATP synthesis. Alternatively, this would imply that other cellular functions are involved in GLP-1(28-36)-mediated ATP production. This could include ATP synthesis through cytosolic glycolysis and will require further investigation.

6.5 Conclusion

In 2015, we are celebrating the 45th anniversary of the discovery of GIP, the first incretin to be identified. There is now a plethora of successful agents targeting the incretin pathway, mainly focused on GLP-1, for effective treatment of patients with type 2 diabetes. As compared to other anti-diabetic therapies, incretin-targeted therapies were deemed attractive due to their stipulated safety in terms of cardiovascular benefits. However GLP-1 biology is complex and its cardiovascular mechanism of action remains a mystery, despite decades of scientific research. Recently, preliminary results on the much-anticipated TECOS (Trial Evaluating Cardiovascular Outcomes with Sitagliptin) were announced. Investigators found that for the primary composite of cardiovascular outcomes, including CV death, nonfatal MI, nonfatal stroke, or hospitalization for unstable angina, sitagliptin, compared to placebo, was noninferior and not superior when added to usual diabetes care. This finding suggests that preventing degradation of GLP-1 with a DPP-4i might not be physiologically relevant for producing large improvements in cardiovascular outcomes. Could an interpretation of these findings be such that the formation of metabolites is required and physiologically important, at least for cardiovascular functions? This
question still remains unanswered, but is an important question that will govern the use of the different classes of incretin therapeutics, because CV safety is integral to overall management of type 2 diabetes. This thesis has laid the foundation for the study of CV actions of GLP-1(28-36). Investigations should continue to unravel its mechanism of action and determine its physiological relevance. The emerging evidence for biological actions of yet another metabolite, GLP-1(32-36), should be considered when designing new experiments.
References


14. Mehta, R.H., et al., *Sustained ventricular tachycardia or fibrillation in the cardiac catheterization laboratory among patients receiving primary percutaneous coronary


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Appendices

• **Appendix 1.** Immunofluorescence microscopy for cellular localization of sAC in the adult mouse heart.

• **Appendix 2.** Protein expression of sAC in distinct cardiac cell types

• **Appendix 3.** Immunofluorescence microscopy for cellular localization of sAC and GLP-1(28-36) in mouse caSMCs.

• **Appendix 4.** GLP-1(28-36) co-localizes with mitotracker in mouse caSMC.
Appendix 1. Immunofluorescence microscopy for cellular localization of sAC in the adult mouse heart. Fixed-frozen sections from an adult mouse heart (5μm, 4 sections/animal, N=1) were stained with Anti-ADCY10 (sAC) antibody (Red) (Abcam), developed specifically for IHC-frozen sections as per the manufacturer’s protocol. Co-immunolabeling was performed using cardiomyocyte-specific sarcomeric α-actinin (Green). sAC expression was localized to both cardiac myocytes (triangles) and coronary vessels (arrows), the latter being in mural cells most likely to represent vascular smooth muscle cells (VSMC). AlexaFluor (AF)-488 and AF-644 (Invitrogen, Molecular Probes®) were used as secondary antibodies to α-Actinin and sAC respectively. Image acquired by an Olympus FluoView confocal microscope, 40X lens.
Appendix 2. Protein expression of sAC in distinct cardiac cell types. Western blots for sAC protein with monoclonal R21 antibody (CEP Biotech) using cellular lysates extracted from different mouse cardiac cell types. Ventricular and atrial CMs were isolated from 1-3 days old mouse neonatal pups. Mouse coronary SMCs were isolated from wild-type 8-10 weeks old C57Bl/6J mice. Cytosolic heart extracts from adult C57Bl/6J mice served as positive control for sAC. GAPDH served as loading control. 20ug of protein was loaded per lane. sAC-tr represent a 50 kDa active form of sAC in mammalian cells. CM (Cardiac myocyte), SMC (Smooth muscle cell).
Appendix 3. Immunofluorescence microscopy for cellular localization of sAC and GLP-1(28-36) in mouse caSMCs. Fixed mouse caSMCs were stained with Anti-ADCY10 (sAC) antibody (Red) (Abcam), as per the manufacturer’s protocol. Co-immunolabeling was performed using biotinylated GLP-1(28-36) or control SCRAM(28-36) (Green). AlexaFluor (AF)-467 and Streptavidin-AF488 conjugate (Invitrogen, Molecular Probes®) were used as secondary antibodies to sAC and biotinylated peptides respectively. Nuclei were stained with Hoescht. Image acquired by an Olympus FluoView confocal microscope, 40X lens. Scale bar 20µm.
Appendix 4. GLP-1(28-36) co-localizes with mitotracker in mouse caSMC. Co-immunostaining of mouse caSMCs with 100nM Mitotracker CMXRos (Red) and 50µg/mL each of biotinylated or unlabelled GLP-1(28-36) (Green). Biotinylated GLP-1(28-36) was detected using streptavidin-conjugated AlexaFluor (AF)-488 secondary antibody. Nucleus was stained with hoescht (1 µg/µl). Image acquired by an Olympus FluoView confocal microscope, 60X lens.