ANGIOTENSIN II PROTEOMIC SIGNATURE IN HUMAN PROXIMAL TUBULAR CELLS AS A PREDICTOR OF RENIN ANGIOTENSIN SYSTEM ACTIVITY IN KIDNEY DISEASES

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy, Graduate Department of the Institute of Medical Science, in the University of Toronto

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Angiotensin II Proteomic Signature in Human Proximal Tubular Cells as a Predictor of Renin Angiotensin System Activity in Kidney Diseases

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ABSTRACT

Angiotensin II (AngII), the major effector of the renin angiotensin system, mediates kidney disease progression by signalling through AT-1 receptor (AT-1R), but there are no specific measures of renal AngII activity. Accordingly, we sought to define an AngII-regulated proteome in primary human proximal tubular cells (PTEC) in order to identify potential AngII activity markers in the kidney. We utilized stable isotope labelling with amino acids (SILAC) in PTECs to compare proteomes of AngII-treated and control cells. Of 4618 quantified proteins, 83 were differentially regulated. SILAC ratios for 18 candidates were confirmed by Selected Reaction Monitoring (SRM) assays. Both SILAC and SRM revealed the nuclear factor erythroid 2-related 2 (Nrf2) target protein, heme oxygenase-1 (HO-1) as the most significantly upregulated protein in response to AngII stimulation. AngII-dependent regulation of HO-1 gene and protein was further verified by qRT-PCR and ELISA in PTECs. In order to extend these in vitro observations, we utilized a systems biology approach. We thus overlaid a network of significantly enriched gene ontology (GO) terms from our AngII-regulated proteins with a
dataset of differentially expressed kidney genes from AngII-treated wild type mice and AT-1R knock-out mice. Five GO terms were enriched both \textit{in vitro} and \textit{in vivo}, and all included HO-1. Furthermore, four additional Nrf2 target proteins were functionally important \textit{in vitro} and \textit{in vivo}. We then studied HO-1 kidney expression and urinary excretion in AngII-treated wild type mice and mice with PTEC-specific AT-1R gene deletion. Deletion of the AT-1R gene in PTECs lowered both kidney expression and urine excretion of HO-1, confirming AngII/AT-1R mediated regulation of HO-1. In summary, our \textit{in vitro} experiments identified novel molecular markers of AngII activity in PTECs and the animal studies demonstrated that these markers also reflect AngII activity in PTECs \textit{in vivo}. These interesting proteins hold promise as specific markers of renal AngII activity in patients and in experimental models.
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CONTRIBUTIONS

Dr. Ana Konvalinka prepared the thesis and is responsible for conceptual and experimental content.

Dr. James W. Scholey and Dr. Eleftherios P. Diamandis provided supervision, guidance and expertise that assisted in successful completion of the work.

Joyce Zhou assisted with Western blots in Chapter 2 and Chapter 5.

Apostolos Dimitromanolakis provided statistical expertise.

Dr. Andrei Drabovich assisted with SRM assay development in Chapter 3.

Fei Fang participated in helpful discussions.

Dr. George Liu assisted with qRT-PCR in Chapter 3.

Dr. Susan Gurley and Dr. Thomas Coffman provided mice with AT-1R proximal tubule knock out and participated in helpful discussions.

Dr. Shao-Ling Zhang assisted with immunohistochemistry in Chapter 5.

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LIST OF ABBREVIATIONS

ACE, Angiotensin converting enzyme
ACE2, Angiotensin converting enzyme 2
AKI, Acute kidney injury
AngI, Angiotensin I
AngII, Angiotensin II
Ang, Angiotensin
APA, Aminopeptidase A
APN, Aminopeptidase N
AT-1R, Angiotensin II type 1 receptor
AT-2R, Angiotensin II type 2 receptor
AQUA, Absolute quantification of abundance
BiNGO, Biological Networks Gene Ontology
cAMP, cyclic AMP
CDC34, Ubiquitin-conjugating enzyme E2 R1
CE, Capillary electrophoresis
CID, Collision induced dissociation
CKD, Chronic kidney disease
CTGF, Connective tissue growth factor
DAG, Diacylglycerol
DMEM, Dulbecco’s Modified Eagle Medium
DNJB4, Heat shock 40 kDa protein 1 homolog
EGFR, Epidermal growth factor receptor
ELISA, Enzyme-linked immunosorbent assay
EM, Enrichment Map
eNOS, Endothelial nitric oxide synthase
ER, Endoplasmic reticulum
ESI, Electrospray ionization
ESRD, End stage renal disease
FBS, Fetal bovine serum; GO, Gene Ontology
FDR, False discovery rate
FTICR, Fourier-transform ion cyclotron resonance
GO, Gene Ontology
GFR, Glomerular filtration rate
HO-1, Heme oxygenase 1, decycling
HPLC, High performance liquid chromatography
ICAM-1, intercellular adhesion molecule 1
ICAT, Isotope-coded tags
IPA, Ingenuity Pathway Analysis
IP3, Inositol triphosphate
iTRAQ, Isobaric tags for relative and absolute quantification
KO, Knock out
LARP4, La ribonucleoprotein domain family member 4
LC, Liquid chromatography
LDL, Low density lipoprotein
LTQ-Orbitrap, Linear ion trap – Orbitrap mass spectrometer
MALDI, Matrix-assisted laser desorption/ionization
MAPK, Mitogen activated protein kinase
MCP-1, monocyte chemoattractant protein 1
MS/MS, Tandem mass spectrometry
mTOR, Mammalian target of rapamycin
NFκB, Nuclear factor kappa B
Nrf2, Nuclear factor (erythroid derived-2)-like 2
PAI-1, Plasminogen activator inhibitor 1
PCP, Prolyl carboxylase
PDCD4, Neoplastic transformation inhibitor
PDGF, Platelet derived growth factor
pERK, Phosphorylated extracellular signal-regulated kinase
PLA2, Phospholipase A2
PLC, Phospholipase C
PLD, Phospholipase D
PTEC, Proximal tubular epithelial cell
PTKO, Proximal tubule specific knock out
Q, quadrupole mass analyzer
qRT-PCR, Quantitative reverse transcriptase polymerase chain reaction
RAS, Renin angiotensin system
RHOB, Rho-related GTP binding protein
ROS, Reactive oxygen species
SD, Standard deviation
SILAC, Stable isotope labeling with amino acids in cell culture
SRM, Selected reaction monitoring
SSB, La autoantigen
2D-DIGE, Two-dimensional fluorescence difference gel electrophoresis
TGF, Transforming growth factor
TIMP-1, Tissue inhibitor of metalloproteinase
TLR-4, Toll-like receptor 4
TOF, time-of-flight mass analyzer
TSP-1, Thrombospondin 1
TxA2, Thromboxane A2
TXNIP, Thioredoxin-interacting protein
VCAM-1, Vascular cell adhesion molecule 1
VSMC, Vascular smooth muscle cell
WT, Wild type
XIC, Extracted ion chromatogram
CHAPTER 1

INTRODUCTION

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**Searching for new biomarkers of renal diseases through proteomics.**


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1.1 Renin angiotensin system

1.1.1. Systemic renin angiotensin system

The contribution of the renin angiotensin system (RAS) to the regulation of arterial blood pressure has long been recognized. Renin, the rate-limiting proteolytic enzyme in the activation of RAS was first discovered over a century ago. The key sites of production of active renin, the so called juxtaglomerular cells are situated near the afferent arteriole of each glomerulus of the kidney. Juxtaglomerular cells synthesize prorenin, the renin precursor which is subsequently cleaved to the active form. Prorenin is secreted into the systemic circulation, where it accounts for 50-90% of circulating renin\(^1\). There is no evidence that prorenin is cleaved to renin in the circulation, and the role of this precursor remains unclear\(^1,2\).

The major physiological stimuli for renin release include renal hypoperfusion due to hypotension or decreased effective circulating volume and increased sympathetic activity. Renin initiates a cascade of events that begins with the cleavage of angiotensinogen to angiotensin I. Angiotensinogen is the only known substrate of renin, and is an \(\alpha\)-2-globulin synthesized constitutively and released into the circulation by the liver\(^3\). Cleavage of angiotensinogen enables intravascular release of angiotensin I, which is a decapeptide cleaved from the N-terminal region of angiotensinogen. Angiotensin I has no known physiological actions, but it is subsequently cleaved by angiotensin converting enzyme (ACE) into the major effector molecule of RAS called angiotensin II (AngII). ACE is a
carboxypeptidase, which is secreted by the endothelium of the lungs and many other vascular beds.\textsuperscript{4,5} AngII is the main effector of the RAS, and has two main systemic effects – systemic vasoconstriction and increased sodium and water absorption by the kidneys. Both of these effects have the consequence of reversing the initial stimuli that led to the activation of RAS, and in physiological terms, serve to restore effective circulating volume and blood pressure.

Systemic vasoconstriction occurs as a result of direct action of AngII on the vascular smooth muscle, but some of its effect may relate to AngII-mediated release of norepinephrine.\textsuperscript{6,7} The vascular actions of AngII are mediated by enhanced phosphatidylinositol turnover.\textsuperscript{8} Receptor mediated action of phospholipase C (PLC) leads to the generation of diacylglycerol (DAG) and inositol triphosphate (IP3). IP3 binds to its receptor on sarcoplasmic reticulum and leads to the release of Ca\textsuperscript{++} in the cytoplasm, which then causes smooth muscle contraction. Diacylglycerol produced from phosphatidylinositol causes the release of arachidonic acid which can be converted to prostaglandins, as well as to metabolites of hydroxyeicosatetraenoic acid.\textsuperscript{9} Prostaglandins oppose some of the vasoconstrictive effects of AngII, while the metabolites of hydroxyeicosatetraenoic acid appear responsible for the release of aldosterone from the adrenal gland, and direct vasoconstrictive effects.\textsuperscript{9} In addition to systemic hemodynamic effects, AngII influences renal hemodynamics and glomerular filtration rate (GFR). AngII causes vasoconstriction of both afferent and efferent glomerular arterioles through the release of thromboxane A2 (TxA2).\textsuperscript{10} Since the efferent arteriole has a smaller caliber than the afferent one, AngII causes disproportionately greater constriction
of the efferent arteriole, thus increasing intraglomerular hydraulic pressure and increasing GFR. Contrary to GFR, renal blood flow (RBF) is significantly diminished. AngII has two additional effects that influence GFR. It causes mesangial cell contraction, resulting in diminished surface area for filtration, and it also sensitizes the afferent arteriole to tubuloglomerular feedback. On balance, these effects tend to preserve GFR, even though RBF declines.

The second main systemic effect of AngII is its role in sodium and water reabsorption. AngII can directly activate sodium and water reabsorption through the Na+-H+ antiporter in the S1 segment of the proximal tubules. This action is mediated mainly by AngII signaling through the angiotensin II type I receptor (AT1R) and by diminishing cyclic AMP (cAMP) production and the consequent removal of inhibitory cAMP effect on Na+-H+ exchange. AngII can also indirectly increase sodium and water reabsorption in the cortical collecting duct by stimulating the release of aldosterone. Recent studies have demonstrated that AngII also has direct effects on increasing activity of other important transporters along the nephron, including: Na+-K+-2Cl- channel in the thick ascending limb of the loop of Henle, Na+-Cl- cotransporter in the distal tubule, and epithelial Na channel in the cortical collecting duct. Ultimately, the increase in sodium reabsorption mediated by AngII helps restore effective circulating volume and blood pressure.

1.1.2. Bioactive peptides of the renin angiotensin system
This relatively simplistic understanding of the systemic RAS has evolved considerably in the last two decades. Most importantly, it has been recognized that local RAS exists in several major organs including the kidney, heart, brain, adrenal gland and vascular endothelium\textsuperscript{18-22}. This tissue-specific activation of RAS can be regulated independent of the systemic RAS, as evidenced by 1000 times higher levels of AngII in the peritubular capillary and the proximal tubule compared to the circulating AngII levels\textsuperscript{23}. The local RAS may be important for tissue-specific regulatory functions. Activation of the local RAS can occur due to nitric oxide or endothelin release\textsuperscript{18}. Identification of additional RAS components challenged the original understanding of the RAS further. One such component is angiotensin converting enzyme 2 (ACE2), another carboxypeptidase capable of cleaving angiotensin I into a nonapeptide Ang (1-9), which is subsequently cleaved by ACE to Ang (1-7), a peptide that has vasodilatory effects, contrary to AngII\textsuperscript{24}. It has also been recognized that there are additional carboxypeptidases capable of cleaving angiotensin I into active peptides, such as chymase, neprilysin and cathepsin G\textsuperscript{25,26}. Besides carboxypeptidases, aminopeptidases capable of cleaving off the amino terminus of angiotensin I have been discovered\textsuperscript{27,28}. As a result of the action of these peptidases, numerous additional peptides with potential activity have been discovered, and they include but are not limited to: angiotensin (1-7), angiotensin (1-5), angiotensin (2-10), angiotensin (2-8) (also known as angiotensin III), and angiotensin (3-8) (also known as angiotensin IV). These additional components have substantially increased
Figure 1.1 depicts a contemporary view of the synthesis and metabolism of angiotensin peptides. Angiotensinogen, the precursor of bioactive peptides of RAS is a 55-60kDa glycoprotein synthesized mainly in the liver and released...
constitutively into the circulation. However, additional sites of angiotensinogen production have been recognized, and they include proximal tubular cells (PTECs), collecting duct and mesangial cells of the kidney, but other major organs including the heart, adipose tissue, adrenal gland, vascular endothelium, and brain also synthesize angiotensinogen. Although not itself biologically active, plasma angiotensinogen levels are poised near the Km for cleavage by renin, with the consequence that alteration of angiotensinogen levels also affects the formation of AngII. Moreover, the redox state of angiotensinogen has been shown to be important in rendering angiotensinogen sensitive to cleavage by renin. The reduced unbridged form of angiotensinogen is present in the circulation in a near 40:60 ratio with the oxidized sulphydryl-bridged form, which preferentially interacts with receptor-bound renin.

Angiotensin I (AngI) is a decapeptide generated by renin-mediated cleavage of angiotensinogen, and is not known to be a bioactive peptide, but it is the substrate for generation of a large array of important peptides. AngII, an octapeptide formed by carboxypeptidase action on AngI is the most important bioactive peptide of the RAS. This is a dynamically regulated peptide that is formed and degraded rapidly and that acts in both endocrine and paracrine fashion. Interestingly, AngI can also be metabolized by nephrilysin into angiotensin (1-7), another peptide with important functions that is increasingly being recognized as a vasodilatory, antiproliferative and anti-fibrotic peptide, which antagonizes the actions of AngII. AngI also undergoes metabolism by ACE2 into angiotensin (1-9), which is subsequently cleaved to angiotensin (1-7) by ACE. ACE2 is a homologue of
ACE, and a recently discovered carboxypeptidase with systemic and local actions that appear to counterbalance the effects of AngII. ACE2 can generate angiotensin (1-7) from AngI or from AngII. ACE2 has 42% genomic homology with ACE and is less widely expressed, having been demonstrated in the heart, kidney and testis\(^{41-43}\). While ACE2 only appears to metabolize AngI and AngII, ACE is also capable of metabolizing angiotensin (1-7), angiotensin (2-10), angiotensin (3-10), angiotensin (4-10) and angiotensin (5-10). ACE2 is not inhibited by ACE inhibitors, suggesting that ACE2 has a significantly different functional site\(^ {44,45}\).

There are additional peptidases that metabolize AngII into angiotensin (1-7), and one of these enzymes is prolyl carboxylase (PCP)\(^ {46}\). In addition to angiotensin (1-7), another heptapeptide, alamandine has been shown to form by ACE2-mediated metabolism of angiotensin A\(^ {47}\). Angiotensin A is formed by beta-decarboxylation of Aspartate-34 in AngII, which yields alanine as the first N-terminal residue. The existence of alamandine has recently been demonstrated using mass spectrometry in human blood and in the heart tissue\(^ {47}\). Moreover, alamandine could be formed directly from angiotensin (1-7) and this preliminary work suggests that its actions are anti-hypertensive and anti-fibrotic in a rat\(^ {47}\). The carboxy-terminal processing of AngI thus leads to formation of a plethora of potentially important bioactive peptides that require further study.

In addition to carboxy-terminal metabolism of AngI, this peptide is also metabolized from the N-terminus by aminopeptidases. Cleavage of AngI by aminopeptidase A (APA) leads to formation of angiotensin (2-10) or (des-Asp1)-angiotensin I, a nonapeptide implicated in vasopressor responses in hypertensive
APA also mediates the conversion of AngII to angiotensin (2-8) (also known as angiotensin III). Angiotensin III has recently been demonstrated to cause natriuresis and oppose the effects of AngII. Its half-life in the circulation is short. Aminopeptidase N (APN) can cleave angiotensin III to generate angiotensin (3-8) (also known as angiotensin IV). Angiotensin IV has inconsistent effects, at least in the kidney. Some lines of evidence suggest that its actions are analogous to AngII. It has a short half-life in the circulation, much like angiotensin III. Recent lines of evidence support the idea that both angiotensin III and angiotensin IV can be generated by mechanisms that bypass formation of AngII, thus suggesting their potential importance in physiological processes, rather than as break-down products of AngII. The list of angiotensin peptides has expanded recently, with new players such as angiotensin (4-8), angiotensin (5-8), angiotensin (5-10), and angiotensin (6-10) measured in human blood. Their physiological or pathophysiological roles however are unknown. Despite the complexity and the number of players involved, AngII remains the most influential bioactive peptide of the RAS, with numerous well-described hemodynamic and non-hemodynamic effects.

The bioactive peptides of RAS bind to and activate distinct receptors. AngII signals through angiotensin II receptor type I (AT-1R) and angiotensin II receptor type II (AT-2R). The alternate peptide form, angiotensin A, has the same affinity for the AT-1R as AngII, but a higher affinity for the AT-2R. Unlike AngII, angiotensin (1-7) signals through a G-coupled receptor Mas. Alamandine’s actions appeared to be independent of AT-1R, AT-2R or Mas receptor. Instead,
this peptide signals through a Mas-related G-Protein coupled receptor, MrgD. Angiotensin III has recently been shown to be the preferred ligand for AT-2R in the tubules. Angiotensin IV is a ligand of the angiotensin II type IV receptor\textsuperscript{55,56}.

1.1.3. Angiotensin II signaling and its receptors

AngII signals through two main receptors: AT-1R\textsuperscript{57} and AT-2R\textsuperscript{58}. These receptors are both G-protein coupled, with seven transmembrane domain motifs. Although both receptors bind AngII with similar affinity, their functions are quite different. Furthermore, most of the cellular effects of AngII appear to be mediated by AT-1R. For example, AngII-induced salt and water reabsorption is mediated by AT-1R\textsuperscript{59}. AT-1R is widely expressed, and has been demonstrated in the kidney, lung, heart, brain, vasculature, adrenal gland and liver. AT-1R is composed of 359 amino acids, and has been mapped to chromosome 3 in humans. The extracellular domain of the receptor is characterized by three glycosylation sites, and mutation of these sites has no effect on agonist binding\textsuperscript{60}. G protein interactions occur on the transmembrane domain at the NH2 terminus and the first and the third extracellular loops. Along with several residues located on the extracellular region of the receptor, four cysteine residues of AT1-R form disulfide bridges and are essential for AngII binding\textsuperscript{61}. The AT1-R cytoplasmic tail contains many serine/threonine residues, which are phosphorylated by G protein receptor kinases or GRKs. Modifications within these functional sites may be responsible for the altered receptor function. AngII acutely increases activity of AT-1R,
however chronic AngII exposure leads to downregulation of AT-1Rs. AngII binding to AT-1R elicits a series of signaling cascades that have physiologic and pathologic effects. Traditionally, these signaling events have been divided into G protein coupled and non-G protein coupled signaling.

G protein coupled signaling mediates one of the main acute functions of AngII - contraction. G protein mediated AT-1R signaling is displayed in Figure 1. When activated by an agonist, AT-1Rs couple to \( \text{G}_{\alpha_{q/11}}, \text{G}_{\alpha_{12/13}}, \text{and G}_{\beta\gamma} \) complexes, which activate downstream effectors including phospholipase C (PLC), phospholipase A2 (PLA2), and phospholipase D (PLD). Antagonists of G protein coupled signaling have also been identified. These regulators of G protein signaling (RGS) constitute a protein group with several isoforms of which RGS2 has been particularly important in \( \text{G}_{\alpha_q} \) inhibition. Activation of PLC produces inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG) within seconds. IP3 binds to its receptor on sarcoplasmic reticulum, opening a channel that allows calcium efflux into the cytoplasm. \( \text{Ca}^{2+} \) binds to calmodulin and activates myosin light chain kinase (MLCK), which phosphorylates the myosin light chain and enhances the interaction between actin and myosin, causing smooth muscle cell contraction. To counter-regulate MLCK, cells have myosin light chain phosphatase (MLCP), which is inhibited by Rho kinase, leading to sustained contraction. DAG activates PKC, which serves to increase the pH during cell contraction by phosphorylating the \( \text{Na}^+ / \text{H}^+ \) pump, and also participates as an effector in the Ras/Raf/MEK/ERK pathway. These downstream molecules contribute to the vasoconstrictive properties of AT-1R activation and lead to
AngII’s growth promoting effects. AngII has been shown to result in phosphorylation of PLA2, which leads to production of arachidonic acid (AA) and its metabolites. The derivatives of AA function in maintaining vascular tone and in

**Figure 1.2.** G protein coupled signaling of AngII by activation of AT-1R. Abbreviations: PC, phosphatidylcholine; PLD, phospholipase D; PA, phosphatidic acid; PIP2, phosphatidylinositol bisphosphate; PLC, phospholipase C; PKC, protein kinase C; DAG, diacylglycerol; IP3, inositol trisphosphate; MLCK, myosin light chain kinase; PLA2, phospholipase A2; PG, prostaglandins; EET, epoxyeicosatrienoic acid; HETE, hydroxyeicosatetraenoic acid; COX, cyclooxygenase; LT, leukotrienes; LO, lipooxygenase; TxA2, thromboxane A2; NO, nitric oxide. Adapted with permission from Mehta and Griendling. *Am J Physiol Cell Physiol* 292: C82–C97, 2007.

NADPH oxidation (63). The cyclooxygenase-derived prostaglandins, such as PGI2 and PGE2 are vasodilatory, and are counteracted by PGH2 and TxA2. AT-1R activation can also lead to activation of PLD, which is
responsible for breaking down phosphatidylcholine to choline and phosphatidic acid, which is subsequently converted to DAG. The protagonists of sustained contraction have been implicated in hypertension.

AngII has been also demonstrated to cause activation of mitogen-activated protein kinases (MAPK) by signaling through the AT-1R. These MAPKs include extracellular signal regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), and p38MAPK. ERK has been the best characterized of the MAPKs. ERK is phosphorylated within 5 minutes of AT-1R activation by AngII, and its phosphorylation appears dependent on PLC\(^7\). ERK is negatively regulated by phosphatase MAP kinase phosphatase-1. ERK has been implicated in contraction by making more Ca\(^{2+}\) available to the cells\(^6\). It was also implicated in anti-apoptosis, cell hypertrophy and protein synthesis\(^7\). ERK and the other MAPKs regulate apoptosis, inflammation and response to environmental stress in vascular and other cells. JNK and p38MAPK are stress-responsive kinases. Unlike ERK and p38MAPK, activation of JNK is independent of epidermal growth factor receptor (EGFR) recruitment\(^7\). AngII-stimulated activation of JNK and p38MAPK depends on G\(\alpha_{12/13}\) -mediated activation of Rho/Rho kinase, with resultant activation of the small G protein Rac and reactive oxygen species (ROS) production\(^7\). Activation of the JNK pathway by AngII can also occur via Gq-mediated activation of PKC-\(\delta\), and subsequent stimulation of Pyk-2 and PDZ-RhoGEF-mediated Rho activation\(^7\). AngII mediated activation of p38MAPK occurs predominantly via NADPH oxidase that produces ROS\(^7\). p38MAPK activates another kinase known as Akt, which has pleotropic effects on the cell.
AT-1R mediated activation of MAPKs can thus result in multiple adverse effects on the cells that will further be discussed in the subsequent sections.

AngII-mediated activation of AT-1R is a powerful inducer of NADPH oxidase, with the resultant production of ROS that include superoxide and hydrogen peroxide\textsuperscript{77-79}. The exact mechanisms of NADPH oxidase activation are unknown, although it may be dependent on AngII-induced phosphorylation of p47, and subsequent action of NOX1/2. AngII also induces expression of NOX4. ROS can activate p38MAPK and lead to endothelial dysfunction (mainly by reducing nitric oxide), and also promote inflammation by influencing the release of pro-inflammatory cytokines\textsuperscript{80,81}. Transcription factors that include NF\kappa B, AP-1 and nuclear factor (erythroid derived-2)-like 2 (Nrf2) are activated by ROS, and have proinflammatory or anti-oxidant roles\textsuperscript{82-84}.

The complexity of AT-1R signaling is further evident in the ability of this receptor to form homo- or heterodimers with other types of receptors. Homodimerisation of AT-1Rs may be constitutive and appears to occur prior to receptor expression on the cell membrane\textsuperscript{85}. AT-1R can form heterodimers with bradykinin B2 receptor, \(\beta\)2-adrenergic receptor, and dopamine D2 receptor\textsuperscript{86-88}. It has also been shown that AT-2Rs can bind to AT-1Rs and interfere with their function. The importance of oligomerization has not been established. In addition to oligomerization, AT-1Rs can recruit other receptors, particularly tyrosine kinases and allow signaling through these recruited receptors. The most important examples include EGFR, platelet-derived growth factor receptor (PDGFR), and insulin receptor. AngII can activate these receptors independently of their agonist
(as in the case of the former two receptors), or can potentiate signaling through the receptors in the presence of the agonist (as is the case with the insulin receptor). Transactivation of EGFR was demonstrated to be a critical event in development of AT-1R-mediated fibrosis in mice infused with AngII\textsuperscript{89}. The importance of these effects will be discussed more in the sections below.

It should be recognized that non-G protein coupled signaling of AT-1R is also important. AT-1R can recruit β-arrestins, which associate with the clathrin, resulting in receptor internalization and cessation of G protein signaling. Approximately 25\% of the receptors internalized are recycled back to plasma membrane, whereas the remaining 75\% are degraded by lysosomes. However, β-arrestins are not simply trafficking proteins, but can also act as scaffold to bring about signaling proteins that lead to the initiation of second wave of signaling. β-arrestins can also involve other tyrosine kinases in their signaling\textsuperscript{90}. Recent study demonstrated that the majority of genes transcribed in HEK293 cells with a stably expressed AT-1R after stimulation with AngII or a biased G protein independent agonist were regulated by G protein coupled signaling, but β-arrestin mediated, G protein-independent signaling also resulted in differential regulation of a minority of genes\textsuperscript{91}. Interestingly, G protein-independent biased agonist SII AngII potentiated β2-adrenergic receptor-induced gene expression. G protein-independent signaling has been implicated in processes such as protection from apoptosis, protein synthesis and migration\textsuperscript{92-94}. This type of signaling is actively explored in order to define potential novel therapeutic strategies that can modulate the function of AT-1Rs.
AT-2R mediated signaling of AngII has been much less well understood than the dominant AT-1R signaling. AT-2R is located on chromosome X. Consisting of 363 amino acids, AT-2R is highly expressed in fetal tissue, including fetal aorta, gastrointestinal mesenchyme, connective tissue, skeletal system, brain, and adrenal medulla. The expression however appears to be absent or diminished in these tissues in adults, suggesting that this receptor plays an important role in development. In adults, AT-2R is expressed at the low level in kidney, lung, and liver, although its significance has not been determined. AT-2Rs have been found to be upregulated in some pathological conditions, including chronic heart failure. Curiously, the ratio of expression of AT-1R-to-AT-2R is lower in aorta of female spontaneously hypertensive rats compared to male ones, suggesting sex-specific regulation of expression. In addition to AngII, other ligands can signal through AT-2R including AngIII, AngIV, angiotensin (1-7), and angiotensin (2-10). AT-2R is a seven transmembrane domain receptor with only 34% sequence identity to the AT-1R. AT-2R is believed to have anti-proliferative and pro-apoptotic changes, thus counteracting AT-1R mediated signaling.

Although the function of AT-2R is generally believed to be counter-regulatory to the AT-1R, there are reports that suggest AT-2R could even mimic the function of AT-1R.

Although AT-2R is also a G protein coupled receptor, its signaling has not been well established. Studies have shown that AT-2Rs couple to G\(_{i2/3}\) and G\(_{i1}\) proteins. AT-2R stimulation in endothelial cells can lead to NO production by endothelial nitric oxide synthase (eNOS). Activation of eNOS can occur
either directly by stimulating the AT-2R or indirectly via stimulation of bradykinin B2 receptor by bradykinin\textsuperscript{106,107}. In the indirect case, endogenously produced bradykinin is released after stimulation of the AT-2R. NO subsequently stimulates guanylyl cyclase to produce cGMP, which mediates vasodilation\textsuperscript{108}, natriuresis\textsuperscript{109} and the inhibition of renin production\textsuperscript{110}. AT-2R also antagonizes AT-1R signaling by activating serine, threonine and tyrosine phosphatases\textsuperscript{111,112}.

Similar to the AT-1R, AT-2R can form homo or heterodimers. It forms heterodimers with AT-1R or with bradykinin B2 receptor. It appears that AT-2Rs preferentially form homodimers, but formation of heterodimers depends not only on the receptor number, but also requires AT-2R stimulation. In conclusion, AT-2R is a homologue of AT-1R that may function to either counter the effects of AT-1R, or in some circumstances to mimic the AT-1R actions.

1.1.4. The role of angiotensin II in hypertrophy, inflammation and fibrosis

Given the complexity of AngII signaling, it is not surprising that this peptide has pleiotropic effects on cells and tissues, including cellular hypertrophy, tissue inflammation and organ fibrosis (Figure 1.3). AngII can induce growth of several renal cell types as well as vascular smooth muscle cells (VSMCs) and cardiac myocytes, through stimulation of growth and synthesis of extracellular matrix. Growth induced by AngII appears to result by both hypertrophy and/or proliferation, depending on the cell type and context. AngII appears to have a direct or indirect effect on cell growth. Direct growth is stimulated through
interference with the cellular cycle.

**Figure 1.3.** AngII signaling through AT-1R induces pro-inflammatory and profibrotic growth factors and cytokines. Adapted with permission from Ruster and Wolf. *J Am Soc Nephrol* 2011: 22; 1189 – 1199.

Another indirect mechanism of AngII-mediated cell growth may also occur secondary to hypertension. For example, in rats, infusion of AngII leads to hypertension and VSMC hypertrophy\(^{113}\). It is likely that mechanical stress plays an important role in hypertension-induced hypertrophy. Multiple studies support the observation that AngII causes hypertrophy of cardiac myocytes\(^{114-116}\). Hypertrophy appears to be mediated by AT-1R, as AT-1R blockers prevent cardiac hypertrophy in rats\(^{117}\).

Cell growth and hypertrophy induced by AngII are mediated by MAP
kinases, p38MAPK, ERK and JNK, and the JAK/STAT pathway, which all lead to changes in transcription of cellular proteins.\textsuperscript{60} Production of ROS by AngII leads to activation of p38MAPK, and can also result in hypertrophy. ROS have been demonstrated to be important in inducing growth in the kidney, and are part of the adaptive process of compensatory hypertrophy after nephron loss.\textsuperscript{118} ROS induces ERK and MAPKs linked to downstream hypertrophy. Renal growth has been invariably linked to increased GFR, which in turn may be a consequence of intrarenal AngII production. Similar to VSMCs, growth of PTECs of the kidney is associated with G1 cell cycle arrest in response to AngII.\textsuperscript{119}

This AngII-mediated growth arrest is linked to phosphorylation-mediated activation of p27\textsuperscript{kip1}.\textsuperscript{120,121} p27\textsuperscript{kip1} is a cyclin-dependent kinase inhibitor. Cyclin-dependent kinases are suppressed in the presence of the inhibitor, thus preventing cells from progressing in the cell cycle. The evidence implicating p27\textsuperscript{kip1} in AngII-mediated hypertrophy came from VSMCs.\textsuperscript{122} VSMCs grow by hypertrophy in response to AngII, and their total protein content appears to increase due to enhanced synthesis rather than impaired degradation.\textsuperscript{123} The evidence of AngII-mediated p27\textsuperscript{kip1} activation in renal tubules comes from in vivo animal models,\textsuperscript{124} where it was linked to ROS accumulation. Tubulointerstitium occupies the largest portion of the kidney, and so growth of this compartment contributes most to kidney hypertrophy. Nonetheless, other renal cells are susceptible to AngII-induced hypertrophy. Mesangial cells are fundamentally similar to VSMCs, so it is not surprising that they undergo hypertrophy in response to AngII.\textsuperscript{125-128} Recent studies demonstrate a connection between AngII,
oxidative stress and mesangial cell hypertrophy and fibronectin synthesis\textsuperscript{129}. Podocytes, specialized visceral epithelial cells in renal glomeruli, develop hypertrophy in response to high glucose, which is AngII-dependent\textsuperscript{130}.

Inflammation is one of the hallmarks of AngII signaling. AT-1R has particularly been implicated in promoting proliferation, inflammation and fibrosis (Figure 1.3)\textsuperscript{131-133}. By signaling through AT-1R, AngII upregulates many proinflammatory genes such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1) through the activation of several intracellular signaling systems, including the nuclear factor-\kappa B (NF\kappa B)\textsuperscript{134}, MAPK cascade, Rho proteins, and redox pathways\textsuperscript{131,135,136}. AngII induces plasminogen activator inhibitor (PAI)-1 and tissue inhibitor of metalloproteinase (TIMP)-1\textsuperscript{137} through AT-1R.

Inflammation is a process that involves activation of the endothelium of the blood vessels and expression of diverse adhesive molecules that orchestrate extravasation of leukocytes. AngII can lead to vascular permeability by inducing the production and secretion of vascular endothelial growth factor (VEGF)\textsuperscript{138}. AngII affects monocytes, macrophages, VSMCs, and endothelial cells by activating NF\kappa B and the downstream inflammatory molecules including VCAM-1, ICAM-1, E-selectin, and chemokines such as MCP-1, IL-6 and IL-8\textsuperscript{139,140}. By inducing these inflammatory cytokines, AngII results in local infiltration of inflammatory cells such as monocytes and macrophages. AT-1R mediated signaling leads to activation of toll-like receptor 4 (TLR4), which promotes
oxidative injury, apoptosis and inflammation\textsuperscript{141,142}. AT-1R blockade reduces injury post-myocardial infarction through downregulation of TLR-4, in experimental \textit{in vivo} models\textsuperscript{143}. AngII is also capable of contributing to T-cell infiltration. T-cells possess their own RAS, and contain AT-1Rs\textsuperscript{144}. AngII can trigger T-cell cytoskeletal rearrangements, and production of cytokines which recruit other T-cells\textsuperscript{144,145}. Tissue infiltration by T-cells is at least in part responsible for initiation of hypertension, as documented in animal models\textsuperscript{146}.

Additionally, AngII promotes vascular dysfunction by inducing COX2 and ROS. The proinflammatory effect of AngII in atherosclerosis has been established. In apolipoprotein E deficient mice, AngII causes increased atherosclerosis and aneurysm formation\textsuperscript{86,139,147}. Furthermore, a link between pro-atherogenic cytokine IL-18 and AngII signaling pathways has been established in VSMCs. AngII intensifies the effect of IL-18 via NF\kappa B pathway activation, and it also has a direct effect on IL-18 signaling by inducing IL-18$\alpha$ receptors mRNA via STAT3\textsuperscript{148}. In conclusion, AngII affects a variety of different cells to cause inflammation which may have profound physiologic and pathophysiologic consequences.

Fibrosis is the final common pathway of organ injury. AngII is capable of inducing fibrosis in the heart, VSMCs, and the kidney (Figure 1.3). AngII-driven activation of ERK1/2, JNK, and p38MAPK can result in fibrosis in VSMCs\textsuperscript{149,150}. In the kidney, there is substantial evidence that AngII can induce epithelial-to-myofibroblast transformation (EMT), leading to renal fibrosis, the final common pathway of renal injury. Multiple pathways have been implicated in this process. \textit{In
vitro and in vivo animal studies have demonstrated fibrosis following AngII stimulation by signaling via TGF-β dependent and independent mechanisms, through Smads$^{126,151,152}$, via EGFR/AT-1R cross-talk$^{89}$, via ROCK/MAPK pathways$^{153}$, via Notch and Snail mediated signaling$^{154}$, and curiously via Mas-1 and Ang 1-7$^{155}$. Nox-2 has been shown to induce EMT in renal allografts, a process dependent on AngII, cyclosporine, or direct TGF-β1 activation$^{156}$.

The interaction between AngII and TGF-β is worth discussing further. TGF-β is a pro-fibrotic cytokine which can be directly activated by AngII via p38MAPK or thrombospondin-1. AngII leads to increased TGF-β transcription and translation, at least in renal PTEC$^{157}$, and it can also increase transcription of TGF-β receptors, such as TGF-β type 2 receptors$^{158}$. AngII can also indirectly increase TGF-β transcription via MCP-1$^{159}$, endothelin$^{160}$ or osteopontin$^{161,162}$. Release of TGF-β leads to phosphorylation of Smad2 and Smad3, the recognized mediators of fibrosis. AngII can also affect Smad activation directly, and this typically happens early in fibrosis. TGF-β is however believed to be the main mediator of Smad activation in chronic processes, which ultimately lead to fibrosis.

Connective tissue growth factor (CTGF) is a growth factor related to TGF-β, which is also regulated by AngII, and which represents another important mediator of fibrosis. CTGF belongs to the CCN family of early response genes. It is involved in multiple processes including migration, angiogenesis, proliferation, apoptosis and fibrosis$^{163,164}$. In vitro and in vivo studies demonstrated that CTGF is upregulated by AngII in the kidney and its upregulation is AT-1R
dependent\textsuperscript{165, 166}. Although it was initially thought to be downstream of TGF-\(\beta\), CTGF can also be induced independently of TGF-\(\beta\)\textsuperscript{131}. CTGF expression in tissue is linked to collagen I and fibronectin upregulation and to fibrosis.

Additional molecules have been implicated in AngII-induced fibrosis. Endothelin-1 leads to fibrosis of all renal compartments that could be prevented by endothelin-1 receptor blocker \textit{in vivo}, in animal models of renal injury\textsuperscript{167, 168}. PAI-1 is an important serine protease inhibitor implicated in pathologic deposition of extracellular matrix, which is induced at the mRNA and protein level by AngII. Furthermore, AngII can affect tissue expression of matrix metalloproteases such as TIMP-2 and MMP-2. AngII can thus induce fibrosis by altering the balance of extracellular matrix production and degradation.

The process of fibrosis is complex, and recent surprising insights shed light on the role of EGFR transactivation in initiation and progression of fibrosis in response to AngII. Although the connection between AngII and EGF had been suggested several decades ago, Lautrette and colleagues recently demonstrated that mice unable to activate EGFR were protected from glomerulosclerosis, tubular atrophy and fibrosis, and mononuclear cell infiltration induced by chronic AngII infusion\textsuperscript{89}. The EGFR ligand responsible for this effect was demonstrated to be TGF\(\alpha\). Release of this molecule from a larger precursor by the action of TACE (TNF\(\alpha\) converting enzyme) appears critical for AngII-mediated activation of EGFR. AngII infusion appears to result in translocation of TACE to the cell membrane, where it has the ability to cleave proTGF\(\alpha\) to TGF\(\alpha\), which subsequently activates EGFR, leading to downstream events, such as activation
of PI3K, mammalian target of rapamycin (mTOR) and ERK1/2. The relevance of this model was demonstrated in mice with renal ablation, and appears to be independent of the ability of AngII to increase blood pressure. It thus appears plausible that in animal models of renal disease, EGFR transactivation by AT-1R may be critical for progression to fibrosis.

In summary, AngII exerts its effects on multiple cells and tissues, and its actions can lead to hypertrophy, inflammation and fibrosis.

1.2 Renin angiotensin system and kidney diseases

1.2.1. Epidemiology of chronic kidney diseases

Chronic kidney disease (CKD) is a worldwide health problem. The prevalence of CKD in the US has been estimated to be over 9.6% in non-institutionalized adults\textsuperscript{169,170}. CKD is estimated to affect between 1.9 million and 2.3 million Canadians\textsuperscript{171}. CKD leads to major morbidity and mortality, and over 1.4 million people worldwide require renal replacement therapy as a result of end stage kidney disease (ESRD), according to World Health Organization estimates\textsuperscript{172}. The frequency of CKD continues to increase worldwide.

The leading causes of ESRD in Canada include diabetes mellitus (35%) and renal vascular disease including hypertension (18%). Diabetes mellitus and hypertension are the leading causes of CKD and ESRD worldwide as well, with higher contribution of hypertension in the black population. CKD and ESRD lead
to high morbidity and mortality. Cardiovascular risk increases dramatically with CKD, and further rises proportionally to the decline in GFR. Death from cardiovascular causes is 8-fold higher in individuals with CKD compared to healthy population (and is much higher than death from cancer). Furthermore, death from cardiovascular causes is 10 – 30 times higher in patients with ESRD compared to the general population\textsuperscript{173}. Survival of patients with ESRD treated with dialysis for 5 years is approximately 34\% (United States Renal Data System Annual Report 2009). ESRD is extremely costly. In Canada, hemodialysis accounts for the most common mode of renal replacement therapy, and its cost is approximately $60,000 per patient annually. The one-time cost for kidney transplantation is $23,000 plus $6,000 per year for necessary anti-rejection therapy (data from Kidney Foundation of Canada). CKD is thus prevalent, carries high morbidity and mortality, and the cost of renal replacement therapy is high.

\subsection{1.2.2. Intra-renal renin angiotensin system}

There is evidence that both diabetic and non-diabetic CKD result in activation of the RAS\textsuperscript{174,175}. The mainstay of therapy in fact, focuses on blockade of the RAS, which has been demonstrated to slow down progression of kidney disease\textsuperscript{176-179}. Interestingly, it is the activation of the intra-renal RAS that has been linked to progression of kidney disease rather than the systemic RAS.

The most compelling evidence for the existence of intra-renal RAS comes from micropuncture studies in rats which demonstrate that the tubulointerstitial
AngII concentration is around 5-10 nmol/L, close to 1000 times higher than in plasma. These tubulointerstitial AngII levels correlated with development of hypertension in animal models. Other studies demonstrated even higher AngII levels in both glomerular and tubulointerstitial fluid compartments of the kidney. All of the components of the RAS are expressed in the kidney. Angiotensinogen is the substrate for all bioactive peptides of the RAS. Although constitutively synthesized and secreted by the liver into the circulation, angiotensinogen is also produced in the kidney. The major sites of kidney production include PTECs, collecting duct cells, and mesangial cells. The major site of renal angiotensinogen production is PTEC. Elegant studies demonstrated that overexpression of the angiotensinogen gene in the PTEC resulted in hypertension and proteinuria. The condition was reversed with RAS blockade. The same transgenic mice were more susceptible to renal injury when rendered diabetic. These observations were supported by clinical studies demonstrating that states of elevated RAS activity such as diabetes mellitus and hypertension display increased angiotensinogen expression in the kidney. Furthermore, while diabetic patients are typically responsive to ACE inhibitors which slow down progression of their renal disease, their circulating levels of renin and AngII are low, suggesting that renal AngII is increased leading to suppression of systemic renin by negative feedback. A surprising study published recently elegantly demonstrated that the liver-derived angiotensinogen is the only substrate for renal AngII, and accounts for proximal tubular AngII production in animals with a glomerular sieving defect. It thus remains unclear whether both liver-derived and kidney-derived
angiotensinogens are cleaved to AngII and what their respective contributions are under physiological and pathophysiological states.

As discussed previously, the cleavage of angiotensinogen to AngI by renin is the rate-limiting step in the formation of AngII. The major sites of renin production are the juxtaglomerular cells in the kidney. However, additional sites of renin production have been discovered. Proximal tubular cells and collecting duct cells also synthesize renin. Podocytes have been shown to express renin under pathological conditions. Prorenin can be cleaved to renin by cathepsin B in the kidney. Although the role of prorenin remains unclear, prorenin receptor has been identified in the kidney and demonstrated to bind both prorenin and renin. The expression of this receptor seems particularly prominent in the mesangial cells and podocytes. This receptor has been suggested to mediate the progression of glomerular damage in several animal models of glomerular disease including diabetic nephropathy and Goldblatt renovascular hypertensive model.

In addition to renin, the second enzyme necessary for the formation of AngII is ACE. ACE mediates the cleavage of AngI to AngII. ACE is expressed in the kidney, particularly in PTECs and distal tubular cells. Although the expression has also been demonstrated in mesangial cells and podocytes, it is found there at much lower levels than in the tubules. ACE can also be expressed by infiltrating macrophages, in cases of renal injury. There is controversy over the expression of ACE in pathologic states such as diabetic nephropathy. It appears that ACE is increased in glomeruli of human biopsies with
diabetic nephropathy, while its tubular expression is decreased\textsuperscript{196}. Hyperglycemia
does not consistently lead to increase in ACE expression, at least in mesangial
cells and podocytes of rat\textsuperscript{197}, and so the mechanism of ACE upregulation remains
unknown. Other human renal diseases such as IgA nephropathy, membranous
nephropathy and hypertension lead to increased glomerular and tubulointerstitial
ACE expression\textsuperscript{198,199}. Intra-renal ACE was elegantly demonstrated to be one of
the critical regulators of hypertension. Absence of renal ACE was linked to
decreased AngII-induced hypertension in mice\textsuperscript{13}. The lack of hypertensive effect
of AngII was further explained by modulation of the expression and
phosphorylation of a number of sodium channels along the nephron. This study
proposes a model for key importance of intra-renal ACE (as well as
angiotensinogen) in the formation of intra-renal AngII. According to the model,
even systemically delivered AngII will have no effect on the kidney unless it leads
to generation of more AngII through the local activation of renin and ACE, and
enzymatic cleavage of angiotensinogen, presumably by acting through the AT-1R.
AngII formed in this way will then lead to activation of a large number of channels
along the nephron with the resulting increase in sodium and water reabsorption.
ACE inhibition has long been known to slow down progression of both diabetic
and non-diabetic renal disease, and this recently proposed model may explain the
paradigm, at least in part. The questions that remain unanswered relate to the
reason for the protective effect of renal ACE knock out; is systemic AngII simply
degraded faster as a result of overactivity of other peptidases, or is the kidney
unable to generate more AngII due to the deficiency of ACE?
ACE2 is the homologue of ACE with actions that are shown to be counter-regulatory to the actions of ACE. ACE2 is expressed in normal glomeruli and tubules\textsuperscript{36,200,201}. Mice deficient in ACE2 display increased sensitivity to AngII-induced hypertensive injury\textsuperscript{202}. ACE2 loss (either through genetic manipulation or pharmacological blockade) exacerbates diabetic renal injury and increases proteinuria in diabetic mouse models\textsuperscript{203,204}. It remains unclear whether the protective mechanisms associated with ACE2 are linked to increased angiotensin (1-7) levels, or lower levels of AngII. The ratio of ACE:ACE2 further appears important and is increased in diabetic nephropathy, potentially leading to increased AngII levels and kidney disease progression\textsuperscript{205}. While the importance of intra-renal ACE2 is still being unraveled, recombinant ACE2 may represent a promising therapeutic agent that could tip the balance away from AngII generation.

Other enzymes participate in the catalytic breakdown and formation of bioactive molecules of RAS, and many of them are expressed in the kidney. For example, chymase represents an alternative enzyme capable of breaking down AngI to AngII. Its expression in the kidney was convincingly demonstrated only in mesangial cells, as well as infiltrating mast cells\textsuperscript{198,206}. The role of chymase blockade for prevention of AngII generation remains an unexplored area. Aminopeptidases APA and APN are capable of cleaving the N-terminal sequence of AngI (Figure 1.1), and they were demonstrated at the brush border of PTECs, as well as in mesangial cells and podocytes\textsuperscript{207-209}. Another endopeptidase called neprilysin was found to be responsible for cleavage of AngII to angiotensin (1-7),
as well as for the cleavage of bradykinin. Neprilysin is expressed in tubules and glomeruli\textsuperscript{210,211}, however the role of its blockade has been challenging to study since its blockers were found to block ACE as well. The role of these enzymes in the pathophysiology of CKD is still being elucidated. Taken together, these studies establish that AngII can be generated within the kidney, and that the bioactivity of AngII may prove to be important for establishing risk of kidney disease progression and may represent a marker to guide therapy with RAS blockade.

1.2.3. Markers of intra-renal renin angiotensin system activity

Clinically, blockade of the RAS has demonstrated effectiveness in slowing down progression of kidney disease, but no adequate specific markers of the activity of RAS exist. The lack of specific markers is problematic because some clinical studies suggest that more aggressive or dual RAS blockade may be beneficial in proteinuric CKD, independent of blood pressure\textsuperscript{212-214}. However, the enthusiasm for dual RAS blockade has been tempered by reports of adverse events when dual blockade is applied broadly to patients with CKD\textsuperscript{215,216}. It thus follows that markers of RAS (and more specifically AngII) activity would be of great clinical utility. Accordingly, markers of RAS in the kidney may guide RAS blockade in patients with CKD.

Direct measurements of AngII in the kidney are problematic because of its short half-life, and its sequestration in different compartments of the kidney. Most
of the studies addressing biomarkers of RAS activity have thus focused on measuring the levels of the substrate angiotensinogen. There is some rationale for doing this. It has been recognized that plasma angiotensinogen levels are poised near the Km for cleavage by renin, with the consequence that alteration of angiotensinogen levels also affects the formation of angiotensin II. Furthermore, it was recently recognized that angiotensinogen conformation could change in response to the redox state, with the oxidized angiotensinogen being more susceptible to cleavage by renin. Several studies have reported increased urinary angiotensinogen excretion in patients with CKD, and in a few studies angiotensinogen excretion correlated with CKD progression. Most of these studies suffer from lack of control for salt intake and hypertension, both of which have been linked to urine angiotensinogen excretion rate. Additionally, most of the studies have been retrospective and thus susceptible to other potential confounders. Finally, demonstration that the liver-derived angiotensinogen may be the primary source of intra-renal AngII, while the angiotensinogen excreted in urine is kidney-derived added additional complexity and uncertainty about the significance of urine angiotensinogen as a marker of RAS activity. Taken together, the utility of urine angiotensinogen excretion as a marker of intra-renal RAS activity has not been established.

1.3 Proteomics-based strategies for characterization of cellular proteomes

1.3.1. Basics of mass spectrometry
Since its inception, almost 30 years ago, the goal of mass spectrometry (MS) has been to characterize all proteins. In principle, MS measures the mass-to-charge ratio (m/z) of gas phase ions. Rather than measuring the actual molecular mass of a compound, MS measures individual ions and the true unit of this measurement is kg per Coulomb. Each MS instrument consists of three main components: an ionization source, a mass analyzer and a detector. The ionization source converts analyte molecules into gas phase ions. Ionization techniques that allow almost any non-volatile and thermally labile compound to be converted into a gas phase include electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). Development of these two ionization techniques was awarded with the Nobel Prize in Chemistry in 2002. Another technique for ionization is called atmospheric pressure chemical ionization.

ESI generates ions by passing a sample in solution through a small capillary that is at a potential difference relative to a counter electrode at voltages between +500 and +4500V. Such passage of solvent generates an aerosol of charged droplets consisting of solvent and analyte molecules. The charge of the mixture can be positive or negative depending on the polarity of the applied voltage. Ions ultimately become free of the solvent and make their way into the mass analyzer of the MS. The charged ions are mainly generated by protonation or deprotonation. Most molecules with acid/base properties have several sites of protonation/deprotonation. This results in a predictable spectrum envelope of an analyte, and the ability to create molecules with lower m/z values compared to the
singly charged ions. The advantages of ESI include: 1) ability to ionize molecules of any size; 2) ability to preserve non-covalent bonds ("soft" ionization technique) thus allowing protein-protein complexes, protein-DNA, protein-drug complexes to be studied; 3) ability to couple MS and liquid separation techniques. However, ESI has two shortcomings. First, the sample is continually consumed, while the mass analyzers do not work continuously thus wasting part of the sample. Second, ESI is susceptible to ion suppression effects by either high salt concentration in the sample or by more highly abundant ions in the mixture. ESI-based MS has the ability to analyze higher molecular weight molecules compared to MALDI, which creates only singly protonated ions.

Ions in MALDI are produced by pulsed-laser irradiation of the sample. The sample is not liquid, but solid and co-crystallized with matrix that can absorb the wavelength of light emitted by the laser. Here too, gas phase ions are formed and travel toward the analyzer. As mentioned, only singly protonated ions are formed thus limiting the size of molecules that can be analyzed by this technique. The advantages of MALDI rest on its high sensitivity (providing data from sub-femtomole amounts of sample), formation of ions as discrete event (so there is no wastage of sample), and high tolerance to salts and buffers (which enables direct analyses of tissues and cells). The main disadvantages include the fact that only some MS analyzers can be coupled to it, and that it has low accuracy at m/z ratios below 500 Da. Some additional concerns have been raised, including poor reproducibility and sensitivity to sample processing techniques\textsuperscript{224}. A variant of MALDI is called surface-enhanced laser desorption-ionization (SELDI), and it has
similar features as MALDI.

Once ions are formed, they are analyzed by the mass analyzer. Analyzers can be categorized based on their accuracy and precision. The precision of the measurement is analogous to resolution. There are two general types of analyzers: beams and traps. In beam analyzers, ions form a beam and pass through the analyzing field to the detector. In trapping analyzers, ions are trapped in the analyzer after being formed or injected there. There are further several types of mass analyzers which include: 1) time-of-flight (TOF), 2) quadrupole (Q), 3) ion traps (linear ion trap, LTQ or quadrupole ion trap (IT)), 4) Fourier-transform ion cyclotron resonance (FTICR), and 5) Orbitrap. 1) TOF is a prototypical beam analyzer. It separates ions based on velocity. The velocity of an ion is inversely proportional to the square root of its m/z. That means that smaller and more highly charged ions will travel faster to the detector, and that m/z can be determined by measuring the time it takes to hit the detector. TOF is ideally paired with MALDI because of the requirement of pulsed source of ions. 2) Quadrupoles separate ions as a result of their motion in a dynamic electric field, which is dependent directly on the m/z of the ions. Mass analysis is a function of voltages applied to four rods of this analyzer, rather than kinetic energy, as in TOF analyzers. Quadrupole instruments are ideally suited for quantification. 3) While quadrupoles have electric fields in two dimensions, ion traps have the electric field in three dimensions. The ions become unstable by increasing the voltage in ion traps in order to obtain the mass spectrum. Ion traps have accuracy and resolution comparable to quadrupoles, but their sensitivity is somewhat better at higher m/z.
ratios. 4) FTICR uses a magnetic field to determine the m/z of an ion. The cyclotron frequencies of the ions trapped in FTICR are measured and converted to m/z. This analyzer is characterized by a high degree of accuracy and very high resolution, but at the expense of throughput. 5) Orbitrap is the newest type of mass analyzer, in which the ions are trapped and orbit around spindle-like electrode and oscillate harmonically along its axis with a frequency characteristic of their m/z ratios. Orbitrap has mass accuracy and resolution capabilities similar to FTICR. Hybrid instruments have been designed in order to capture the strengths and capabilities of different analyzers. They include Q-Q-Q, Q-Q-LTQ, Q-TOF, TOF-TOF, LTQ-FTICR, and finally LTQ-Orbitrap. LTQ-Orbitrap hybrid has become particularly popular because it combines robustness, sensitivity and MS/MS capability of the LTQ with the very high mass accuracy and high-resolution capabilities of Orbitrap. Furthermore, this instrument has demonstrated good dynamic range (the range over which ion signal is linear with analyte concentration), allowing improvements in the number and quality of the peptide measurements. LTQ-Orbitrap has also become the ideal instrument to use for unbiased "shot-gun" proteomics, quantitative shot-gun proteomics, and even top-down proteomics.

The next phase of ion analysis typically involves tandem mass spectrometry (MS/MS). In the first MS phase, desired ions are isolated (these are called precursor or parent ions). These ions are then fragmented in a predictable fashion into fragments that are termed product ions. The most popular method for fragmentation is called collision induced dissociation (CID), which results in
precursor peptide backbone fragmentation along C-N bond by colliding it with an inert gas. This breakage results in the formation of –b and –y ions. Two other newer methods of fragmentation include electron capture dissociation (ECD) and electron transfer dissociation (ETD), which result in backbone fragmentation at the N-Cα bond. These methods result in the formation of –c and –z ions. The tandem mass spectra generated through these processes are further searched by different algorithms which allow peptide identification.

There are two main approaches used in peptide identification: de novo sequencing and database searching. De novo sequencing is an older sequencing method applied when there is no knowledge of sample at hand. This method depends critically on the quality of the data generated. The most commonly used identification method nowadays is database searching. The reason for the success of this approach is that only an infinitesimal fraction of the possible peptide amino acid sequences actually occur in nature. A peptide fragment spectrum might not have all the information to unambiguously derive the entire amino acid sequence, but it might have enough information to match it to a peptide sequence in database based on observed and expected fragment patterns. Several searching algorithms have been developed for MS/MS spectral data and these include: Mascot, Sequest, PeptideSearch, X!Tandem, and Andromeda. The list of proteins generated is typically accompanied by a score based on the peptide number and confidence in identified peptides.

1.3.2. Fractionation methods for proteomics analyses of complex samples
The most commonly used method for analysis of proteins in complex samples like cellular lysates, biological tissues, or biofluids is “bottom-up” proteomics approach. This approach refers to the characterization of proteins by analysis of peptides released from proteins after proteolysis. Shot-gun bottom-up proteomics is bottom-up proteomics performed on a mixture of proteins with the goal of characterizing as many proteins in the mixture. In a typical shot-gun proteomic experiment, proteins are cleaved by an enzyme such as trypsin (which cuts behind each arginine and lysine) into peptides. The peptide mixture obtained is extensively fractionated in order to resolve as many peptides as possible and to improve identification of low-abundance proteins. There are multiple fractionation approaches that have been used.

The most frequently employed methods for fractionation are gel electrophoresis, capillary electrophoresis (CE) and liquid chromatography (LC). Gel electrophoresis can be one-dimensional (1DE), two-dimensional (2DE), or two-dimensional fluorescence difference gel electrophoresis (2DE-DIGE). 1DE separates proteins based on mass, CE separates them based on isoelectric point, and 2DE separates them in two dimensions using both mass and isoelectric point. The resolution of 2DE is poor, and this technique has a narrow dynamic range. CE has a much higher resolution, being able to resolve 400–2,000 peptides per run. CE can be coupled to the MS and has been used extensively in proteomic studies of renal disease. Unfortunately, this technique misses small peptides. A variation of two-dimensional separation has been developed which is called two-
dimensional fluorescence difference gel electrophoresis (2DE-DIGE) and which relies on fluorescent dyes for more accurate quantification. Liquid chromatography can be used multi-dimensionally, and two-dimensional LC has gained popularity due to a number of advantages including its resolving power. In this method, the peptides are separated by chromatography, based on a property such as charge, followed by another chromatographic separation based on another property such as hydrophobicity. This powerful technique demonstrates higher reproducibility and excellent fractionation, but it requires more time compared to 2DE. Two-dimensional LC coupled to MS/MS is termed multi-dimensional protein identification technology (MudPIT). This is the type of approach that was employed in this study of PTEC proteome.

1.3.3. Approaches to characterization of cellular proteomes

Given the complexity of analyzing biofluids, and the difficulty in identifying the low-abundance proteins which have the most potential as biomarkers, researchers have turned to more simplified systems which reflect the disease process of interest. The important techniques that have been described include: tissue proteomics, proximal fluid proteomics (which in case of kidney would be urine or interstitial fluid), cyst or biopsy-based proteomics, and finally cell proteomics. In particular, studies in Oncology have pioneered several cell-based biomarker
discovery approaches. These approaches take advantage of immortalized cell lines or cells isolated from patients and expanded in vitro. Some notable advantages of cell-based approaches include the specificity of the disease/tissue studied without contamination by neighbouring or distant tissue proteomes. Confounding by comorbidities, medications, sex, or age is eliminated (in case of immortalized cells) or minimized (in case of primary cells). Finally, the wide dynamic range of protein concentrations in blood or urine is reduced. These advantages come at the expense of some shortcomings. The main shortcomings include the in vitro nature of these studies, which may be too simplified to be relevant in vivo. Immortalized cell lines have dramatically altered metabolic machinery compared to primary cells or normal tissues. On the other hand, primary cells are difficult to grow and can only be passaged a limited number of times in culture. Nonetheless, these approaches have expanded to numerous diseases and have yielded analytes with great potential.

Most of the current biomarkers are secretory proteins, and numerous studies have examined cellular secretomes in search for novel biomarkers. In one such study a shotgun proteomics approach was applied to identify novel markers of breast cancer from conditioned media of three cancer cell lines. Over 1,100 proteins were identified using LC-MS/MS approach and LTQ analyzer. Spectral counting was used for quantification. One third of proteins discovered were extracellular based on Gene Ontology (GO). Several proteins previously linked to breast cancer were identified. Numerous proteins not previously linked to breast cancer were also discovered. Another ambitious study analyzed conditioned
media (secretome) of 6 pancreatic cell lines and compared it to a normal pancreatic cell line\textsuperscript{226}. In addition, the secretome was compared to pancreatic juice proteome of pancreatic cancer patients. LC-MS/MS approach and LTQ-Orbitrap mass spectrometer was used. Over 3000 proteins were identified and the top 5 candidate proteins were validated in plasma of pancreatic cancer patients. These 5 proteins (AGR2, OLFM4, SYCN, COL6A1, and PIGR) further demonstrated superior performance on the receiver operator curve (ROC) to CA19.9, the traditional marker of pancreatic cancer. These markers await further validation in larger studies as potential candidates for early detection of pancreatic cancer. A similar study from the same group studied proteins involved in lung cancer. Four different cell lines representing non-small cell lung cancer and small cell lung cancer were mined using a similar approach\textsuperscript{227}. Secretome analysis revealed >1,800 non-redundant proteins. By comparing these data to other studies, and looking for the most biologically plausible markers with some specificity to lung tissue, and presence in serum, the list of candidates was reduced to five, and included: ADAM-17, osteoprotegerin, pentraxin 3, follistatin, and tumor necrosis factor receptor superfamily member 1A. These top candidates were validated in sera of patients with lung cancer, as they differentiated cancer patients from normal controls. The data collected are still being mined for markers associated with lung cancer.

More recent studies of cellular secretomes have pushed the boundaries further with novel protein labeling technique to allow accurate quantification of the entire cellular proteomes – stable isotope labeling of amino acids in cell culture.
SILAC is a method of labeling amino acids such as arginine and lysine with heavy non-radioactive isotopes. This technique has markedly improved the ability to quantify proteomes in cell culture, and it will be discussed in detail in the following section. With the ability to more accurately quantify proteins and the emergence of high resolution, high-accuracy MS instruments, the cellular secretome studies gained even more power and popularity. For example, one of the studies aimed to discover improved markers of trisomy 21, and it examined SILAC-labeled amniocytes from women with fetuses that had trisomy 21 as well as women with chromosomally normal fetuses.\textsuperscript{228} Proteomes of both amniocyte supernatants and cell lysates were examined by LC-MS/MS on LTQ-Orbitrap. Close to 5000 proteins were identified and quantified. This deep proteome analysis allowed examination of representative pathways, which enhanced our knowledge of pathophysiology of trisomy 21. Finally, nine proteins were verified by SRM in independent amniocytes, and two of them (SOD1 and NES) showed consistent differential regulation between chromosomally normal and trisomy 21 samples.

An elegant study by Barderas and colleagues examined the secretome of colorectal cancer cells in search for markers as well as mechanisms of colorectal cancer metastasis.\textsuperscript{229} They utilized SILAC-labeling to quantitatively compare secretomes of a highly metastatic colorectal cancer cell line and a non-metastatic cell line. Analyses were carried out on LTQ-Orbitrap Velos, and the authors used stringent criteria to exclude false positives. They were able to quantify >1000 proteins, and considered 155 to be differentially regulated between metastatic and
non-metastatic cell lines. Although they demonstrated that three of the differentially regulated proteins (GDF15, S100A8/A9, and SERPINI1) measured in sera discriminated between colorectal cancer patients and normal controls, further validation in patients with colorectal cancer metastasis and those with localized colorectal cancer was not pursued. Nonetheless, mechanistic insights were gained by using siRNA and blocking antibodies to delineate the proteins most responsible for migration and colonization capacity. Furthermore, a panel of six proteins demonstrated correlation with tissue gene expression and patient outcome. There are numerous other secretome studies, and many of them have led to insights into the potential mechanisms as well as biomarkers of disease.

With improved quantitative methods and increased resolution and accuracy of MS instruments, the ability to analyze cellular lysates for low-abundance proteins involved in disease pathophysiology has improved. If the interest of a study lies primarily in the discovery of potential biomarkers that will be subsequently monitored in biofluids, secretome analysis will yield a significantly higher proportion of secreted proteins (30-40% compared to ∼5% from lysate analysis). Both classical and non-classical pathways of secretion tend to be represented in the secretome. However, this increase in the proportion of secretory proteins comes at the expense of several fold fewer quantified and identified proteins, and an incomplete picture of the intracellular processes. There are numerous examples of cell lysate proteomic analyses with the goal of finding novel biomarkers or pathophysiologic insights into a disease process. The advantages of whole cell lysate versus secretome is illustrated in a recent study.
that uncovered a potentially novel mechanism of prostate cancer progression to androgen-independent state\textsuperscript{230}. Using SILAC-labeling of prostate cancer androgen-independent and androgen-dependent cell lines and LC-MS/MS on LTQ-Orbitrap, the authors quantified >3000 proteins and determined 88 to be differentially regulated between the two cell lines. One of the candidates, HMGCS2 stood out based on differential regulation in cell lysates, and it was found to be involved in ketogenesis. Exploration of other proteins in the ketogenesis pathway led to discovery of additional biomarkers of advanced prostate cancer, as validated in human tissue samples at protein and mRNA level. Since HMGCS2 is a mitochondrial enzyme with no signal sequence and unlikely to be secreted, the role of ketogenic pathway would likely not have been identified by performing secretome analysis. Cell lysate proteomic analysis thus enables the most complete examination of pathophysiological processes that affect protein expression levels.

1.3.4. Quantitative proteomics techniques

MS as a technique is not quantitative. Thus in order to accurately assess the amount of one or hundreds and even thousands of proteins present in a complex sample, several ways of quantifying the proteome had to be developed. In general, quantitative proteomics techniques can be divided into label-free and those with labeling. Labeling techniques are more accurate, but also more expensive, and require powerful instruments with excellent resolution and
expertise to process the complex data. Label-free quantitative methods will be discussed first.

The label-free quantitative proteomics methods can be further subdivided into spectral counting and measurement of the signal intensity (also referred to as the area under the curve). Spectral counting literally means counting the MS/MS peptide spectra. This method is based on the premise that peptides which are more abundant will be sequenced more often and will generate a larger number of MS/MS spectra. By extrapolation, proteins containing those more abundant peptides will be also more abundant. Spectral counting is more accurate for quantification of abundant proteins and when used with MS instruments that have high resolving power. Spectral counting has evolved from summing spectra to adjusting this number by normalization factor(s). An estimate of the protein’s abundance can be calculated using the protein abundance index (PAI). PAI adjusts the number of observed peptides in an experiment by the number of observable tryptic peptides for each protein. In this way, spectral count is adjusted for the protein length (as longer proteins will have more tryptic peptides that could be sequenced). PAI was later modified to the exponential form of PAI minus 1 (emPAI), which is directly proportional to the abundance of protein in the sample. emPAI has shown high correlation with the actual protein amount, and it is simple to implement with high robustness. The problem with spectral counting is that different peptides have different physicochemical properties, which affect their ionization ability and introduce bias into the MS measurements. To adjust for this, another index has been introduced called absolute protein expression
APEX is a modified form of spectral counting which takes into account the number of observed peptide spectra for a protein adjusted for the probability of peptide to be observed by the MS instrument. The probability is calculated by a machine learning algorithm and is based on the peptide length and amino acid composition. APEX has been validated in biological experiments, and is accurate over 3-4 orders of magnitude of protein concentrations\textsuperscript{232}.

The second type of label-free quantitative proteomics is based on measurement of the signal intensity. This is an accurate approach based on measurement of the chromatographic peak area (also referred to as extracted ion chromatograms or XIC) of each peptide in LC-MS runs. This method has demonstrated excellent linear correlation with the actual concentration of peptide in complex mixtures at concentration ranges of 10fmol – 1000pmol. This method is based on the measurement of ion abundances at specific retention times for ionized peptides without the use of spiked in standard. The peptides are quantified at the MS\textsuperscript{1} level, and their identity is confirmed at the MS/MS level. Although conceptually simple, this technique depends on instrument resolution, biological and technical variability between samples. Other factors that need to be considered are retention, background noise, and the availability of powerful software packages to “clean-up” the noise. Two improvements to the simple signal intensity measurement include Top3 and intensity-based absolute quantification (iBAQ). Top3 is based on the XICs of the three most abundant peptides of a protein. iBAQ is a novel label free method implemented as part of MaxQuant software, that was demonstrated to have excellent correlation with the
actual protein amount\textsuperscript{233}. Mass spectrometers traditionally operate in data-dependent mode, so that only top 6–10 precursor peaks are subsequently fragmented by CID. Novel data-independent mode of MS runs, whereby all precursor ions are fragmented promises to be the most accurate method for label-free quantification using signal intensities. This approach allows excellent proteome coverage and does not compromise precise quantification. In general, label-free methods cannot accurately estimate amounts of low-abundance proteins, but they are easy to implement, cost little, and are relatively robust when it comes to medium and high-abundance proteins.

The labeling proteomic techniques consist of chemical labeling, metabolic labeling and spiked-in standards. The first reported chemical labeling approach was isotope-coded affinity tag (ICAT)\textsuperscript{234}. ICAT contained a thiol-reactive group, a linker region and a biotin tag. It came in two flavours – heavy (+8Da) or light. This tag is added at the protein level and it reacts with the sulphydryl group of cysteine residues. The biotin tag could then be used to enrich for the labeled, quantifiable peptides. Relative quantification is performed based on the mass shift between heavy and light labeled peptides at the MS\textsuperscript{1} level. The problem with this technique is incomplete labeling, and exposure of only a fraction of the proteome to quantification due to the absence of cysteine residues from a large fraction of proteins.

Currently the most popular chemical labeling methods include isobaric tags for relative and absolute quantification (iTRAQ)\textsuperscript{235} and tandem mass tags (TMT)\textsuperscript{236}. Both of these methods target N-terminal amines and ε-amines of the
lysine residues, and are isobaric tags (equal mass). These tags consist of a reporter group (with mass of 114 – 117 Da), a balancer group (28-31 Da), and an amine-reactive group. All isobaric tags would be balanced so that they have identical mass (145Da) and so the retention time of peptides with different labels would not change. Upon CID, these tags would fragment in a similar fashion to peptides, so that the balancer group is lost and the reporter group is charged and can be detected. The actual quantification is based on the cleaved reported ion which is found in the low MS/MS spectrum that does not interfere with peptide identification. The relative intensity of the peptides is deduced from the ratio of the intensities of their reported ions. iTRAQ allows quantification of 4 to 8 samples simultaneously, while TMT allows 2 to 6. The problems with this approach include incomplete labeling, labeling at the level of peptide, and interference with the reporter ion and contaminants. The newer adaptation of iTRAQ is mTRAQ, which incorporates isotopic labeling at the level of the peptide in addition to isobaric tags.

The chemical labeling methods have paved the way to metabolic labeling. There are several types of metabolic labeling which include $^{15}$N and SILAC. SILAC is a metabolic labeling method for use in cell culture or even in complete model organisms. It is based on incorporation of a non-radioactive metabolically labeled essential amino acids into entire proteomes. These amino acids typically include arginine and lysine (although others such as leucine have been used) and they contain “light” labels (i.e. the ones found in nature, such as $^{12}$C and $^{14}$N) or “heavy” labels (i.e. $^{13}$C and/or $^{15}$N). In principal, the two cell
populations are grown in the light or heavy medium for 5 doubling times, at which point their proteomes are close to 100% labeled. The cell lysates can then be mixed and analyzed together from the first step of protein isolation, without any additional steps required as in chemical labeling. This allows minimization of errors introduced by fractionation and further analysis. Relative quantification is performed in MS$^1$, since the same peptides labeled with distinct label cause a predictable shift in mass. Quantification is based on signal intensity. Two to three different conditions can be compared at the same time, but multiple conditions increase the complexity of data analysis. This method offers extreme labeling efficiency and has become the gold standard for quantifying the entire cellular proteomes. SILAC also has versatility and can be used to study cell turnover (pulsed-SILAC), as well as to relatively quantify protein amounts in human tissues by spiking-in heavy labeled standards (super-SILAC). On the other hand, it is expensive, requires fast, high accuracy and resolution instruments, sophisticated software and expertise in the procedures.

The final method of quantification includes absolute quantification by spiked-in standards. This method involves spiking in the known amount of stable isotope-labeled reference standards and looking for the co-elution (detection at the same retention time) of the peptides from the sample and their labeled reference standards after MS analysis. One such strategy is called absolute quantification of abundance (AQUA), and it involves purchasing synthesized peptides which contain stable isotope-labeled amino acids and/or tags. These peptides represent the unique (proteotypic) peptides for specific proteins of
interest that need to be quantified in a biological sample. Another strategy that uses the same principle is termed QconCAT. The QconCAT technology uses recombinant DNA techniques to construct synthetic proteins in which large numbers of internal standard peptides are concatenated (or expressed together in the same construct). The synthetic protein can be expressed and purified in stable isotope-labeled form from a suitable host (Escherichia coli). The QconCAT protein is then added to a sample and the reference peptides for the desired proteins are generated in situ during the protease digestion step. This method allows addition of protein at an early step of sample preparation, thus minimizing processing artifacts. Nonetheless, AQUA strategy has become increasingly popular because of the ability to pair it with SRM, a targeted quantitative proteomic approach. SRM is a method typically performed on a triple quadrupole (Q-Q-Q) instrument which can be used for relative or absolute quantification. The principle of this method is based on spiking known amounts of proteotypic AQUA peptides into a complex mixture of interest. The peptides of interest are then monitored by the Q-Q-Q instrument. The peptides of interest will co-elute with their heavy peptides, thus allowing both confident identification and quantification of these peptides. Assumption is made that the amount of measured proteotypic peptide represents the amount of the protein of interest. With state-of-the-art SRM assays, up to 100 peptides representing 100 medium-to-high abundance proteins (10ng/mL – 1mg/mL) can be measured simultaneously in the unfractionated digest of proteins, while achieving coefficients of variation (CV) under 20%. This technique is becoming ideal for hypothesis-driven investigations where numerous proteins
require quantification in a short time, and without the need for developing protein-specific antibodies.

1.3.5. Limitations and challenges of current proteomics studies

There are several limitations of proteomics-based approaches. First, given the sensitivity of the proteome to a variety of factors, design of proteomic studies is critically important. For example, it is extremely difficult to define a homogeneous patient population for a study. Even small variations in patient selection, such as medications or age, may result in proteome differences that do not reflect the disease state, thus making it difficult to arrive at general conclusions or translate candidate biomarkers into clinical practice\textsuperscript{238}. The same rule applies to \textit{in vitro} studies – the selection of experimental conditions and cell types used should be such that the differences between the states examined should only reflect the question at hand (e.g. the drug or hormone treatment). Additionally, samples should be processed in such a way to minimize variability between the analyses of cases and controls.

Apart from issue of experimental design and uniformity, shot-gun proteomic studies are susceptible to false positive and false negative findings. The false positives should be addressed by keeping the false discovery rate (FDR) low, by generating multiple biological replicates, and performing verification and validation of results, whenever possible. The false negatives can be addressed by exhaustive fractionation, by the use of high resolution and high accuracy
instruments, as well as adequate software for data analysis (including searches performed against the most complete and current databases), and by having longer gradients on the instrument for peptide elution, and at least 2 injections of the same sample on the MS instrument. Another major shortcoming is the use of data-dependent analysis mode, in which the MS selects the top peaks for further analysis. This approach will limit the discovery of low abundance proteins. Although not yet routinely implemented, the use of data-independent mode where all precursor peaks are fragmented will improve coverage of low-abundance proteins. Other technical shortcomings include differential ionization capacity of distinct peptides, so that some peptides will never be sequenced by the MS instrument. Finally, when it comes to protein labeling and quantification, the typical problems may include incomplete labeling, errors introduced during sample processing (these are minimized in SILAC experiments), and introduction of contaminants (such as bovine serum in supernatant analysis, but could be human hair, skin proteins, saliva etc.). The key to reduce all these problems is to have a solid experimental design, to follow a consistent protocol, to perform multiple replicates and to subject samples to extensive fractionation and use the most robust instruments and software.

1.5. Systems biology approaches

1.5.1 Definition of systems biology
A proposed definition for systems biology is the study that examines how non-linear interactions between the components lead to functional properties of the system that are not present in the components themselves\(^{239,240}\). For example, if biology studies the functioning of an organism such as a human, systems biology would aim to explain how much of that function comes about from the interactions between the various organs in human. Because it is determined by layers of distinct components, each of which has a direct or indirect effect on another, systems biology must be complex, and can only be simplified to a degree. To study systems biology, both *in vitro* and *in vivo* studies are necessary, as the components must be discovered in the simplified *in vitro* system, while their contribution to function can only be assessed *in vivo*\(^{241}\).

Why should one study systems biology? Over the last century the dominating approaches in science have been reductionist. These approaches have indeed allowed us to understand the structure and function of a variety of proteins, and have allowed us to identify potential players in various diseases. However, these approaches have not helped achieve mastery in understanding human physiology or pathophysiology. Furthermore, most human diseases are not easily explained by dysfunction in single proteins, but are rather a result of non-linear interactions between multiple proteins. Even monogenic diseases (caused by a mutation in a single gene) have polygenic consequences\(^{242}\). It follows that there is a need to integrate information in order to gain insight into the fundamental processes that govern disease states or outcomes.

The initial studies in systems biology were termed “data-poor”\(^{243}\). In these
approaches, mathematical modeling was used to represent the energy or mass balance, and various laws governing the movement of substances and driving forces behind these movements. There were multiple input parameters monitored, and the process was labour intensive, but ultimately led to testable hypotheses.\(^\text{242}\) The advent of high-throughput technologies, and the completion of genome sequencing for multiple organisms have led to “data-rich” systems biology studies. The large sources of data needed to be integrated in some way, giving rise to the field of bioinformatics. Complete genome sequencing resulted in the final number of possibilities for any molecular hypothesis. Additionally, the advent of other technologies such as mass spectrometry for the study of proteome, and microarray-based analysis has allowed researchers to combine granular, multi-layered data from their own or previously conducted experiments. Systems biology approaches thus aim to address more holistic perspective, unlike the reductionist approaches represented in traditional biomedical research.

1.5.2. Bioinformatics approaches to understanding the networks in a cell or tissue

The aim of a systems biology approach is to decipher the fundamental biological processes, pathways and interactions that result in certain function or outcome. Large-scale data such as proteomics or gene expression data can be represented and integrated using networks. Networks represent relationships (physical interactions, genetic interactions, common process etc.). The purpose of these
networks is to allow us to group, understand, integrate and visualize high-throughput data. Networks are well-defined mathematical objects. Each node in a network typically represents a protein (although node can also represent a gene, or a Gene Ontology (GO) term). The size or colour of the node could be informative, for example represent the cellular location or the strength of evidence in support of the protein in question etc. Nodes are connected with edges which typically represent interactions between the proteins, although they could represent some shared attribute. The thickness and the colour of edges have meanings. For example, the thickness of the edge could relate to robustness of data supporting the interaction between two proteins, while the colour could indicate the experiment the data is derived from, the type of interaction etc. Networks are thus mathematically derived means of interpreting, integrating or visualizing high-throughput data.

Integration of data and creation of networks is relevant and can add information in several different ways. First, network analysis can simplify and visually clarify relationships between different proteins. For example, it can group interacting proteins according to the biological processes that they mediate, or the pathways to which they belong. Second, the analysis patterns achieved are unbiased and can lead to generation of novel hypotheses based on the observed relationships\textsuperscript{244}. Third, by combining different data layers, such as for example, protein-protein and protein-DNA interactions, we can begin to understand the control mechanisms fundamental to the observed changes\textsuperscript{245}. For example, transcription factors important in the regulation of groups of genes can be
elucidated. Similarly, expression data from different laboratories or from analogous models could be combined or overlaid. Those regions in the network that overlap between different experimental sets are more likely to be correct and thus biologically relevant. Networks can thus also serve to validate the results of an experiment. An elegant example of this is a study by Merico and colleagues\textsuperscript{246}, who utilized a newly developed tool called the Enrichment Map (EM), that allowed visualization of non-redundant gene sets enriched in patients with colon cancer, and overlaid them with known colon cancer genes from DiseaseHub database, which integrates genotype-phenotype information. This led to an understanding of the fundamental and functionally important gene-sets common to both data sets. Finally, protein function could be elucidated from network data. One can infer that the function of an unannotated protein may be similar to that of its neighbours. Similarly, a protein in the biological process mediating for example, CKD progression, could be further studied in its relationship to CKD progression, even if it had not previously been linked to CKD.

While these types of network analyses could be extremely useful and lead to novel hypotheses and observations, there are caveats that must be kept in mind. GO is a large Consortium intended to standardize names of genes/gene products so that previously generated data can be more easily used to ask novel questions. There are three types of ontologies: biological process, molecular function, and cell component. In addition, ontologies are further systematically annotated. Ontologies are represented as directed acyclic graphs, which means that the most distal nodes are the most specific, and that a certain gene
automatically inherits parent nodes, once it is assigned to a distal node. Gene or protein interactions are not represented in GO. The evidence for annotation can come from various sources. It could be experimentally derived, computationally derived, or other. This is important, since it implies that the evidence behind all annotations is not the same. The analysis of enriched GO terms in a given experimental set of genes is devoid of context, and may not be true in all tissues, cells, or diseases. Furthermore, such enrichment analysis does not take into account the actual magnitudes or directions of changes in gene expression, but rather only the number of genes that are annotated to a certain GO term. Only certain statistical tests are considered appropriate for testing for significance of over- or under-representation of a certain term, and these typically include hypergeometric test, chi-squared, and binomial test (although the latter does not take into account how the probability changes when a gene is selected without replacement from a gene set). Adjustment for multiple hypothesis testing is mandatory, and precludes analyses that include the entire experimental sets, but rather demands a pre-selection of significant genes. The reference set has to be carefully selected. Finally, the results of an enrichment analysis are hypothesis-generating, and can be used to support certain observations, but cannot serve as de facto proof of a concept. These principles apply to any enrichment or pathway analysis.

There are multiple software packages available today that can be used to generate networks. One such popular software is called Cytoscape. Cytoscape is an open access software with extensive capabilities\textsuperscript{245}. It allows analysis,
modeling and visualization of molecular and genetic data. It can be used to analyze datasets available from literature or user defined data sets. It can perform statistical network analysis. Additionally, it is excellent for performing GO or pathway enrichment analysis, and it has extended functionality through freely available modules called “plugins”. Cytoscape has been widely cited and used in high-impact publications. It has recently been used to demonstrate the profound effect on proteins interacting at the slit diaphragm between podocytes in culture and podocytes during development\textsuperscript{247}. Another powerful algorithm implemented in Cytoscape predicted impact of single or multiple gene knockouts \textit{in silico}, by combining transcriptome and ChIP-seq data\textsuperscript{248}. Another software for analyzing pathways and networks is Ingenuity Pathway Analysis (IPA) (Ingenuity® Systems, \url{www.ingenuity.com}). This is commercially available software that is not as powerful as Cytoscape but that has some advantages. IPA is extremely user-friendly, which makes it attractive to use. Additionally, when searching for canonical pathways over-represented among the genes/proteins of interest, it can provide a simplified and intuitive picture of significantly overrepresented relationships. IPA uses entirely different algorithms to search for enriched pathways or interacting proteins. The IPA knowledge base is curated and includes data from publically available databases, but also includes studies from medical literature that both confirm or refute particular interactions or protein/gene presence in a pathway. Standard statistical analyses are performed for both pathways and networks obtained from IPA, in order to assess the significance of the interaction patterns. As will be described later, I utilized systems biology
approaches to both define the effects of AngII on biological processes and to link my findings to *in vivo* datasets.

1.5 **Objectives of the present study**

1.5.1. **Rationale**

CKD leads to major morbidity and mortality, and over 1.4 million people worldwide require renal replacement therapy as a result of ESRD. Moreover, renal replacement therapy is extremely costly.

RAS has been implicated in the progression of CKD, but is there evidence behind RAS activity in the kidney? There is abundant evidence pointing to the existence of intra-renal RAS, as well as its involvement in the progression of CKD. Irrefutable evidence for the existence of intra-renal RAS came from the studies that demonstrated close to 1000 times higher AngII in the kidney compared to systemic AngII. Furthermore, the level of AngII in the kidney correlated with development of hypertension in animal models. AngII is the main effector of RAS. Several cell types in the kidney, including PTECs, mesangial cells, and collecting duct cells contain the substrate angiotensinogen and the enzymes renin and ACE which are necessary to cleave the substrate in order to generate AngII. AngII exerts multiple adverse hemodynamic and non-hemodynamic effects on renal cells that are mediated by the type 1 angiotensin II receptor (AT-1R). The hemodynamic effects include: systemic vasoconstriction and hypertension,
increase in glomerular arteriolar resistance, increase in glomerular capillary pressure and permeability, reduction in medullary blood flow. Some of the non-hemodynamic effects include hypertrophy, inflammation, and renal fibrosis. It has been demonstrated that intra-renal AngII may be more important for progression of renal injury than systemic AngII\textsuperscript{249-252}. Finally, a recent study demonstrated that intra-renal ACE was critical for the development of hypertension even in face of systemic AngII infusion\textsuperscript{13}. It follows that understanding of intra-renal AngII activity may represent the ideal window into the activity of RAS and the risk of CKD progression. However, AngII has a short half-life, and is difficult and impractical to measure at a tissue level. How could we then obtain a measure of the bioactivity of AngII, and understand the fundamentally perturbed processes occurring as a result of heightened AngII activity?

There is evidence that both diabetic and non-diabetic CKDs result in activation of the RAS\textsuperscript{174,175}. The mainstay of current CKD therapy focuses on blockade of the RAS, which has been demonstrated to slow down CKD progression\textsuperscript{176-179}. However, apart from reducing blood pressure to target levels, there is no specific guideline for the extent of RAS blockade in patients with CKD. This is problematic because some clinical studies suggest that more aggressive or dual RAS blockade may be beneficial in proteinuric CKD, independent of blood pressure\textsuperscript{212-214}, even though the choice of agents and dosing remain uncertain. Recently, enthusiasm for dual RAS blockade has been tempered by reports of adverse events when dual blockade is applied broadly to patients with CKD\textsuperscript{215,216}. Currently there is no specific measure of RAS bioactivity in the kidney.
Although several renal cell types contain complete RAS, PTECs appear to be the main sites of intra-renal AngII accumulation\textsuperscript{253,254}. The function of PTECs is regulated by both circulating and locally formed AngII\textsuperscript{255,256}. All major components of RAS have been demonstrated in PTECs\textsuperscript{257-260}. AT-1R is highly expressed in PTECs and acts as the main signaling receptor of AngII. Recent \textit{in vivo} studies identified the PTEC as a key site in blood pressure regulation by AngII\textsuperscript{261}. Furthermore, overexpression of angiotensinogen in PTECs of transgenic mice resulted in hypertension and albuminuria. In clinical histopathology, tubular atrophy and interstitial fibrosis are the hallmarks of progression of all CKDs, implicating these cells in the pathophysiology leading to end stage renal disease. Finally, PTECs communicate directly with urine \textit{in vivo} and secrete potentially important proteins that could be detected by examining this biofluid.

The systems biology approaches are attractive as they allow an unbiased examination of the AngII activity in the kidney. Several high-throughput strategies exist, and proteomics emerges as the most promising one for several reasons. Proteins mediate biological functions. There are many more proteins than genes, and distinct protein isoforms or PTMs are not reflected in genomic or transcriptomic studies. Furthermore, the correlations between changes in expression of genes and proteins are modest, at best. Improvements in MS technology in the last decade have allowed accurate and deep examination of complex proteomes, and quantification-based strategies have improved dramatically, further allowing accurate quantification of entire proteomes. Finally, proteins are most amenable to being measured in proximal biofluids such as
urine. Unfortunately, urine proteomic studies to date have been disappointing, and discovered mostly non-specific high-abundance proteins, or defined a number of peptide analytes too large to optimize and monitor routinely in large cohorts of patients. Additionally, these studies have mostly been semi-quantitative in nature. Given the complexity of urine, and many unsolved technical issues pertaining to sample collection and processing, an alternative approach to defining an AngII proteome may be desirable. Many cellular proteomes defined in vitro led to promising cancer biomarkers and novel insights into disease pathophysiology. SILAC technology would allow accurate quantitative analysis of the entire cellular proteome. We thus aimed to define the proteome of AngII activity in kidney cells.

The goal of this work was to study the AngII-regulated proteome of PTEC in order to define the 'AngII signature' (in other words, the list of proteins significantly regulated by AngII). Better understanding of AngII-regulated proteins will help gain insight into the mechanisms of RAS-mediated renal damage, and could further help define novel markers of CKD progression, and responsiveness to RAS blockade.

1.5.2. Hypotheses

Our hypotheses are as follows:

1) In vitro studies of PTECs will define AngII-regulated proteome and yield new insights into biology of RAS;

2) A subset of the AngII-regulated proteome will reflect AngII activity in vivo.
1.5.3. Objectives

To address the above hypotheses, we developed the following objectives:

1) To define an AngII regulated proteome in primary human PTECs in vitro
   • To address this first objective, we decided to use SILAC-based labeling of primary PTECs in culture. Such completely labeled PTECs were then stimulated with AngII, while control PTECs were unstimulated.
   • Both supernatant and cell lysates were collected, and subjected to alkylation, reduction and trypsin digestion followed by extensive fractionation by strong cation exchange liquid chromatography followed by LC-MS/MS on LTQ-Orbitrap mass spectrometer.
   • Five biological replicates were performed.

2) To verify and confirm AngII-regulated proteins
   • To address the second objective, we used a combination of ELISA and qRT-PCR technology in order to verify up- or downregulation of specific protein/mRNA in response to AngII stimulation in vitro.
   • We also employed targeted proteomic technique called SRM to confirm the differential regulation of specific proteins in PTECs in response to AngII stimulation in vitro.
• The completion of this objective led to a list of proteins confirmed/verified as differentially regulated by AngII in PTECs in vitro.

3) To utilize systems biology approaches to better understand the processes critically important in PTEC responses to AngII

• Differentially regulated AngII proteins were subjected to enrichment analysis using BiNGO plugin of Cytoscape to define those GO terms significantly enriched among these proteins.

• Separate analysis of pathways and protein interaction networks was performed by IPA to define overrepresented pathways and networks among those differentially regulated proteins.

4) To utilize systems biology approaches to validate our findings in an independent experiment from a different laboratory

• Literature searching uncovered a study of mice with AT-1R knock-out, that were treated with AngII infusion and compared to wild type mice also treated with AngII infusion. Kidneys of these mice were subjected to microarray analysis, and those genes differentially regulated between the two groups of mice, following AngII infusion were extracted.

• Taking advantage of the enrichment map plugin of Cytoscape, we examined the overlap between functionally enriched gene-sets from the mouse and from our experiment.
• This allowed us to validate the observation that protein HO-1 was functionally important not only in our PTECs, but also in the mouse kidney response to AngII in vivo.

5) To validate our in vitro approach in an animal model in vivo

• We obtained kidneys of mice with AT-1R knock-out specifically in PTEC, that had been treated with AngII. As control, we obtained wild type mouse kidneys following an identical treatment with AngII.

• We examined by immunohistochemistry whether HO-1 protein was upregulated in PTEC of wild type mice in response to AngII treatment.

• We next examined whether there was differential HO-1 protein expression in the kidneys of mice with AT-1R knock-out compared to wild type mice.

• We then examined whether urine excretion rate of HO-1 in the two mouse groups was different.

• Finally, we examined the correlation between total kidney HO-1 protein expression and urine excretion.
CHAPTER 2:

SILAC-BASED APPROACH TO IDENTIFICATION OF ANGIOTENSIN II-
REGULATED PROTEINS IN HUMAN PRIMARY PROXIMAL TUBULAR CELLS

Sections of this Chapter have been published previously in the Journal of Biological Chemistry:

Ana Konvalinka, Joyce Zhou, Apostolos Dimitromanolakis, Andrei P. Drabovich, Fei Fang, Susan Gurley, Thomas Coffman, Rohan John, Shao-Ling Zhang, Eleftherios P. Diamandis, James W. Scholey
Determination of an Angiotensin II-Regulated Proteome in Primary Human Kidney Cells by Stable Isotope Labeling of Amino Acids in Cell Culture (SILAC)

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2.1. Introduction

AngII is the main effector of RAS. Abundant evidence points to the existence of intra-renal RAS, and its importance in CKD progression. However, the potential markers and the fundamental processes of intra-renal RAS are not known. Although several renal cell types contain complete RAS, PTECs appear to be the main sites of intra-renal AngII accumulation\(^\text{253,254}\). The function of PTECs is regulated by both circulating and locally formed AngII\(^\text{255,256}\). All major components of RAS have been demonstrated in PTECs\(^\text{257-260}\). AT-1R is highly expressed in PTECs and acts as the main signaling receptor of AngII. Recent in vivo studies identified the PTEC as a key site in blood pressure regulation by AngII\(^\text{261}\). Furthermore, overexpression of angiotensinogen in PTECs of transgenic mice resulted in hypertension and albuminuria. In clinical histopathology, tubular atrophy and interstitial fibrosis are the hallmarks of progression of all CKDs, implicating these cells in the pathophysiology leading to end stage renal disease. Finally, PTECs communicate directly with urine in vivo and secrete potentially important proteins that could be detected by examining this biofluid.

We thus aimed to utilize a systems biology approach to define in an unbiased fashion the proteome of AngII-stimulated PTECs. Changes in protein expression in response to AngII may be particularly informative, and SILAC has become the gold standard for quantification of the entire cellular proteomes. SILAC is a metabolic labeling method for use in cell culture\(^\text{262}\) or even in complete
model organisms. It is based on incorporation of a non-radioactive metabolically labeled essential amino acids into entire proteomes. These amino acids typically include arginine and lysine (although others such as leucine have been used) and they contain “light” labels (i.e. the ones found in nature, such as $^{12}$C and $^{14}$N) or “heavy” labels (i.e. $^{13}$C and/or $^{15}$N). In principal, the two cell populations are grown in the light or heavy medium for 5 doubling times, at which point their proteomes are close to 100% labeled. This method offers excellent labeling efficiency. The cell lysates can then be mixed and analyzed together from the first step of protein isolation, without any additional steps required as in chemical labeling. This allows minimization of errors introduced by fractionation and further analysis. Relative quantification is performed in MS, since the same peptides labeled with distinct label cause a predictable shift in mass. Quantification is based on signal intensity.

The aim of this study was to define the AngII-regulated proteome in primary human PTECs. To achieve this, we took primary PTECs at an early passage and separated them into two populations, which were labeled with heavy or light SILAC arginine and lysine for six doubling times. At the end of the labeling, heavy-labeled cells were stimulated with AngII, and light-labeled cells with conditioned media alone. Control experiments and replicates were performed to ensure minimization of false positive hits.
2.2. Materials and Methods

2.2.1. Experimental overview

The experimental scheme is shown in Figure 2.1. Briefly, PTECs at 2nd passage were split into two populations and incubated in heavy (H) or light (L) SILAC media for 6 doubling times. They were then deprived of serum for 18 hours, and subsequently H-labeled cells were stimulated with AngII ($10^{-7}M$) and L-labeled cells with untreated medium for 8 hours. Cell lysates were collected and lysed as described below. The experiment was repeated 4 times. In 3 experiments we added AngII to the H-labeled cells, and in 1 experiment we added AngII to the L-labeled cells (reverse labeling). We performed an additional experiment, in which AngII was added to the H-cells, and we only collected the supernatant. In the final experiment, we labeled cells as H and L, but did not add AngII to either H or L cells for control purposes. In each set of labeling experiments, we determined that AngII led to phosphorylation of extracellular signal-regulated kinase (ERK), as explained below.

2.2.2. SILAC labeling of primary proximal tubular epithelial cells

Primary human renal PTECs were purchased from Lonza Walkersville Inc. (Walkersville, USA). These cells originated from 3 different individuals, from different age groups, both male and female. They were cultured in custom made
Dulbecco’s Modified Eagle Medium (DMEM), free of arginine and lysine (AthenaES), supplemented with 10% v/v dialysed fetal bovine serum (dFBS), 10ng/ml EGF, 5µg/ml transferrin, 5µg/ml insulin, 0.05µM hydrocortisone, 50U/ml penicillin and 50µg/ml streptomycin. Heavy arginine (\(^{13}\)C\(_6\)) and heavy lysine (\(^{13}\)C\(_6\)\(^{15}\)N\(_2\)) were added to DMEM ‘heavy’ bottles used to incubate ‘heavy’ (H) labeled cells, whereas light arginine (\(^{12}\)C\(_6\)) and lysine (\(^{12}\)C\(_6\)\(^{14}\)N\(_2\)) were added to ‘light’ DMEM used to incubate ‘light’ (L) labeled cells, as described previously\(^{262}\). In preparation of the L media, arginine is added at a concentration of 84mg/L and lysine at 146mg/L. H media is prepared with the same amino acid concentrations, but adjusted for their molecular weights (87mg/L of H-arginine and 154mg/L of H lysine). Both media were supplemented with L-proline (Sigma), at the concentration of 150mg/L. Cells were divided into two populations (H and L), and incubated in their respective media for 6 doubling times, and until passage 6. Once labeled, cells were grown in T75 flasks to approximately 80% confluence. They were then serum-deprived for 18 hours, and subjected to 10\(^{-7}\)M (final concentration) AngII or Control (medium alone). Following stimulation, PTECs were incubated for 8 hours. Conditioned medium (supernatant) representing the cellular secretome was collected and stored at -80°C. Cells were then washed 3 times with PBS, harvested with trypsin and snap-frozen at -80°C until further analysis. Cells from preliminary experiments were tested for efficiency of incorporation. All cells were cultured in a humidified incubator at 37 °C and 5% CO\(_2\). All media were freshly made and filtered using 0.22µm syringe filter.
**Figure 2.1.** Experimental scheme. The figure demonstrates a simplified experimental flow including: SILAC labelling, cell treatment, protein digestion, HPLC followed by LC-MS/MS, data analysis by MaxQuant, assignment of AngII/Control protein ratios, selection of differentially regulated proteins, and bioinformatic analyses by Cytoscape and Ingenuity Pathway Analysis. Adapted with permission from Konvalinka et al. *J Biol Chem* 2013; 288: 34: 24834–24847.

2.2.3. **Western blotting for phosphorylated ERK**

A subpopulation of labeled SILAC cells was separated into a 6-well plate with each experiment conducted. These cells were serum-deprived for 18 hours and then stimulated with control media or sequentially with 20uM Losartan for 30 minutes, and/or AngII (10⁻⁷M) for 5 minutes. Western blotting was performed for
pERK and total ERK as previously described\textsuperscript{263}. The primary antibodies against p44/42 ERK and total 44/42 ERK were polyclonal and developed in rabbit (Cell Signaling, USA). The secondary antibody was anti-rabbit antibody developed in goat (Santa Cruz, USA).

2.2.4. Cell lysate and supernatant preparation and fractionation strategies

Cell pellets were thawed on ice and resuspended in 200 µL of 0.2\% w/v acid-labile detergent RapiGest SF (sodium-3-[(2-methyl-2-undecyl-1,3-dioxolan-4-yl)-methoxy]-1-propanesulfonate, Waters, Milford, MA) in 25 mM ammonium bicarbonate, vortexed and sonicated three times for 30 s. All lysates were centrifuged for 20 min at 15,000 rpm at 4°C. Total protein concentration was measured using a Coomassie (Bradford) protein assay reagent (Pierce). H and L samples were mixed in a 1:1 total protein ratio. Proteins in detergent solution were denatured at 60°C, and the disulfide bonds were reduced with 10 mM dithiothreitol. Following reduction, the samples were alkylated with 20 mM iodoacetamide. Samples were then digested overnight at 37°C with sequencing grade modified trypsin (Promega, Madison WI, USA). Trypsin:total protein ratio of 1:50 was used. The supernatants were dialyzed in 1mM ammonium bicarbonate with two buffer exchanges, using a molecular cutoff of 3.5kDa, for 24h. They were subsequently lyophilized and a similar procedure as for the analysis of lysates was followed. After digestion, RapiGest SF detergent was cleaved with trifluoroacetic acid, 1 \% v/v final concentration, and samples were centrifuged at
4,000 rpm. Upon removal of Rapigest, tryptic peptides were diluted to 500 µL with SCX mobile phase A (0.26 M formic acid in 5% v/v acetonitrile; pH 2-3) and loaded directly onto a 500 µL loop connected to a PolySULFOETHYL A™ column (2.1 mm ID × 200 mm, 5 µm, 200 Å, The Nest Group Inc., MA). The SCX chromatography and fractionation were performed on an HPLC system (Agilent 1100) using a 60-min two-step gradient. An elution buffer which contained all components of mobile phase A with the addition of 1 M ammonium formate was introduced at 10 min and increased to 20% at 30 min and then to 100% at 45 min. Fractions were collected every 1 min from the 20 min time point onwards. The resulting 20 fractions (200 uL each) corresponding to chromatographic peaks of eluting peptides were collected.

2.2.5. Tandem mass spectrometry

Peptides in each fraction were identified by LC-MS/MS as previously described. Briefly, peptides were extracted with 10 µL OMIX C18 MB tips (Varian, Lake Forest, CA), eluted in 5µL of 65% v/v acetonitrile, diluted to 85 µL with 0.1% v/v formic acid in pure water, and loaded onto a 3 cm C18 trap column (with an inner diameter of 150 µm; New Objective), packed in-house with 5 µm Pursuit C18 (Varian). Eluted peptides from the trap column were subsequently loaded onto a resolving analytical PicoTip Emitter column, 5 cm in length (with an inner diameter of 75 µm and 8 µm tip, New Objective) and packed in-house with 3 µm Pursuit C18 (Varian, Lake Forest, CA). The trap and analytical columns were operated on
the EASY-nLC system (Thermo Fisher Scientific, San Jose, CA), and this liquid chromatography setup was coupled online to an LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Fisher Scientific, San Jose, CA) using a nano-ESI source (Proxeon Biosystems, Odense, Denmark). Each fraction was run using a 90-min gradient, in duplicates, and analyzed in data dependent mode in which a full MS1 scan acquisition from 450-1450 m/z in the Orbitrap mass analyzer (resolution 60,000) was followed by MS2 scan acquisition of the top six parent ions in the linear ion trap mass analyzer. The following parameters were enabled: monoisotopic precursor selection, charge state screening and dynamic exclusion. In addition, charge states of +1, >4 and unassigned charge states were not subjected to MS2 fragmentation. For protein identification and data analysis, XCalibur software (v. 2.0.5; Thermo Fisher) was utilized to generate RAW files of each MS run.

2.2.6. Data analysis and candidate selection

The resulting raw mass spectra from each pooled fraction were analyzed using Andromeda search engine (MaxQuant software v.1.2.2.2)264, on the non-redundant IPI Human database version 3.62. The raw files from all biological replicates were analyzed simultaneously with MaxQuant. To assess the false positive rate, a reverse hit database was created by MaxQuant. False discovery rate (FDR) of 1% was specified. Up to 2 missed cleaves were allowed and searches were performed with fixed carbamidomethylation of cysteines and variable oxidation of methionine
residues, and N-terminal acetylation. R (+6Da) and K (+8Da) heavy-labels were selected. A fragment tolerance of 0.5 Da and a parent tolerance of 20 Da were used, with trypsin as the digestion enzyme. Re-quantification and matching between runs was selected. Protein was identified with a minimum of one unique peptide. Quantification was performed using unmodified unique and razor peptides, and a minimum of one counted ratio. The median normalized protein ratios of biological replicates were used for further analyses. Perseus software (v.1.2.0.17) was used to calculate Significance A. Benjamini Hochberg correction was applied. Lysate experiments were further analyzed to select differentially regulated proteins, as the supernatant yielded 3-4 times fewer protein identifications. Protein was considered to be significantly differentially regulated if its ratio was significant by Significance A with p<0.01 in ≥2/4 experiments. FDR for proteins calculated with these parameters was 0.0006. This threshold was selected because at this level of significance, and taking into account the total number of proteins (4618), less than 3 proteins should have been found by chance alone. Statistical language R (v.2.13.1) was used for selection of candidates. Proteins that had ratios changing significantly in opposite directions in any of the replicates were eliminated. Additionally, proteins were eliminated if their ratios were above two standard deviations from the mean in the control experiment, where no treatment was applied to either H or L sample. Individual peptide ratios for all differentially regulated candidates were examined manually. If ratios of proteotypic (unique) peptides of the same protein were changing significantly in opposite directions, or if posterior error probability of peptides was >0.05, these proteins were not pursued.
The final list for development of SRM methods included 51 proteins.

The mass spectrometry SILAC data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with the dataset identifier PXD000183.

2.3. Results

2.3.1. Confirmation of adequate cellular response to Angiotensin II

Two control experiments were performed. When AngII engages the AT-1R, PTECs respond by activating a cascade of signaling events, one of which is phosphorylation of ERK. Western blot analysis showed that AngII led to AT-1R-dependent phosphorylation of ERK in comparison to the control cells (Figure 2.2a). While phosphorylation of ERK is one of many downstream events initiated by AngII, this was an important confirmation that PTECs exhibited a biological response to AngII. The second control experiment involved labeling PTECs with H and L media and comparing their proteomes, to ensure that the changes in proteome due to the conditioned media were not attributed to AngII stimulus. The distribution of log2 transformed H/L ratios of these proteins is demonstrated in Figure 2.2b. Standard deviation (SD) of the transformed ratios was 0.33, and this was greater than in the 4 stimulation experiments, as indicated below. This control experiment was thus underpowered for the purpose of determining variance in the
stimulation experiments, while it was still suitable for identifying hypervariable outliers in the absence of stimulation. Hepatocyte nuclear factor 1-beta was the only protein that had a H/L ratio more than 2 SD outside of the mean in the absence of AngII stimulation, and that also subsequently appeared on the list of AngII-regulated proteins. Hepatocyte nuclear factor 1-beta was thus eliminated from the final list of differentially regulated candidates.

In addition to these controlled experiments, we examined the expression of some PTEC transporters. As expected, Na\textsuperscript{+}/K\textsuperscript{+} ATPase subunits were identified with multiple peptides, as were Na\textsuperscript{+}/HCO\textsubscript{3}\textsuperscript{-} co-transporter and Na\textsuperscript{+}/H\textsuperscript{+} exchanger. The expression of these transporters did not change significantly with AngII treatment. We did not study PTECs as a polarized epithelium.
Figure 2.2. Control experiments. A) Representative Western blot demonstrating phosphorylation of ERK after stimulation with AngII (which is AT-1 receptor dependent) only in cells exposed to AngII, but not in those exposed to medium alone (C) or to AngII and AT-1 receptor blocker Losartan (A+L); B) Histogram of log2 transformed H/L ratios for labeled but unstimulated PTECs; C) Histogram of log2 transformed AngII/Control ratios in 4 lysate experiments. Vertical lines represent 1.96*SD. Adapted with permission from Konvalinka et al. J Biol Chem 2013; 288: 34: 24834–24847.
2.3.2. Identification and quantification of Angiotensin II-regulated proteins

We identified 5011 proteins and quantified 4975 in the four cell lysate experiments together with one supernatant experiment (at 1.0% FDR). We then restricted our analyses to the four cell lysate experiments. The distribution of normalized log2 transformed H/L ratios of these proteins is shown in Figure 2.2c. SD of the transformed ratios was 0.22. 4618 proteins were quantified, and 83 of these proteins were differentially regulated by AngII, when applying the selection criteria described in the Data analysis section of Methods, as well as in Figure 2.3. There were 53 up-regulated and 30 down-regulated proteins (Tables 2.1 and 2.2). The candidate with the most consistent and significant expression in all replicates was heme oxygenase 1 (HO-1), which was upregulated in all experiments, including the supernatant. HO-1 is induced by oxidative stress and is a known target of nuclear factor (erythroid derived-2)-like 2 (Nrf2) transcription factor. Notably, there were three other proteins directly regulated by Nrf2: Heat shock 40 kDa protein 1 homolog (DNJB4), Thioredoxin-interacting protein (TXNIP), and Ubiquitin-conjugating enzyme E2 R1 (CDC34). The direction of change in expression of these proteins would support increased Nrf2 mediated transcription. Another protein, La ribonucleoprotein domain family member 4 (LARP4) was significantly upregulated following AngII stimulation. La autoantigen (SSB) can induce rapid Nrf2 translocation to the nucleus, and transcription of downstream genes. SSB appears to be a homologue of LARP4 based on sequence similarity (BLAST
algorithm), functional similarity from distiller algorithm (both affect nuclear transcription) and co-expression.

**Figure 2.3.** Selection of candidate proteins that were reproducibly and differentially expressed following AngII stimulation of PTECs *in vitro*.
Table 2.1. Proteins that were reproducibly and significantly differentially upregulated by AngII with ratios in 4 biological replicates. Column labeled as AngII/Con Ratio 4 indicates the ratios in replicate with reverse labeling. NA indicates that protein was not identified in that replicate. *Ratios with p <0.01. Bold proteins were confirmed by SRM.

<table>
<thead>
<tr>
<th>Protein Name (Gene name)</th>
<th>AngII/Con Ratio 1</th>
<th>AngII/Con Ratio 2</th>
<th>AngII/Con Ratio 3</th>
<th>AngII/Con Ratio 4</th>
<th>Median AngII/Con Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA FLJ77012, highly similar to Homo sapiens interferon responsive gene 15 (IFRG15)</td>
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<td>1.27*</td>
<td>1.30*</td>
<td>NA</td>
<td>1.28</td>
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<td>1.64*</td>
<td>NA</td>
<td>1.52</td>
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<tr>
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<td>NA</td>
<td>1.45*</td>
<td>1.45*</td>
<td>NA</td>
<td>1.45</td>
</tr>
<tr>
<td>Bifunctional arginine demethylase and lysyl-hydroxylase JMJD6 (JMJD6)</td>
<td>NA</td>
<td>1.41*</td>
<td>2.21*</td>
<td>NA</td>
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<tr>
<td>Cervical mucin-associated protein (DBNL)</td>
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<td>1.29*</td>
<td>1.26*</td>
<td>NA</td>
<td>1.28</td>
</tr>
<tr>
<td>Deubiquitinating protein VCIP135 (VCPIP1)</td>
<td>1.78*</td>
<td>0.92</td>
<td>1.02</td>
<td>2.70*</td>
<td>1.40</td>
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<tr>
<td>Cirhin (CIRH1A)</td>
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<td>1.05</td>
<td>2.45*</td>
<td>1.24</td>
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<tr>
<td>Death receptor 5 (TNFRSF10B)</td>
<td>NA</td>
<td>1.45*</td>
<td>NA</td>
<td>1.83*</td>
<td>1.64</td>
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<tr>
<td>Heme oxygenase 1 (HMOX1)</td>
<td>1.23</td>
<td>1.66*</td>
<td>1.50*</td>
<td>1.80*</td>
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<tr>
<td>PP2A subunit A isoform PR65-beta (PPP2R1B)</td>
<td>NA</td>
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<td>1.67*</td>
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<td>Retinol dehydrogenase 10 (RDH10)</td>
<td>NA</td>
<td>1.70*</td>
<td>1.92*</td>
<td>1.66*</td>
<td>1.70</td>
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<tr>
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<td>1.00</td>
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<td>1.25</td>
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<tr>
<td>Ribosomal protein S6 (RPS6)</td>
<td>NA</td>
<td>1.41*</td>
<td>1.13</td>
<td>1.66*</td>
<td>1.41</td>
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<tr>
<td>Protein</td>
<td>Ratio 1</td>
<td>Ratio 2</td>
<td>Ratio 3</td>
<td>Ratio 4</td>
<td>Ratio 5</td>
</tr>
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<td>------------------------------------------------------------------------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
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<td>1.02</td>
<td>1.46*</td>
<td>1.23</td>
<td>1.45*</td>
<td>1.34</td>
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<tr>
<td>DAP kinase-related apoptosis-inducing protein kinase 1 (STK17A)</td>
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<td>1.36*</td>
<td>1.43*</td>
<td>1.43</td>
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<td>ATP-dependent chromatin-remodeling protein (BAZ1A)</td>
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<td>1.56*</td>
<td>1.42*</td>
<td>1.42</td>
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<td>Ubiquitin-conjugating enzyme E2 R1 (CDC34)</td>
<td>NA</td>
<td>1.34*</td>
<td>1.61*</td>
<td>1.40*</td>
<td>1.40</td>
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<tr>
<td>Eukaryotic translation initiation factor 4B (EIF4B)</td>
<td>1.22</td>
<td>1.28*</td>
<td>1.17</td>
<td>1.40*</td>
<td>1.25</td>
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<tr>
<td>ADP ribosylation factor-like protein 7 (ARL4C)</td>
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<td>1.25*</td>
<td>1.51*</td>
<td>1.39*</td>
<td>1.39</td>
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<tr>
<td>Alpha-catenin-related protein (CTNNAL1)</td>
<td>NA</td>
<td>1.40*</td>
<td>1.59*</td>
<td>1.38*</td>
<td>1.40</td>
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<tr>
<td>Laminin B2 chain (LAMB2)</td>
<td>0.75</td>
<td>1.15</td>
<td>1.28*</td>
<td>1.35*</td>
<td>1.22</td>
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<tr>
<td>La ribonucleoprotein domain family member 4 (LARP4)</td>
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<td>1.02</td>
<td>1.26</td>
<td>1.34*</td>
<td>1.30</td>
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<tr>
<td>Apoptosis-associated nuclear protein (PHLDA1)</td>
<td>0.83</td>
<td>1.26*</td>
<td>1.37*</td>
<td>1.34*</td>
<td>1.30</td>
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<tr>
<td>cDNA FLJ61695, highly similar to Gioma tumor suppressor candidate region gene 2 protein (GLTSCR2)</td>
<td>NA</td>
<td>1.24*</td>
<td>1.17</td>
<td>1.33*</td>
<td>1.24</td>
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<tr>
<td>WD repeat-containing protein 75 (WDR75)</td>
<td>NA</td>
<td>1.41*</td>
<td>1.56*</td>
<td>1.32*</td>
<td>1.41</td>
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<tr>
<td>60S ribosomal protein L22-like 1 (RPL22L1)</td>
<td>NA</td>
<td>1.30*</td>
<td>1.47*</td>
<td>1.31</td>
<td>1.31</td>
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<tr>
<td>3-hydroxy-3-methylglutaryl coenzyme A synthase (HMGCS)</td>
<td>1.00</td>
<td>1.38*</td>
<td>1.29</td>
<td>1.30*</td>
<td>1.30</td>
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<tr>
<td>Five-pass transmembrane protein localizing in the Golgi apparatus</td>
<td>0.89</td>
<td>1.27*</td>
<td>1.42*</td>
<td>1.25</td>
<td>1.26</td>
</tr>
<tr>
<td>Protein Description</td>
<td>EIF1</td>
<td>EIF2</td>
<td>EIF3</td>
<td>EIF4</td>
<td>EIF5</td>
</tr>
<tr>
<td>--------------------------------------------------------------------------------------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>Eukaryotic translation initiation factor 1 (EIF1)</td>
<td>1.16</td>
<td>1.32*</td>
<td>1.25*</td>
<td>1.23</td>
<td>1.24</td>
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<tr>
<td>DNAJ homolog subfamily B member 4 (DNAJB4)</td>
<td>NA</td>
<td>1.28*</td>
<td>1.27*</td>
<td>1.23</td>
<td>1.27</td>
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<tr>
<td>BAG family molecular chaperone regulator 1 (BAG1)</td>
<td>NA</td>
<td>1.39*</td>
<td>1.36*</td>
<td>1.21</td>
<td>1.36</td>
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<tr>
<td>DNAFLJ78093, highly similar to Homo sapiens ribosomal protein L29 (RPL29)</td>
<td>0.69</td>
<td>1.42*</td>
<td>1.40*</td>
<td>1.20</td>
<td>1.30</td>
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<tr>
<td>TGF-beta receptor type-2 (TGFB2)</td>
<td>0.50</td>
<td>1.47*</td>
<td>1.37*</td>
<td>1.16</td>
<td>1.26</td>
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<tr>
<td>Importin subunit alpha-2 (KPNA2)</td>
<td>0.97</td>
<td>1.30*</td>
<td>1.32*</td>
<td>1.14</td>
<td>1.22</td>
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<tr>
<td>Aspartate beta-hydroxylase domain-containing protein 1 (ASPHD1)</td>
<td>NA</td>
<td>1.30*</td>
<td>1.56*</td>
<td>1.14</td>
<td>1.30</td>
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<tr>
<td>Olfactory receptor 4A16 (OR4A16)</td>
<td>1.82*</td>
<td>1.32*</td>
<td>NA</td>
<td>1.12</td>
<td>1.32</td>
</tr>
<tr>
<td>Guanine nucleotide exchange factor H1 (ARHGEF2)</td>
<td>1.22</td>
<td>1.24*</td>
<td>1.29*</td>
<td>1.09</td>
<td>1.23</td>
</tr>
<tr>
<td>Putative RNA-binding protein 3 (RBM3)</td>
<td>1.20</td>
<td>1.41*</td>
<td>1.31*</td>
<td>1.05</td>
<td>1.25</td>
</tr>
<tr>
<td>TRAF2 and NCK-interacting protein kinase (TNIK)</td>
<td>0.85</td>
<td>1.25*</td>
<td>1.26*</td>
<td>1.05</td>
<td>1.15</td>
</tr>
<tr>
<td>Abraxas brother protein 1 (FAM175B)</td>
<td>0.90</td>
<td>1.38*</td>
<td>1.41*</td>
<td>1.03</td>
<td>1.20</td>
</tr>
<tr>
<td>Transmembrane protein 41B (TMEM41B)</td>
<td>NA</td>
<td>1.24*</td>
<td>1.61*</td>
<td>1.03</td>
<td>1.24</td>
</tr>
<tr>
<td>CYPLI (CYP51)</td>
<td>1.16</td>
<td>1.28*</td>
<td>1.27*</td>
<td>1.02</td>
<td>1.22</td>
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<tr>
<td>Alpha- and gamma-adaptin-binding protein p34 (AAGAB)</td>
<td>1.03</td>
<td>1.31*</td>
<td>1.58*</td>
<td>0.99</td>
<td>1.17</td>
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<tr>
<td>50 kDa nucleoporin (NUP50)</td>
<td>1.18</td>
<td>1.39*</td>
<td>1.32*</td>
<td>0.96</td>
<td>1.25</td>
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<tr>
<td>Long chain base biosynthesis protein 2b (SPTLC3)</td>
<td>1.20</td>
<td>1.38*</td>
<td>1.42*</td>
<td>0.95</td>
<td>1.29</td>
</tr>
<tr>
<td>Protein Name (Gene name)</td>
<td>AngII/Con Ratio 1</td>
<td>AngII/Con Ratio 2</td>
<td>AngII/Con Ratio 3</td>
<td>AngII/Con Ratio 4</td>
<td>Median AngII /Con Ratio</td>
</tr>
<tr>
<td>--------------------------</td>
<td>-------------------</td>
<td>-------------------</td>
<td>-------------------</td>
<td>-------------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>ATP-binding protein associated with cell differentiation (TXNDC9)</td>
<td>1.04</td>
<td>1.27*</td>
<td>1.30*</td>
<td>0.94</td>
<td>1.16</td>
</tr>
<tr>
<td>Methionine adenosyltransferase 1 (MAN2C1)</td>
<td>NA</td>
<td>1.24*</td>
<td>1.26*</td>
<td>0.94</td>
<td>1.24</td>
</tr>
<tr>
<td>HBV pX-associated protein 8 (RSF1)</td>
<td>1.70*</td>
<td>1.13</td>
<td>1.32*</td>
<td>0.89</td>
<td>1.22</td>
</tr>
<tr>
<td>Dynactin subunit 6 (DCTN6)</td>
<td>1.05</td>
<td>1.33*</td>
<td>1.81*</td>
<td>0.88</td>
<td>1.19</td>
</tr>
<tr>
<td>Tumor necrosis factor receptor superfamily, member 6 isoform 1 variant (FAS)</td>
<td>1.03</td>
<td>1.41*</td>
<td>1.57*</td>
<td>0.86</td>
<td>1.22</td>
</tr>
<tr>
<td>Antigen NY-CO-10 (CWC27)</td>
<td>1.46*</td>
<td>1.30*</td>
<td>1.42*</td>
<td>0.85</td>
<td>1.36</td>
</tr>
<tr>
<td>Smu-1 suppressor of mec-8 and unc-52 protein homolog (SMU1)</td>
<td>1.37</td>
<td>2.03*</td>
<td>1.60*</td>
<td>0.84</td>
<td>1.48</td>
</tr>
</tbody>
</table>

**Table 2.2.** Proteins that were reproducibly and significantly differentially downregulated by AngII with ratios in 4 biological replicates. Column labeled as AngII/Con Ratio 4 indicates the ratios in replicate with reverse labeling. NA indicates that protein was not identified in that replicate. *Ratios with p <0.01. Bold proteins were confirmed by SRM.
<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Gene Name</th>
<th>1</th>
<th>0.74*</th>
<th>0.57*</th>
<th>1.16</th>
<th>0.75</th>
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</thead>
<tbody>
<tr>
<td>Retinoic acid-induced protein 15 (SMYD5)</td>
<td></td>
<td>0.76</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Copine II (CPNE2)</td>
<td></td>
<td>1.16</td>
<td>0.58*</td>
<td>0.50*</td>
<td>1.06</td>
<td>0.82</td>
</tr>
<tr>
<td>Basement-membrane protein 40 (SPARC)</td>
<td></td>
<td>0.86</td>
<td>0.67*</td>
<td>0.63*</td>
<td>1.02</td>
<td>0.76</td>
</tr>
<tr>
<td>Thrombospondin-1 (THBS1)</td>
<td></td>
<td>1.04</td>
<td>0.73*</td>
<td>0.70*</td>
<td>0.95</td>
<td>0.84</td>
</tr>
<tr>
<td>nm23-H7 (NME7)</td>
<td></td>
<td>0.30*</td>
<td>0.65*</td>
<td>0.96</td>
<td>0.91</td>
<td>0.78</td>
</tr>
<tr>
<td>Putative uncharacterized protein APIP (APIP)</td>
<td></td>
<td>0.83</td>
<td>0.25*</td>
<td>0.55*</td>
<td>0.90</td>
<td>0.69</td>
</tr>
<tr>
<td>Glutamate decarboxylase (GLUL)</td>
<td></td>
<td>0.95</td>
<td>0.76*</td>
<td>0.53*</td>
<td>0.81</td>
<td>0.78</td>
</tr>
<tr>
<td>Conserved edge-expressed protein (GET4)</td>
<td></td>
<td>0.93</td>
<td>0.75*</td>
<td>0.65*</td>
<td>0.81</td>
<td>0.78</td>
</tr>
<tr>
<td>Sodium- and chloride-dependent taurine transporter (SLC6A6)</td>
<td>0.91</td>
<td>0.88</td>
<td>0.67*</td>
<td>0.80*</td>
<td>0.84</td>
<td></td>
</tr>
<tr>
<td>LYN protein (LYN)</td>
<td></td>
<td>0.86</td>
<td>0.79*</td>
<td>0.69*</td>
<td>0.80*</td>
<td>0.80</td>
</tr>
<tr>
<td>Rho-related GTP-binding protein (RHOB)</td>
<td></td>
<td>0.94</td>
<td>0.70*</td>
<td>0.70*</td>
<td>0.78*</td>
<td>0.74</td>
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<tr>
<td>Leptin receptor gene-related protein (LEPR)</td>
<td></td>
<td>NA</td>
<td>0.58*</td>
<td>0.77*</td>
<td>0.76*</td>
<td>0.76</td>
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<tr>
<td>RCC1-like G exchanger factor-like protein (WBSCR16)</td>
<td>NA</td>
<td>0.97</td>
<td>0.60*</td>
<td>0.72*</td>
<td>0.72</td>
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<tr>
<td>Kinesin-like protein KIF1C (KIF1C)</td>
<td></td>
<td>1.37</td>
<td>0.94</td>
<td>0.70*</td>
<td>0.71*</td>
<td>0.82</td>
</tr>
<tr>
<td>Epidermal growth factor receptor (EGFR)</td>
<td></td>
<td>1.28</td>
<td>0.59*</td>
<td>0.65*</td>
<td>0.71*</td>
<td>0.68</td>
</tr>
<tr>
<td>65 kDa FK506-binding protein (FKBP10)</td>
<td></td>
<td>NA</td>
<td>0.78</td>
<td>0.70*</td>
<td>0.65*</td>
<td>0.70</td>
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<tr>
<td>Neoplastic transformation inhibitor protein (PDCD4)</td>
<td></td>
<td>0.76</td>
<td>0.71*</td>
<td>0.62*</td>
<td>0.64*</td>
<td>0.68</td>
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<tr>
<td>Transmembrane protein 184C (TMEM184C)</td>
<td></td>
<td>1.01</td>
<td>NA</td>
<td>0.45*</td>
<td>0.62*</td>
<td>0.62</td>
</tr>
<tr>
<td>Thioredoxin-</td>
<td></td>
<td>NA</td>
<td>0.63*</td>
<td>0.84</td>
<td>0.59*</td>
<td>0.63</td>
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</table>
interaction protein (TXNIP)

<table>
<thead>
<tr>
<th>Protein Description</th>
<th>Ratio</th>
<th>p-value</th>
<th>FDR</th>
<th>p-value</th>
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</thead>
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<tr>
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<td>1.06</td>
<td>0.50*</td>
<td>0.55*</td>
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<tr>
<td>Unknown protein, IPI00917920</td>
<td>NA</td>
<td>1.13</td>
<td>0.63*</td>
<td>0.55*</td>
</tr>
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<td>Glycogen synthase kinase 3 beta-interacting protein (NIN)</td>
<td>0.80</td>
<td>0.74*</td>
<td>1.14</td>
<td>0.45*</td>
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<tr>
<td>Putative uncharacterized protein CWC22 (CWC22)</td>
<td>NA</td>
<td>0.36*</td>
<td>0.90</td>
<td>0.38*</td>
</tr>
<tr>
<td>Unknown protein, IPI00879223;IPI00937588</td>
<td>NA</td>
<td>0.76*</td>
<td>0.76*</td>
<td>0.25*</td>
</tr>
</tbody>
</table>

2.3.3. Enriched Gene Ontology terms among Angiotensin II-regulated proteins

We next examined the GO terms (biological processes, molecular functions and cell compartments) overrepresented among the 83 differentially regulated proteins. This list of proteins was analyzed using the Biological Networks Gene Ontology (BiNGO) plugin of Cytoscape. BiNGO plugin is a tool to determine enriched GO terms in a set of genes or proteins. The most significant Gene Ontology (GO) biological process enriched among the 83 AngII-regulated proteins was apoptosis, and the most enriched cellular organelle was endoplasmic reticulum (ER) (Table 2.3). Biological processes linked to apoptosis were also significantly enriched among AngII-regulated proteins from the supernatant. Other processes directly linked to AngII signaling that were significantly enriched
include: regulation of intracellular protein kinase cascade, regulation of leukocyte migration, TGF-β binding, as well as cell cycle and the homeostasis of number of cells. The former three processes are involved in inflammation and fibrosis, while the latter two have to do with hypertrophy and hyperplasia.

2.4. Discussion

In this first important part of the study, our main objective was to define AngII-regulated proteome in primary human PTECs, in order to capture the effect of AngII activity in the kidney. To address our first aim, we utilized SILAC methodology, which is the current standard for accurate relative quantification of entire cellular proteomes by mass spectrometry. We identified over 5000 proteins, most of which were also quantified, thus providing a unique depth of insight into PTEC responses to AngII. The strengths of our approach include: excellent proteome coverage using extensive fractionation, instruments with high sensitivity and accuracy, multiple biological replicates and use of reverse labeling, and control experiments to minimize false positive hits. Additionally, AngII-regulated proteome represents PTEC responses from 3 distinct individuals, thus signifying true biological variability, not typical of transformed cell culture models. To the best of our knowledge, this is the first effort to date to characterize proteomic responses of human kidney cells to AngII stimulation.

Using stringent selection criteria, we uncovered 83 proteins that were reproducibly and differentially regulated by AngII. The top candidate that was
Table 2.3. The most representative/ informative Gene Ontology (GO) terms among the 83 differentially regulated proteins. Corrected p-value was obtained by applying Benjamini Hochberg correction for multiple testing. The whole annotation was used as a reference set. Statistical analysis was performed by BiNGO plugin of Cytoscape. Adapted with permission from Konvalinka et al. J Biol Chem 2013; 288: 24834–24847.

<table>
<thead>
<tr>
<th>Gene Ontology</th>
<th>p-value</th>
<th>Corrected p-value</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptosis</td>
<td>7.16 e⁻⁶</td>
<td>0.004</td>
<td>ARGHEF2, JMJD6, THBS1, PDCD4, APIP, BAG1, PHLDA1, STK17A, TNFRSF10B, FAS, RPS6, RHOB</td>
</tr>
<tr>
<td>Erythrocyte homeostasis</td>
<td>6.56 e⁻⁵</td>
<td>0.013</td>
<td>LYN, JMJD6, HMOX1, RPS6</td>
</tr>
<tr>
<td>Regulation of intracellular protein kinase cascade</td>
<td>9.23 e⁻⁵</td>
<td>0.015</td>
<td>EGFR, HMOX1, LEPR, TNFRSF10B, LYN, THBS1, DBNL, PDCD4</td>
</tr>
<tr>
<td>Positive regulation of signal transduction</td>
<td>2.24 e⁻⁴</td>
<td>0.024</td>
<td>EGFR, HMOX1, LEPR, TNFRSF10B, ADA, LYN, THBS1</td>
</tr>
<tr>
<td>Mitotic cell cycle</td>
<td>2.80 e⁻⁴</td>
<td>0.027</td>
<td>EGFR, ARHGEF2, NDE1, ANAPC3, CDC34, VCP1P1, KPN2, RPS6</td>
</tr>
<tr>
<td>Endoplasmic reticulum</td>
<td>5.15 e⁻⁴</td>
<td>0.032</td>
<td>CYP51, HMOX1, LAMB2, ASPHD1, YIPF5, BST1, RDH10, SPTLC3, FAS, KIF1C, FKBP10, IFRG15, EXT2</td>
</tr>
<tr>
<td>Regulation of leukocyte migration</td>
<td>7.40 e⁻⁴</td>
<td>0.033</td>
<td>HMOX1, ADA, THBS1</td>
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differentially upregulated by AngII in every replicate including the secretome was HO-1. The discovery of HO-1 in PTEC secretome suggested that this protein may be secreted by PTECs, despite the absence of the classical signal sequence (SignalIP algorithm prediction from Human Protein Atlas). HO-1 is an interesting, cytoprotective enzyme, typically upregulated in response to oxidative stress. HO-1 is an enzyme that mediates the breakdown of free heme to iron, carbon monoxide and biliverdin. A variety of stimulants were previously associated with HO-1 upregulation, both at the transcript and protein level. The typical such stimulants include: heme, nitric oxide, nephrotoxins such as cadmium, and growth factors. Prior studies also support our observation that AngII induces HO-1. Nath’s group first described HO-1 upregulation in rat PTECs and rat whole kidneys in response to AngII both in vitro and in vivo\textsuperscript{270}, and demonstrated that heme-dependent stabilization of Nrf2 transcription factor was responsible for HO-1 induction\textsuperscript{271}. AngII-induced HO-1 upregulation was demonstrated in vivo in animal models of kidney disease by other groups\textsuperscript{272-274}. Increased renal immunostaining for HO-1 correlated with AngII and angiotensinogen immunostaining in biopsies of humans with IgA nephropathy\textsuperscript{275}. In experimental models of disease, HO-1 inhibition led to increased susceptibility to injury and death. Transgenic mice lacking HO-1 gene developed tubulo-interstitial iron infiltrates, and also had systemic evidence of rampant inflammation\textsuperscript{276}. A natural human knock out of HO-1 was reported, and this individual had stunted growth, anemia, lymphadenopathy, leukocytosis, increased predisposition to oxidant injury, and widespread iron deposits within PTECs detected on autopsy\textsuperscript{277}. While inhibition or loss of this enzyme
predisposed to injury, overexpression of HO-1 led to protection from renal injury in several models of AKI. The mechanism that leads to this protective effect remains unclear, however it has been postulated that a combination of efflux of iron, vasodilatory effect of carbon monoxide, and antioxidant effect of bilirubin may be responsible. As such, HO-1 represents a potential therapeutic target for patients with CKD. We believe that our study is the first to demonstrate HO-1 upregulation by AngII in primary human kidney cells.

HO-1 is a target of Nrf2. Additional Nrf2 target proteins were differentially regulated by AngII based on the SILAC data, and the direction of change of these proteins supported Nrf2 activation. Nrf2 is a transcription factor that becomes phosphorylated in the cytoplasm in response to oxidative stress, and translocates to the nucleus, where it results in transcription of multiple genes responsible for reduction of oxidative damage, repair and removal of damaged proteins, and activation of phase I–III detoxifying proteins. There is cross talk between Nrf2 and other pathways. The Nrf2 pathway has been involved in the control of NFkB through reduction of IkBα phosphorylation, thereby favouring NFkB degradation. Similar to HO-1, transgenic mice deficient in Nrf2 exhibited increased renal injury in response to toxins\textsuperscript{278}, increased diabetes-induced oxidative stress\textsuperscript{279}, as well as a lupus-like autoimmune nephritis\textsuperscript{280}. Animal models of CKD demonstrated deficiency in Nrf2, or an inability to activate Nrf2 in face of oxidative stress and inflammation\textsuperscript{281,282}. Inducers of Nrf2 however protected animals from diabetic nephropathy (by limiting albuminuria and oxidative damage)\textsuperscript{283}. Despite the potential for Nrf2 agonism in slowing down the progression of renal disease, Nrf2
agonist bardoxolone methyl demonstrated increased proteinuria, weight loss, liver toxicity and mortality in a clinical trial of diabetic nephropathy patients with stage IV CKD. The reasons for this unexpected outcome are still debated and investigated. This study does not diminish the impact of our suggestion that an early Nrf2 activation is one of the most consistent cellular responses to AngII, perhaps as a result of oxidative stress.

We next aimed to put AngII-regulated proteins in the context of their biological function, in order to better understand whether these proteins represent some unifying processes. Apoptosis was the most enriched biological process among AngII-regulated proteins. Endoplasmic reticulum was the most enriched cell compartment. The common thread to these enriched GO terms is oxidative stress leading to endoplasmic reticulum stress and unfolded protein response, which could trigger apoptosis. The cascade leading from oxidative stress to apoptosis was indirectly linked to the activation of RAS in the kidney. AngII was previously shown to induce apoptosis in PTECs, as well as in other kidney cells. HO-1 is an anti-apoptotic protein, upregulated in response to oxidative stress. Could oxidative stress be the primary mechanism for PTEC’s response to AngII? Interestingly, we also saw early profibrotic signals as evidenced by overrepresented TGF-β binding and likely EGFR-mediated signaling. Also, signals for the well documented cellular hypertrophy/hyperplasia in response to AngII was likely mirrored in processes affecting cell cycle and homeostasis of number of cells. These expected findings strengthen our observations. Although previously linked to AngII, the importance of our
observations that apoptosis and oxidative stress dominate early PTEC responses to this powerful peptide is that we performed an unbiased search for differentially regulated proteins, which was not the case in any of the previously published studies. Oxidative stress may thus represent the dominant early mode of injury by AngII.

In this chapter we addressed our first objective, which was to define an AngII-regulated proteome in primary PTECs *in vitro*. We went on to verify and confirm these observations by a combination of ELISA, quantitative qRT-PCR and SRM.
CONFIRMATION AND VERIFICATION OF ANGIOTENSIN II-REGULATED PROTEINS DISCOVERED BY SILAC

Sections of this Chapter have been published previously in the Journal of Biological Chemistry:

Ana Konvalinka, Joyce Zhou, Apostolos Dimitromanolakis, Andrei P. Drabovich, Fei Fang, Susan Gurley, Thomas Coffman, Rohan John, Shao-Ling Zhang, Eleftherios P. Diamandis, James W. Scholey
Determination of an Angiotensin II-Regulated Proteome in Primary Human Kidney Cells by Stable Isotope Labeling of Amino Acids in Cell Culture (SILAC)


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3.1. Introduction

One of the trade-offs of high-throughput approaches is the high false positive rate. Even with the most stringent shot-gun proteomics approaches, the requirement is to typically verify the most important results by using a different quantitative method. Enzyme-linked immunosorbent assay (ELISA) is one of the most sensitive and accurate assays available today for the absolute measurement of a protein concentration in a biofluid or cell/tissue lysate. Unfortunately, ELISA measurement depends on the presence of antibodies to the protein of interest, and it is costly and difficult to develop thus limiting its use for verification to few top protein hits. Although the expression levels of any protein may not correlate to its expression levels at the mRNA level, in some cases this correlation should be sought. In the example of Nrf2 target proteins, if we postulate that their expression was altered as a result of increased Nrf2 migration to the nucleus and binding to the promoter elements of these genes, then their mRNA levels should also be altered and should correlate with their protein expression. We used stringent criteria to select our candidates. We set the initial protein identification FDR to 1%, and in the selection of differentially regulated proteins, we set FDR to 0.06%, thus minimizing the risk of false positive hits. Nonetheless, we recognized the importance of proper verification, and we performed HO-1 protein measurement by ELISA in freshly cultured primary PTEC lysates, after exposure to AngII. In addition, we examined three proteins found to be regulated by AngII at the level of mRNA by qRT-PCR. These included HO-1, TXNIP, and DNJB4.
In order to confirm AngII-regulated expression of additional proteins discovered from SILAC data, we took advantage of SRM. SRM is a targeted proteomic approach that can be used to accurately quantify multiple proteins in a small amount of biological sample and without the need for protein-specific antibodies. SRM is a method typically performed on a triple quadrupole (Q-Q-Q) instrument, which can be used for relative or absolute quantification. The principle of this method is based on spiking known amounts of proteotypic AQUA peptides into a complex mixture of interest. The peptides of interest are then monitored by the Q-Q-Q instrument. We took advantage of SILAC labeled PTECs, which contained the heavy-labeled versions of all peptides, thus eliminating the need for purchasing additional AQUA peptides. The peptides of interest co-elute with their heavy peptides, thus allowing both confident identification and quantification of these peptides. Assumption is made that the amount of measured proteotypic peptide represents the amount of the protein of interest. An SRM assay includes the following steps: sample preparation and digestion of proteins in the biological fluid, liquid chromatography (LC) separation of peptides, ionization of peptides with nanoelectrospray ionization (nanoESI), filtering of peptides of interest in the first quadrupole (Q1), fragmentation of peptides in the second quadrupole (Q2), filtering of peptide fragments in the third quadrupole (Q3), measurement of several fragment ion intensities, and integration of the ion signals. With state-of-the-art SRM assays, up to 100 peptides representing 100 medium-to-high abundance proteins (10ng/mL – 1mg/mL) can be measured simultaneously in the unfractionated digest of proteins, while achieving CV under 20%. This technique
is becoming ideal for hypothesis-driven investigations where numerous proteins require quantification in a short time, and without the need for developing protein-specific antibodies.

We thus aimed to perform relative quantification (i.e. obtain ratios of AngII treated-to-control peptides) of a fraction of our 83 candidate proteins in SILAC labeled PTEC lysates. In this chapter we address our second objective, namely the verification and confirmation of AngII-regulated proteins.
3.2. Materials and Methods

3.2.1. Primary human proximal tubular cell cultures

PTECs employed for SRM-based quantification were derived from different aliquots of SILAC-labeled, AngII and control-treated PTECs. The cell lysate aliquots were mixed in a 1:1 heavy-to-light total protein ratio, and then underwent reduction, alkylation and digestion with trypsin, as described in the previous chapter. After trypsin digestion, no SCX-based fractionation was performed. Instead, peptide mixtures were vortexed, then separated into aliquots containing 10 – 30µg of total protein (at a concentration of approximately 1µg/µL). The aliquot analyzed was desalted by zip-tipping and analyzed by LC-MS/MS on the triple quadrupole. Remaining aliquots were stored at -20°C.

Primary human PTECs used for qRT-PCR and ELISA experiments were grown in DMEM with glucose concentration 4.5g/l (Invitrogen). The medium was enriched with 10% v/v fetal bovine serum (FBS), and cells were otherwise treated the same as in SILAC experiments. Fully confluent cells in 6-well plates, at passage 5 or 6, were serum deprived for 18 hours, and then stimulated with control or AngII for 4 hours (for qRT-PCR) and 8 hours (for ELISA and qRT-PCR). They were then washed with PBS, detached with trypsin, and stored at -80°C until further analysis.

3.2.2. SRM method development and sample preparation for Angiotensin II-regulated proteins
We aimed to develop SRM methods for all AngII-regulated proteins that had definitive evidence of differential regulation at the peptide level. Individual peptide ratios for all 83 differentially regulated candidates were examined manually. If ratios of proteotypic (unique) peptides of the same protein were changing significantly in opposite directions, or if posterior error probability of peptides was >0.05, these proteins were not pursued further by SRM. The final list for development of SRM methods included 51 proteins.

Having obtained the most stringent list of proteins for SRM, we utilized PTPAtlas of the Peptide Atlas (http://db.systemsbiology.net) to select 5 most highly observable peptides for each of the top 51 proteins based on the occurrence of +2 ions. Fully tryptic and doubly charged peptides with 7–20 amino acids were chosen. Peptides with methionine, tryptophan, and N-terminal cysteine residues were avoided, whenever possible. All peptides were also analyzed with the Basic Local Alignment Search Tool (BLAST) to ensure that peptides were unique to each protein. A list of 194 peptides corresponding to the top 41 proteins (the remaining 10 proteins were not readily amenable to SRM) was uploaded to Skyline software and was used to design in silico survey SRM methods. Transitions containing y ions from y+3 to last ion-1 for both heavy and light peptides were selected (approximately 7 light and 7 heavy transitions per peptide). In addition, 9 intense peptides from 6 high abundance proteins that our group had previously monitored were included to serve as internal loading controls. An equimolar mixture of light and heavy SILAC-labelled PTECs (13C₆,
$^{15}$N$_2$ L-Lysine, +8 Da, and $^{13}$C$_6$ L-Arginine, +6 Da) was used to experimentally test 2645 transitions. In the first step of method development, 9-10 peptides and about 100 transitions were included into each survey SRM method and run in a non-scheduled mode with 20 ms scan time per transition, on a 60-minute gradient. Q1 was set to 0.2 Thompson (Th) and Q3 to 0.7 Th. Given the low abundance nature of most candidate proteins, and the difficulty in finding peaks in non-scheduled SRM methods, we pursued 18 proteins only. Proteins involved in processes deemed to be important by GO analyses, such as apoptosis, regulation of intracellular kinase activity, regulation of leukocyte migration, and transforming growth factor β binding were selected. Additionally, the two most consistently observed high abundance peptides with congruent H/L ratios (SYELPDGQVITIGNER peptide of β-actin, and INVYYNEATGGK peptide of tubulin β-4B chain) were monitored as internal controls. All 9 high-abundance peptides had similar ratios. Thus, in the second step, 3 peptides and under 50 transitions were included in each of 10 survey SRM methods and run in a non-scheduled mode with 20 ms scans per transition. Q1 was set to 0.4 Th and Q3 to 0.7 Th. We looked for co-elution of all light and all heavy transitions for each peptide. Retention times, relative intensities of peptides, and the three most intense and selective transitions per peptide were recorded (Table 3.1). Transitions with fragment m/z higher than precursor m/z were preferable, but transitions with lower m/z were not excluded if they had high intensity (especially at proline residue). As a reference to exclude possible interferences, we used SRM signal of SILAC labelled heavy cells.
Table 3.1. Peptides and transitions monitored for each protein using SRM. Light and heavy precursors and product ions are shown. Only the top three most intense and specific transitions for each peptide are shown.

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<td>ALTVPELTQVQFDAK</td>
<td>834.46</td>
<td>1057.58</td>
<td>24.92</td>
<td>31.69</td>
</tr>
<tr>
<td>TUBB</td>
<td>ALTVPELTQVQFDAK</td>
<td>834.46</td>
<td>1283.67</td>
<td>24.92</td>
<td>31.69</td>
</tr>
</tbody>
</table>

3.2.3. Mass spectrometry by triple quadrupole mass spectrometer
Peptides were separated by 60-min C18 reversed-phase liquid chromatography (EASY-nLC, Proxeon, Odense, Denmark) and analyzed by a triple-quadrupole mass spectrometer (TSQ Vantage, Thermo Fisher Scientific Inc., San Jose, CA) using a nanoelectrospray ionization source, as previously described\textsuperscript{290-292}. Reproducibility of SRM signal was ensured by running a QC solution of 0.1 fmol/µL BSA every 5 runs. Raw files recorded for each sample were analyzed using Pinpoint software (Thermo Fisher Scientific Inc., San Jose, CA), and CSV files with peptide areas were extracted.

**3.2.4. Data analysis**

Pinpoint was used for identification and visualization of transitions, as well as manual verification of co-elution of heavy and light transitions. Transitions represent the precursor and product ions. Skyline was subsequently used for reporting of peptide ratios. Pinpoint and Skyline ratios were concordant. Peptide H/L ratios extracted from Skyline were divided by the H/L ratio of internal standards (to control for the differences in mixing). Protein ratios were calculated as an average of all peptide ratios for each protein. If different peptides from the same protein had distinctly different intensities, only the highest intensity peptide was included in the calculation. Collision energy was calculated in Skyline by using the equation: \( CE = 0.03 \times \text{precursor } m/z + 2.905 \text{\textsuperscript{293}}. \)

**3.2.5. Heme oxygenase-1 (HO-1) measurement in cell lysates by ELISA**
Total HO-1 human ELISA DuoSet (R&D Systems DYC3776) was used to assay HO-1 protein expression in PTEC lysates. PTECs were prepared separately, and grown in non-SILAC media as described above, but ultimately treated the same way as the SILAC cells. The lysates of control and AngII-treated cells were compared after 8 hours of incubation. Reagents were made according to the manufacturer’s instructions. Lysis buffer was made as recommended and contained: 1mM EDTA, 0.5% Triton X-100, 10µg/mL leupeptin, 10µg/mL pepstatin, 100µM PMSF, 3µg/mL aprotinin in PBS, pH 7.2-7.4. Cells were washed two times with PBS, and then solubilized at 1x10⁷ cells/mL in lysis buffer and allowed to sit on ice for 15 minutes. They were then stored at -80°C until use. Before use, samples were centrifuged at 2000g for 5 minutes, and supernatant was collected. Total protein was measured by Coomassie assay. TDS Wilson software was used to plot and analyse optical density measurements. HO-1 protein measurements were adjusted for total protein concentration. To minimize inter-plate variation, HO-1 protein measurements were expressed as ratio of AngII-to-Control for each plate. A total of 9 replicates were performed.

3.2.6. Measurement of mRNA of Nrf2 target genes by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cultured primary PTECs using a kit (RNeasy Mini; Qiagen Canada, Mississauga, ON, Canada). 1µg of RNA was then reverse
transcribed into first-strand complementary DNA (cDNA). mRNA expression levels for *Hmox1* (Hs01110250_m1), *Txnip* (Hs00197750_m1), *Dnajb4* (Hs00199826_m1) were quantified by real-time PCR (TaqMan) using a sequence detection system (ABI Prism 7900; Applied Biosystems, Foster City, CA, USA) as previously described\(^2\). *GAPDH* (4352934E) was used as internal control. Specific human primer sets were purchased from Applied Biosystems (Foster City, CA, USA).

### 3.2.7. Statistical analysis

All values are reported as mean ± SEM, unless stated otherwise. Statistical comparison between experimental groups was performed using a two-tailed Student’s t test. P-values <0.05 were considered statistically significant, unless stated otherwise. Benjamini Hochberg correction for multiple testing was applied, where indicated. GraphPad Prism 5 software was used for other statistical tests (GraphPad, La Jolla, CA, USA).

### 3.3. Results

#### 3.3.1. Confirmation of 18 Angiotensin II-regulated proteins

We utilized SRM assays to confirm differential protein expression in response to AngII stimulation in PTECs. With the presence of both heavy-labeled and light-labeled proteotypic peptides, confident identification and quantification of peptides
is enabled. SRM assays were developed for 18 candidate proteins. Proteins involved in processes deemed to be important by GO analyses, such as apoptosis, regulation of intracellular kinase activity, regulation of leukocyte migration, and TGF-β binding were selected. SILAC-labeled PTEC lysates were used to monitor co-eluting heavy and light peptide transitions of all 18 proteins. Heavy and light peak areas were used to calculate AngII-to-Control ratios. These ratios were further adjusted by internal peptides, which served as loading controls. Peak areas of peptides of HO-1, neoplastic transformation inhibitor (PDCD4), thrombospondin-1 (TSP-1), and Rho-related GTP binding protein (RHOB) are demonstrated in Figure 3.1. All 18 proteins demonstrated concordant ratios to SILAC experiments (Table 3.2). We demonstrated using SRM that 18 of our candidates had concordant ratios to those obtained by SILAC, thus confirming their differential expression in PTECs upon AngII stimulation.

3.3.2. Verification of Angiotensin II-mediated upregulation of HO-1 protein

HO-1 was consistently upregulated in the presence of AngII in our SILAC experiments, and this upregulation was confirmed by SRM assays. We thus proceeded to verify HO-1 protein upregulation by ELISA in PTECs. PTECs were cultured to passage 6 in standard medium. They were then serum-deprived for 18 hours and subsequently stimulated with AngII (10^{-7}M) or control for 8 hours. AngII-stimulated cell lysates exhibited significantly higher HO-1 protein expression
compared to controls (p=0.012 for 1-sample t-test (mean = 1.16, 95%CI = 1.04, 1.27)) (Figure 3.2a). We thus confirmed that AngII leads to upregulation of HO-1 protein in primary PTECs.

3.3.3. The effect of Angiotensin II on mRNA levels of HO-1, TXNIP and DNAJB4

We next examined whether HO-1 and two other Nrf2 target proteins were regulated by AngII at the level of transcription by qRT-PCR. PTECs at passage 6 were serum starved, and then treated with control or AngII for 4 hours. Hmox1 (gene name for HO-1) was significantly upregulated following AngII stimulation (p = 0.018, (mean difference = 0.43, 95%CI = 0.09, 0.76)) (Figure 3.2b). Two additional Nrf2-target genes were examined. Txnip was significantly downregulated by AngII treatment (p=0.025, difference between means=0.190 ± 0.055, 95%CI=0.038, 0.342) (Figure 3.3a), a result concordant with SILAC protein expression data. Dnajb4 was not regulated at the level of transcription in AngII-treated PTECs (p=0.33) (Figure 3.3b). These data suggest that AngII regulates HO-1 and TXNIP mRNA levels either by influencing their transcription or affecting their stability.
Figure 3.1. Top 3 heavy and light transitions generated by Pinpoint to allow manual inspection and confirmation of Heavy-to-Light ratios: TSP-1 peptide GGVNDFQGVLQNVR (Upper left); PDCD4 peptide SGVPVLAVSLALEGK (Upper right); C) RHOB peptide IQAYDYLECSAK (Lower left); HO-1 peptide TEPELLVAHAYTR (Lower right). The top 3 transitions listed are light (Control treated), and the bottom 3 transitions are heavy (Ang II treated). Transition intensities are shown in brackets. Adapted with permission from Konvalinka et al. J Biol Chem 2013; 288: 34: 24834–24847.
Table 3.2 Comparison of SILAC and SRM ratios for the proteins monitored. Adapted with permission from Konvalinka et al. *J Biol Chem* 2013; 288: 34: 24834–24847.

<table>
<thead>
<tr>
<th>Protein</th>
<th>SILAC AngII/Con Ratio (median)</th>
<th>SRM AngII/Con Ratio (mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heme oxygenase-1 (HO-1)</td>
<td>1.58</td>
<td>2.12</td>
</tr>
<tr>
<td>Neoplastic transformation inhibitor (PDCD4)</td>
<td>0.68</td>
<td>0.79</td>
</tr>
<tr>
<td>Retinol dehydrogenase 10 (RDH10)</td>
<td>1.70</td>
<td>1.50</td>
</tr>
<tr>
<td>Rho-related GTP-binding protein RhoB (RHOB)</td>
<td>0.74</td>
<td>0.69</td>
</tr>
<tr>
<td>Thioredoxin-interacting protein (TXNIP)</td>
<td>0.63</td>
<td>0.63</td>
</tr>
<tr>
<td>DnaJ homolog subfamily B member 4 (DNJB4)</td>
<td>1.27</td>
<td>1.40</td>
</tr>
<tr>
<td>ADP ribosylation factor-like protein 7 (ARL4C)</td>
<td>1.39</td>
<td>2.61</td>
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<tr>
<td>La ribonucleoprotein domain family member 4 (LARP4)</td>
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<td>2.09</td>
</tr>
<tr>
<td>Protein EFR3 homolog A (EFR3A)</td>
<td>0.55</td>
<td>0.81</td>
</tr>
<tr>
<td>Guanine nucleotide exchange factor H1 (ARHGEF2)</td>
<td>1.23</td>
<td>1.13</td>
</tr>
<tr>
<td>3-hydroxy-3-methylglutaryl coenzyme A synthase (HMGCS1)</td>
<td>1.30</td>
<td>2.00</td>
</tr>
<tr>
<td>Apoptosis-associated nuclear protein (PHLDA1)</td>
<td>1.30</td>
<td>4.62</td>
</tr>
<tr>
<td>Deubiquitinating protein VCP135 (VCPIP1)</td>
<td>1.40</td>
<td>1.17</td>
</tr>
<tr>
<td>Importin subunit alpha-2 (KPNA2)</td>
<td>1.22</td>
<td>1.27</td>
</tr>
<tr>
<td>Retinoic acid-induced protein 15 (SMYD5)</td>
<td>0.75</td>
<td>0.86</td>
</tr>
<tr>
<td>Thrombospondin-1 (TSP-1)</td>
<td>0.84</td>
<td>0.74</td>
</tr>
<tr>
<td>Transmembrane protein 41B (TMEM41B)</td>
<td>1.24</td>
<td>1.56</td>
</tr>
<tr>
<td>Cervical mucin-associated protein (DBNL)</td>
<td>1.28</td>
<td>1.21</td>
</tr>
</tbody>
</table>
Figure 3.2. HO-1 protein and mRNA expression in PTECs. A) HO-1 protein expression in PTEC lysates is measured by ELISA. The results are expressed as ratio of control. Nine replicates were included. *p = 0.012 for 1-sample t-test (mean = 1.16, 95%CI = 1.04, 1.27). B) HO-1 mRNA expression is shown relative to control at 4 hours post stimulation with Control and Ang II. Twelve replicates were performed. *p = 0.018 (mean difference = 0.43, 95%CI = 0.09, 0.76). Data are represented as mean ± SEM.

A)

![Graph showing HO-1 protein expression](image)

B)

![Graph showing HO-1 mRNA expression](image)
Figure 3.3. *Tn*ip and *Dnajb4* mRNA expression in PTECs A) *Tn*ip mRNA expression relative to *Gapdh* in response to Control OR AngII stimulation for 4 hours. *p*=0.025, difference between means=-0.190 ± 0.055, 95%CI=-0.038, -0.342. B) *Dnajb4* mRNA expression relative to *Gapdh* in response to Control or AngII stimulation for 4 hours. *P*=0.33, difference between means -0.184 ± 0.182 (95%CI=-0.57, 0.20). Three replicates were performed.
3.4. Discussion

In this part of the study we addressed our second objective, namely the confirmation and verification of the AngII-regulated proteins discovered by SILAC. For confirmation using SRM, we were limited by the low-abundance nature of most of our candidates. We thus elected to focus on proteins involved in processes deemed to be important by GO analyses, such as apoptosis, regulation of intracellular kinase activity, regulation of leukocyte migration, and TGF-β binding. We also included proteins with consistent up- or downregulation across replicates, such as RDH10 and ARL4C, as well as several of Nrf2 target proteins such as HO-1, TXNIP, DNJB4, and possibly LARP4. All 18 proteins monitored by SRM assays demonstrated concordant expression between SRM and SILAC, thus confirming their differential expression in PTECs in response to AngII.

Several interesting observations related to the proteins confirmed by SRM can be made. First, Rho/Rhö associated kinase signaling is involved in AngII-mediated processes in several cell types. RHOB was found to be upregulated by AngII in a dose-dependent manner in adrenocortical cells at mRNA level\textsuperscript{295}. RHOB is known to mediate apoptosis and to be upregulated in conditions of oxidative stress and ionizing radiation. Interestingly, this protein was found in normal human urine. ARHGEF2 activates Rho GTPase by promoting GDP to GTP transition, and in this way it participates in G-protein coupled signaling. This particular protein is required for RhoA and RIP2 dependent activation of NFκB signaling. Interestingly, in tubular cells, ARHGEF2 was a necessary cofactor for TNFα-induced proliferation\textsuperscript{296}. TNFα proliferative signaling was found to be
dependent on EGFR/ARHGEF2/Rho activation, although TNF-\(\alpha\) was also capable of inducing inflammatory signaling by activating NF\(\kappa\)B in EGFR-independent manner. In this way ARHGEF2 may be one of the players implicated in wound healing and fibrogenesis in PTEC.

Other AngII regulated proteins confirmed by SRM were also implicated in important processes such as fibrosis. PHLDA1 is a protein initially found to be involved in apoptosis. It was demonstrated to mediate the anti-apoptotic effects of insulin-like factor-1. This protein was also found to be induced by endoplasmic reticulum stress\(^{297} \). Recently, endoplasmic reticulum stress was demonstrated to result in EMT in primary human PTEC. EMT was mediated by PHLDA1, also known as TDAG51\(^{298} \). This interesting protein thus appears to link endoplasmic reticulum stress and early fibrosis in PTEC. Another confirmed profibrotic protein was TSP-1. TSP-1 is an interesting adhesive glycoprotein that mediates cell-cell and cell-matrix interactions. It is capable of binding to fibrinogen, fibronectin, collagens and integrins. TSP-1 is a major activator of TGF\(\beta\)1 and it has many links to AngII. In brain cells, TGF\(\beta\)1 expression was AT-1R dependent and mediated by increased AngII via TSP-1 activation\(^{299} \). Furthermore, TSP-1 antagonist blocked cardiomyopathy induced by diabetes mellitus and elevated AngII in rats\(^{300} \). In renal mesangial cells AngII induced activation of latent TGF\(\beta\)1 was dependent on p38MAPK and JNK signaling via TSP-1\(^{301} \). Finally, in a mouse model of unilateral ureteral obstruction AngII infusion resulted in intense tubular deposition of TSP-1, which was linked to tubulointerstitial fibrosis\(^{302} \). TSP-1 may thus represent an important cellular signal for AngII induced fibrosis.
Other AngII regulated proteins have been linked to apoptosis, and they include the following: PDCD4, which interacts with EIF4A2 and is decreased by IL-2 cytokine; KPNA2 which interacts with apoptosis signal-regulating kinase 1 (ASK1) after stimulation with AngII; DBNL, which is an actin-binding adaptor protein involved in T-cell signaling; RHOB; and PHLDA1.

Proteins involved in cholesterol processing and transport include ARL4C and HMGCS1. The former transports cholesterol to the membrane for ABCA1-associated removal. The latter is an enzyme involved in the formation of HMG-CoA, a substrate for HMG-CoA reductase, important in cholesterol synthesis.

We verified the differential expression of HO-1 and other Nrf2-related proteins in PTECs after exposure to AngII. HO-1, TXNIP, and DNJB4 were confirmed to be differentially regulated by SRM. Furthermore, HO-1 and TXNIP were also differentially regulated by AngII at the level of transcription. TXNIP is an interesting protein that binds to thioredoxin and prevents it from reducing peroxiredoxin, thus blocking its anti-oxidant properties. Thioredoxin is a major intracellular thiol-reducing and ROS-scavanging protein. It reduces hydrogen peroxide by activating peroxiredoxin, and once oxidized it can be reduced by thioredoxin reductase. TXNIP has been implicated in the pathogenesis of diabetes mellitus type II. TXNIP promotes oxidative stress in vascular endothelium. It is not surprising then that Nrf2 has recently been demonstrated to diminish transcription of TXNIP in the hearts of diabetic mice by binding directly to its promoter. Nrf2 also reduced TXNIP expression in the hearts of non-diabetic animals, as evidenced by higher TXNIP expression in Nrf2 knock-out mice.
compared to wild type mice. It thus appears that AngII treated PTECs respond by activating Nrf2 which leads to changes in expression of a number of Nrf2 target proteins.

In conclusion of this chapter, we confirmed 18 proteins differentially regulated by AngII and discovered by SILAC. These 18 proteins represent the “AngII signature” proteins. Additionally, we strengthened the evidence behind the importance of HO-1 and Nrf2 in PTEC response to AngII. AngII signature proteins may in fact represent markers of distinct processes induced by AngII within PTECs, and possibly within the kidney.
CHAPTER 4

SYSTEMS BIOLOGY APPROACHES UNCOVER ANGIOTENSIN II

REGULATION OF NRF-2 TARGET PROTEINS

Sections of this Chapter have been published previously in the Journal of Biological Chemistry:

Ana Konvalinka, Joyce Zhou, Apostolos Dimitromanolakis, Andrei P. Drabovich, Fei Fang, Susan Gurley, Thomas Coffman, Rohan John, Shao-Ling Zhang, Eleftherios P. Diamandis, James W. Scholey

Determination of an Angiotensin II-Regulated Proteome in Primary Human Kidney Cells by Stable Isotope Labeling of Amino Acids in Cell Culture (SILAC)


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4.1 Introduction

The aim of a systems biology approach is to decipher the fundamental biological processes, pathways and interactions that result in certain function or outcome. Large-scale data such as proteomic or gene expression data can be represented and integrated using networks. Networks represent relationships (physical interactions, genetic interactions, common process etc.). The purpose of these networks is to allow us to group, understand, integrate and visualize high-throughput data. Networks are well-defined mathematical objects. Each node in a network typically represents a protein (although node can also represent a gene, or a Gene Ontology (GO) term). The size or colour of the node could be informative, for example represent the cellular location or the strength of evidence in support of the protein in question etc. Nodes are connected with edges which typically represent interactions between the proteins, although they could represent some shared attribute. The thickness and the colour of edges have meanings. For example, the thickness of the edge could relate to robustness of data supporting the interaction between two proteins, while the colour could indicate the experiment the data is derived from, the type of interaction etc.

Networks are thus mathematically derived methods to interpret, integrate or visualize high-throughput data.

Integration of data and creation of networks is relevant and can add information. First, network analysis can simplify and visually clarify relationships between different proteins. For example, it could group interacting proteins according to the biological processes that they mediate, or the pathways to which
they belong. Second, the analysis patterns achieved are unbiased and can lead to generation of novel hypotheses\textsuperscript{244}. Third, by combining different data layers, such as for example, protein-protein and protein-DNA interactions, we can begin to understand the control mechanisms fundamental to the observed changes\textsuperscript{245}. For example, transcription factors important in the regulation of certain genes could be elucidated. Similarly, expression data from different laboratories or from analogous models could be combined or overlaid. Those regions in the network that overlap between different experimental sets are more likely to be correct and thus biologically relevant. Networks can thus also serve to validate the results of an experiment. An elegant example of this is a study by Merico and colleagues\textsuperscript{246}, who utilized a newly developed tool called the Enrichment Map (EM), that allowed visualization of non-redundant gene sets enriched in patients with colon cancer, and overlaid them with known colon cancer genes from DiseaseHub database, which integrates genotype-phenotype information. This led to an understanding of the fundamental/functionally important gene-sets common to both data sets.

Finally, protein function could be elucidated from network data. One can infer that the function of an unannotated protein could be similar to that of its neighbours. Similarly, a protein in the biological process mediating for example, CKD progression, could be further studied in its relationship to CKD progression, even if it had not previously been linked to CKD.

We next aimed to use systems biology approach to gain better understanding of the functional features of our SILAC-derived dataset and to visually simplify these features. In this chapter, we address the third and fourth
objectives, namely we use systems biology approaches to better understand the processes critically important in PTEC responses to AngII, and we use bioinformatics methods to validate our data. To this end, we employed Cytoscape software and IPA.
4.2. Materials and Methods

4.2.1. Selection of Angiotensin II-regulated proteins for network analyses

We aimed to better understand the networks and pathways associated with proteins differentially regulated by AngII. We hypothesized that even relatively small changes in protein expression across a large number of proteins may lead to distinct physiological effects. Proteins that had H/L ratios >1.2 or <0.8 (which was ≥2 standard deviations from the mean) in any one biological replicate in SILAC experiments were included in the enrichment analysis.

4.2.2. Determination of enriched Gene Ontology (GO) terms by BiNGO and Enrichment mapping by Cytoscape

BiNGO plugin\textsuperscript{269} was used to perform the enrichment analysis. BiNGO plugin is a tool to determine which GO terms (biological processes, molecular functions, and cell compartments) are overrepresented in a set of genes or proteins. The gene names of proteins selected as above were pasted into the program. Full GO was used as a reference set. Benjamini Hochberg correction for multiple hypothesis testing was applied. The process was repeated separately for upregulated and downregulated proteins. Enriched GO terms were then visualized using EM plugin in Cytoscape (v.2.8.2)\textsuperscript{245}. Cytoscape is an open source software that allows statistical analysis and visualization of various gene or protein sets. EM is a plugin for visualization of non-redundant enrichment results generated by a different
software or plugin. Gene sets, such as pathways and Gene Ontology terms, are organized into a network called the "enrichment map". In this way, mutually overlapping gene sets cluster together, making interpretation easier. To generate an EM from BiNGO results, the following statistical thresholds were applied: FDR of 0.1, q-value of 0.05, Jaccard coefficient of 0.25 (default parameters for moderately conservative estimates).

4.2.3. Enriched functional protein groups common to Angiotensin II response in vitro and in vivo

One of the objectives of our study was to validate our data by using a bioinformatics approach, and to capture significant functional groups in our protein set that would be reproducible in a similar experiment performed in a different laboratory. We thus took advantage of the post-analysis feature in EM, which allows comparison of enriched GO terms between distinct groups of genes or proteins. This tool had been described and applied by Merico and colleagues\textsuperscript{246}. We searched the literature and identified a high-throughput study analogous to our own and performed in mice in vivo. Mice with global AT-1R knock out (KO) and wild type (WT) mice were infused with AngII\textsuperscript{303}. The kidneys of these mice were then harvested and gene expression was examined by microarray analysis. We took the list of genes differentially expressed between the two groups of mice and we overlaid it on top of our enrichment map of AngII-regulated proteins in PTEC. Post-analysis feature of EM was used. Hypergeometric Test cutoff (the
probability to find an overlap of k or more genes between a signature gene-set (mouse gene-set) and an enrichment gene-set (our AngII-regulated protein-set) by chance) was set to 0.05. This approach enabled us to appreciate the functionally important gene sets in vitro and in vivo.

4.2.4. Overrepresented pathways and networks

We next aimed to examine the overrepresented pathways and protein interaction networks using a different software. Ingenuity Pathway Analysis (IPA) (Ingenuity®Systems, www.ingenuity.com) was used to define overrepresented pathways and interaction networks. IPA allows simple and intuitive visualization of enriched canonical pathways and gene/protein interaction networks. IPA is extremely user-friendly, which makes it attractive to use. IPA uses entirely different algorithms from Cytoscape to search for enriched pathways or interacting proteins. IPA’s knowledge base is curated and includes data from publically available databases, but also included studies from medical literature that both confirm or refute particular interactions or protein/gene presence in a pathway. Standard statistical analyses are performed for both pathways and networks obtained from IPA, and they are indicative of the significance of the findings. Our input here included both up- and downregulated proteins from SILAC–labeled and AngII treated PTECs. Fold change was also included as a parameter. The degree of change upon stimulus is not taken into account by IPA, but it allows the user to visualize the direction and fold regulation of proteins displayed in networks.
4.3. Results

4.3.1. Angiotensin II-regulated proteins define enriched GO terms

We aimed to better understand the networks and pathways associated with proteins differentially regulated by AngII. This list of differentially regulated proteins from any experimental replicate was analyzed using the BiNGO plugin, separately for up- and downregulated proteins. Cytoscape is a bioinformatic software that allows complex network analysis and visualization. BiNGO plugin is a tool to determine enriched GO terms in a set of genes or proteins. Using BiNGO we defined the enriched GO terms, separately for proteins upregulated and downregulated in response to AngII. The enriched terms were then visualized by using the Enrichment Map (EM) plugin[^246], a tool for functional enrichment visualization, which minimized redundancy thus making results easier to interpret. Cellular response to AngII stimulation is visualized in Figure 4.1a. Each circle represents a significantly enriched GO term, with red colour indicating significance. The inner circle represents proteins upregulated by AngII, while the outer circle represents those downregulated. Green edges indicate that upregulated proteins are shared by the two GO terms they connect, while blue edges indicate that downregulated proteins are shared. Regulation of immune response, cell proliferation, and response to stress are enriched processes among proteins upregulated by AngII, while wound healing and regulation of cell migration are enriched among both up and downregulated proteins. Regulation of lipoprotein particle clearance was an enriched process amongst proteins.
significantly downregulated by AngII (Figure 4.1b), suggesting that AngII may play a direct role in regulating clearance of low-density lipoproteins (LDL) in PTECs.

4.3.2. Nrf2 target proteins define key functional groups in kidney response to Angiotensin II both in vitro and in vivo

We aimed to capture significant functional groups in our protein set that would be reproducible in a similar experiment performed in a different laboratory. Mice with global AT-1R knock out (KO) and wild type (WT) mice were infused with AngII. The kidneys of these mice were then harvested and gene expression was examined by microarray analysis. By taking the list of genes that were differentially expressed between the two groups of mice, and overlaying it on top of the EM presented in Figure 4.1a, we generated Figure 4.1c, which has one distant node containing significant genes from the mouse experiment. The pink edges represent overlap between the mouse gene-set and the enriched gene-sets from our experiment. Five GO terms were thus enriched in both gene sets: regulation of response to stimulus (p=0.027), regulation of immune response (p=0.011), response to stress (p=0.011), organelle lumen (p=0.013), and membrane enclosed lumen (p=0.011). Genes responsible for significance of the term “regulation of immune response” included: Hmox1, Cfb, Bcar1, C1r, C1in, Tlr3, and Fyn. Three of these genes (Cfb, C1r and C1in) are directly related to the complement system. Bcar1, Tlr3, and Fyn are important for adaptive and innate immunity. Genes responsible for significance of the term “response to stress” are shown in Figure 4.1c. Red arrows point to genes in the Nrf2 pathway. Hmox1 (encoding HO-1) is one of these genes, and is common to all five enriched nodes
shared between the two datasets. Hmox1 was >8 fold upregulated in WT compared to AT-1R KO mice following AngII infusion. The systems biology approach demonstrated that HO-1 protein and Nrf2 pathway are functionally important in response of the kidney to AngII.

4.3.3. Nrf2 and mTOR pathways and top protein networks

We next used IPA to assess the overrepresented pathways amongst our 83 AngII-regulated proteins. The top five enriched canonical pathways included: mTOR, p70S6K, Phospholipase C, anti-proliferative role of TOB in T-cell signaling, and super-pathway of cholesterol biosynthesis. mTOR pathway is displayed in Figure 4.2a. Nrf2 oxidative stress pathway was also significantly enriched (p=0.039) (Figure 4.2b). Protein networks with scores ≥34 (equivalent to FDR ≤0.06%) included NFκB, PDGF, TGFβ, and ubiquitin-related networks (Figure 4.3a-c).
Figure 4.1. Enrichment map (EM) of enriched GO terms amongst proteins differentially regulated by AngII in Cytoscape. EM is depicted in the left upper corner of each image, with a square indicating the zoomed-in area in the right panel. Each circle represents an enriched GO term, with red colour indicating significance. The inner circle represents proteins upregulated by AngII, while the outer circle represents those downregulated. Green edges indicate that upregulated proteins are shared by the two GO terms they connect, while blue edges indicate that downregulated proteins are shared. A) The zoomed in image on the right demonstrates that ‘response to stress’ is an enriched process in proteins upregulated by Ang II, while ‘wound healing’ is enriched in both up and downregulated proteins. B) A zoom-in on nodes significantly enriched amongst proteins downregulated by AngII. Processes related to regulation of lipoprotein clearance are dominant. C) Genes differentially regulated in kidneys of mice following AngII infusion are overlaid on top of the original EM in A. The pink edges represent overlap between the mouse gene-set and the enriched gene-sets from our experiment. Genes responsible for significance of the GO term ‘response to stress’ are displayed. Five genes with red arrows pointing to them are in the Nrf2 pathway. Adapted with permission from Konvalinka et al. *J Biol Chem* 2013; 288: 34: 24834–24847.
Figure 4.2. Ingenuity Pathway Analysis (IPA) generated canonical signalling pathways. A) mTOR pathway. Grey-coloured proteins are regulated by AngII in our dataset. B) Nrf2 pathway. TXNIP is also regulated by Nrf2, but is not shown in this image. Red proteins are upregulated by AngII in our dataset, while green ones are downregulated. White nodes were not identified as differentially regulated in our dataset.

A)
B)
Figure 4.3. Significant interaction networks of Ang II-regulated proteins generated by IPA. A) Proteins interacting with TGFβ and PDGF, ERK and caspase. B) Protein network with NFκB hub. C) Proteins interacting with ubiquitin. Red proteins are upregulated by AngII in our dataset, and the intensity of colour indicates greater upregulation in response to AngII, while green proteins were downregulated in response to AngII. White nodes were not identified as differentially regulated in our dataset.

A)
4.4 Discussion

In this chapter, we address the third and fourth objectives, namely we use systems biology approaches to 1) better understand the processes critically important in PTEC responses to AngII, and to 2) validate our SILAC data. First,
we utilized Cytoscape and BiNGO plugin to understand the biological processes and molecular functions among proteins perturbed by AngII in primary PTECs. We expanded the number of proteins to include those with evidence of differential regulation in any replicate, in order to capture a “footprint” of the key processes affected by AngII. Regulation of immune response, cell proliferation, and response to stress were enriched processes among proteins upregulated by AngII, while wound healing and regulation of cell migration were enriched among both up and downregulated proteins. Not surprisingly, inflammation and early wound healing/fibrogenesis were important cellular responses to AngII. Proteins downregulated by AngII were involved in processes of lipoprotein and cholesterol clearance. In vivo, increased exposure of tubules to non-albumin proteins such as iron, transferrin, LDL and complement components has the potential to further promote tubular injury. LDL may be toxic to renal cells and hyperlipidemia has been linked to a more rapid CKD progression. Although the mechanism remains uncertain, some in vitro evidence suggests that mesangial cells have LDL receptors and that stimulation of these receptors leads to increased secretion of fibronectin, collagen, PAI-1, and ROS. Furthermore, Zager et al noted an increase in PTEC cholesterol content after acute kidney injury, as a potentially cytoprotective effect to maintain plasma membrane integrity. Our analysis suggests that AngII may be involved in the regulation of LDL clearance from PTEC.

We next aimed to validate our findings by using a systems biology approach. We also intended to capture significant functional groups in our protein
set that would be reproducible in a similar experiment performed in a different laboratory. We examined which enriched functional groups of proteins from our experiment were also significant in a dataset of differentially expressed kidney genes from the AngII-infused WT mice and AT-1R KO mice. Five functional groups were common to both datasets, suggesting common and potentially fundamental findings across two experimental platforms. HO-1 was the most prominent individual protein regulated by AngII in our SILAC experiments, and it was also present in all five functional groups of genes. Furthermore, Nrf2 target genes were functionally important in AngII-infused mice, suggesting that this pathway plays an important role in PTEC in vitro as well as whole kidney in vivo responses to AngII. Another interesting and informative signal was related to immune response. Immune response was functionally important in kidney reaction to AngII. As discussed previously, AngII causes inflammation, and can recruit immune cells. The signal for immune cell recruitment appears to be an important early signal, and these infiltrating cells together with complement may cause subsequent damage to the kidney. Our findings also suggest that complement components may be produced locally by the PTECs in response to AngII. These findings demonstrated several significantly enriched important functional groups were common in vitro and in vivo and thus provided support for our SILAC-based approach for discovery of AngII-regulated proteins. Finally, these key functional protein groups appear to be activated by AT-1R, as suggested from the mouse model.
The examination of important pathways and interacting proteins further consolidated our conclusions regarding the key PTEC responses to AngII. The top canonical pathway was mTOR/ p70S6K. mTOR/70kDa ribosomal S6 kinase are serine threonine kinases that promote protein translation, cell growth, and cell phenotype transition. There is evidence that these kinases can play a role in fibrosis. EGFR transactivation by AngII-activated AT-1R leads to mTOR activation, and has been implicated as a critical mediator of fibrosis in animal models of renal disease. AngII-mediated activation of AT-1R was previously linked to downstream mTOR activation, and these events can lead to fibrosis in tubular cells. AngII also activates mTOR/p70S6K in cardiomyocytes, VSMCs, and endothelial cells, leading to hypertrophy, remodeling and insulin resistance. This suggests that PTECs stimulated with AngII demonstrate early signals of cell growth and fibrosis.

Nrf2 canonical pathway was again demonstrated to be enriched among our AngII-regulated proteins. The algorithms used by IPA are distinct from those used by BiNGO and EM. Arriving at the same conclusion by applying these different methods gives further support to the idea that this pathway is important in PTEC and kidney response to AngII.

Finally, we examined protein interaction networks that were overrepresented among our AngII-regulated proteins. The top networks were congruent with prior understanding of AngII effects on the kidney, particularly its role in proliferation, hypertrophy, hypertension, inflammation and fibrosis.
Network that included profibrotic growth factors such as TGF-β and PDGF was significantly overrepresented, providing further evidence that our in vitro model was able to replicate well-described and chronic effects of AngII on the kidney. Additionally, NFκB-related network of proteins was overrepresented, thus demonstrating that inflammatory responses of PTECs were important. Ubiquitin-related proteins are known to participate in biological processes that involve apoptosis, cell division, response to stress, immune response and inflammation. These data further strengthened our in vitro approach by demonstrating that despite studying early effects of AngII on PTECs we were able to detect processes implicated in CKD.

Overall, in this chapter we demonstrated that processes such as response to stress, wound healing and lipoprotein clearance were enriched among our AngII-regulated proteins. We further validated our in vitro approach by using systems biology. Finally, we showed that hypertrophy, inflammation and fibrosis were significantly enriched among our AngII-regulated proteins, an observation congruent with previously established understanding of the AngII effects on the kidney.
CHAPTER 5

VALIDATION OF ANGIOTENSIN II-MEDIATED UPREGULATION OF HEME OXYGENASE-1 PROTEIN IN A MOUSE MODEL

Sections of this Chapter have been published previously in the Journal of Biological Chemistry:

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Determination of an Angiotensin II-Regulated Proteome in Primary Human Kidney Cells by Stable Isotope Labeling of Amino Acids in Cell Culture (SILAC)


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5.1. Introduction

Utilizing an unbiased, quantitative proteomics-based approach we uncovered 83 AngII-regulated proteins in primary human PTECs in vitro. We then confirmed and verified 18 AngII-regulated proteins in PTECs by using SRM, ELISA and qRT-PCR. HO-1 emerged as the top hit from the initial discovery approach, and it was subsequently confirmed and verified. We next utilized a systems biology approach, and demonstrated that HO-1 upregulation was functionally important in kidneys of mice, and that it was likely AT-1R dependent.

Based on the mouse study comparing kidney gene expression in AngII-treated WT and AT-1R global KO mice, HO-1 gene expression was dramatically induced by AngII treatment and this increase appeared to be AT-1R-dependent. Given the 8-fold upregulation at the level of mRNA, it is likely that whole kidney HO-1 protein expression was also increased in WT compared to KO mice in response to AngII. Rather than testing the hypothesis that HO-1 protein expression in the whole kidney was increased in response to AngII-mediated AT-1R signaling, we wanted to also examine whether PTECs played a key role in AngII-mediated HO-1 upregulation in the kidney. PTECs were used in prior in vitro experiments to establish the proteomic AngII signature, and we aimed to understand whether this experimental design would be informative of at least some protein changes in the whole kidney. An ideal setting to test our hypothesis that HO-1 expression in the kidney is dependent predominantly on AngII-mediated AT-1R signaling in PTECs would be in mice with PTEC-specific AT-1R KO.
One of the reasons for selecting PTECs for the study of AngII signature proteins was that fact that these cells communicate with urine directly and secrete proteins which would have the potential to inform intra-renal AngII bioactivity in vivo. HO-1 was found to be differentially regulated in our PTEC secretome analysis, providing evidence that this protein may be found in urine, and may ultimately serve as a marker of intra-renal AngII bioactivity.

In this chapter, we examined whether HO-1 protein expression was increased following AngII infusion in the kidneys of mice with PTEC-specific AT-1R knock out (PTKO), whether this increase was AT-1R dependent, and finally, whether we could measure HO-1 in urine. Here we address our final objective, namely we demonstrate the relevance of our in vitro findings in vivo.
5.2. Materials and Methods

5.2.1. Generation of mice with AT-1 receptor knock out specifically in proximal tubular cells

Mice with a conditional Agtr1a allele were generated as described previously\textsuperscript{261,316}. Briefly, a targeting vector was generated containing a PGK-Neo cassette for positive selection with loxp sites engineered in positions flanking exon 3 of the Agtr1a gene. Correctly targeted 129 ES cell clones were identified by Southern analysis and the PGK-Neo cassette was removed by transient transfection with Flp recombinase. In the presence of Cre recombinase, a null allele was generated. Appropriately modified ES cell lines were expanded and injected into blastocysts to generate chimeras. Germ-line transmitting chimeras were bred with wild-type 129/SvEv mice (Taconic) to generate inbred 129/SvEv Agtr1aflox/+ mice. The Pepck-Cre transgene was back-crossed more than 10 generations onto the 129/SvEv background and these 129/SvEv PEPCK Cre mice were bred with 129/SvEv Agtr1aflox/flox mice to generate proximal tubule knock out (PTKO) mice. The pattern of transgene expression in proximal tubule was verified by inter-crossing with the ROSA26R reporter line. Agtr1a gene expression, quantified using qRT-PCR, was reduced by 45% in Cre+Agtr1aflox/flox (PTKO) mice compared to Cre-Agtr1aflox/flox controls (p=0.01). In order to confirm deletion of AT-1A receptor protein, specific binding of \textsuperscript{125}I-[Sar1, Ile8] AngII was examined using quantitative autoradiography. Non-glomerular, AT1-specific \textsuperscript{125}I-
AngII renal cortical binding was markedly reduced by \( \approx 40\% \) in kidneys from PTKO mice compared to controls; whereas the extent of glomerular binding was virtually identical between the groups. PTKO and other mice were bred and maintained in the animal facility at the Durham VA Medical Center according to NIH guidelines. One mouse from the PTKO group was excluded from analyses because it had the smallest kidneys, and the highest levels of urine and tissue HO-1 expression meeting the criteria of an outlier, by Dixon’s Q-test at \( \alpha=0.05 \). We concluded that this mouse was sick due to factors unrelated to genetic manipulation or treatment.

5.2.2. **Infusion with Angiotensin II infusion**

Angiotensin II (Sigma A9525) was dissolved in sterile saline (0.9% NaCl) and infused via osmotic minipump for 15 days at 1000ng/kg/min. Animals were sacrificed and kidneys were harvested, weighed, decapsulated and immediately frozen in liquid nitrogen. 24-hour urine collection was performed on day 13 during AngII infusion. Urine samples were stored at -80°C.

5.2.3. **Immunohistochemistry of HO-1 in mouse kidney tissue**

Fresh frozen mouse kidneys were embedded in OCT compound and cut into sections at 5 microns and stained with HO-1 polyclonal antibody (Stressgen Assay, Cedarlane Corporation SPA-895-D). Santa Cruz ABC kit (SC-2018) IHC
protocol was followed. Briefly, sections were incubated with primary antibody overnight at 4°C. The sections were then washed with three changes of PBS for 5 minutes each and incubated for 30 minutes with biotinylated secondary antibody at approximately 1 µg/ml. Sections were subsequently washed again with three changes of PBS for 5 minutes each. Sections were subsequently incubated with AB enzyme reagent for 30 minutes and washed with three changes of PBS for 5 minutes each. Finally, sections were incubated in 2 drops peroxidase substrate until desired stain intensity developed. Sections were lastly washed in deionized H₂O for 5 minutes. Permanent mounting medium was added immediately, and sections were covered with a glass coverslip and observed by light microscopy.

5.3.3. HO-1 protein quantification in mouse kidneys by Western blot

Mouse kidneys were weighted and then cut into small pieces. Tissue was then washed with ice-cold PBS two times. Eight µL per mg of renal tissue of modified RIPA buffer (150mM sodium chloride, 50mM Tris-HCl (pH 7.4), 1mM EDTA, 1% v/v Triton-X 100, 1% w/v sodium deoxycholic acid, 0.1% v/v SDS) was added. Tissues were then sonicated two times for 10 seconds, and incubated on ice for 30 minutes. Following centrifugation at 14,000 rpm for 10 minutes, supernatant was transferred to a tube with loading buffer and placed into boiling water for 5 minutes. Proteins were then run on a 7% SDS-PAGE and stained with HO-1 mouse antibody (Abcam 13243).

5.3.4. HO-1 urine excretion in mice by ELISA
Mouse urine samples were thawed overnight at 4°C, and then centrifuged for 20 minutes at 1000g. Pellets were discarded and urine samples were collected and assayed as per manufacturer’s instructions. Mouse HO-1 ELISA kit (USCN Life Science Inc. E90584Mu) was used to measure urine HO-1 protein levels. Urine creatinine was measured by the Jaffé colorimetric method\textsuperscript{317}. TDS Wilson software was used to plot and analyse optical density measurements. Final HO-1 concentrations were expressed as pg/µmol Creatinine.

5.3.4. Statistical analysis

All values are reported as mean ± SEM, unless stated otherwise. Statistical comparison between experimental groups was performed using a two-tailed Student’s t test. P-values <0.05 were considered statistically significant, unless stated otherwise. Statistical language R (v. 2.13.1) was used for linear regression. GraphPad Prism 5 software was used for other statistical tests (GraphPad, La Jolla, CA, USA).

5.3. Results

5.3.1. Validation of AT-1 receptor dependent Angiotensin II-mediated upregulation of HO-1 in mouse kidney
Mice with AT-1R PTKO had been generated and characterized previously261. These mice displayed a normal phenotype. AT-1R PTKO mice and control littermates (WT) were infused with AngII (1000ng/kg/min) for 15 days. In response to AngII infusion, AT-1R PTKO mice had significantly lower blood pressure compared to AngII-infused WT mice261.

We wanted to examine whether HO-1 expression was detectable in kidneys of WT mice infused with AngII, and whether this expression was more dominant in the PTECs of the same mice. We thus performed immunohistochemistry (IHC) staining for HO-1 of AngII-treated WT mouse kidneys. IHC demonstrated increased HO-1 expression in cortex relative to medulla (Figure 5.1a). Intense cortical staining was observed predominantly in the proximal tubules (Figure 5.1b). To quantify renal HO-1 expression, we performed Western blotting of kidney tissues. Blot images and densitometry are presented in Figure 5.2a. WT mice showed a clear trend toward higher HO-1 renal expression levels compared to AT-1R PTKO mice (p=0.11).

5.3.2. HO-1 protein urine excretion rate is directly correlated with HO-1 kidney content and is dependent on Angiotensin II signaling through AT-1 receptor

We demonstrated that AngII treatment led to increased HO-1 staining in PTECs compared to adjacent distal tubules and glomeruli of WT mice, and that these
mice displayed a clear trend toward higher HO-1 renal expression levels compared to AngII-treated AT-1R PTKO mice. We next evaluated urine HO-1 protein excretion in WT and AT-1R PTKO mice. Urine had been collected on day 13 of AngII infusion. Box and whiskers plot showing HO-1 urine protein excretion adjusted for urine creatinine is displayed in Figure 5.2b. A clear separation between the two groups is depicted, with a trend toward higher HO-1 excretion in the WT compared to PTKO animals (p=0.12). Furthermore, there was a positive linear correlation between tissue and urine HO-1 protein level (Figure 5.3), so that for every unit increase in tissue HO-1, there was a 194pg/µmolCr increase in urine HO-1 (p=0.048).
Figure 5.1. HO-1 IHC staining of kidneys from WT mice treated with AngII. Representative fresh frozen sections are shown. **A**) Cross-sectional view of the kidney at 2X magnification. Increased cortical HO-1 staining relative to medulla is demonstrated. **B**) 40X magnification; increased HO-1 staining is noted in proximal tubules (red arrows), while neighboring distal tubules (blue arrows) demonstrate less staining. Adapted with permission from Konvalinka et al. *J Biol Chem* 2013; 288: 34: 24834–24847.
**Figure 5.2.** *In vivo* quantification of HO-1 protein in mice with PTEC-specific AT-1R KO.  
**A)** Western blotting for HO-1 in wild type mice (WT) and mice with PTEC-specific AT-1R knock out (KO) treated with a 15-day infusion of AngII. Densitometry readings for HO-1 adjusted for β-actin are represented as mean±SEM. P=0.11, diff between means=0.43±0.24 (95%CI: -0.11, 0.97).  
**B)** Urine HO-1 measurement by ELISA and adjusted for creatinine in WT and KO mice. Horizontal lines represent 1st, 2nd, and 3rd quartiles, and whiskers the range. P=0.12, diff between means=132.8±78.2 (95%CI: -44.2, 309.7). Same eleven mice were used in both experiments. Adapted with permission from Konvalinka et al. *J Biol Chem* 2013; 288: 34: 24834–24847.
**Figure 5.3.** Correlation between HO-1 kidney tissue expression (densitometry units of HO-1/β-actin) and HO-1 urine protein excretion (pg/µmol Creatinine). Linear regression line is displayed. p=0.048; β= 194.3; 95%CI: 27.1, 361.5. Adapted with permission from Konvalinka et al. *J Biol Chem* 2013; 288: 34: 24834–24847.

5.4. **Discussion**

In this chapter, we examined whether 1) HO-1 protein expression was increased following AngII infusion in the kidneys of mice with PTEC-specific AT-1R knock out (PTKO), 2) whether this increase was AT-1R dependent, and finally, 3) whether we could measure HO-1 in urine. In this chapter we addressed our last
objective, namely we demonstrated that our AngII-regulated proteome defined in *vitro* reflected the effects of AngII on PTECs *in vivo*.

In previous chapters we defined AngII-regulated proteins by using SILAC in PTECs *in vitro*. HO-1 protein was our top candidate, and we went on to confirm and verify upregulation of this protein in response to AngII in PTECs. Furthermore, systems biology approaches demonstrated that HO-1 was one of the key functional genes upregulated after treatment with AngII in kidneys of mice *in vivo*. Based on these observations, we went on to examine whether AngII effects in PTECs *in vitro* were relevant in mice with PTEC-specific AT-1R KO *in vivo*. These mice were chosen because they fully recapitulated our *in vitro* model, and because they enabled us to test whether at least some of the effects of AngII were AT-1R dependent.

First, we demonstrated that HO-1 protein expression was predominant in PTECs of WT mice treated with AngII. We next demonstrated that mice with AT-1R PTKO had a trend toward lower HO-1 kidney protein expression, suggesting that AngII upregulates HO-1 in mouse kidneys via AT-1R, and that PTECs contribute substantially to kidney HO-1 content. Literature suggests that HO-1 is only weakly expressed in the healthy kidney (The Human Protein Atlas, http://www.proteinatlas.org/), and its expression is localized to proximal and distal tubules, the loop of Henle, and medullary collecting tubules. Upon injury, HO-1 is upregulated in the tubules. Despite this broad tubular expression of HO-1, we found a trend in difference in HO-1 expression between AT-1R PTKO and WT mice following AngII infusion even when genetic manipulation was limited to
proximal tubules. Moreover, AT-1R gene deletion with Cre recombinase resulted in only 50% reduction in gene expression, which likely also accounted for the lack of significance in HO-1 expression between the two mouse groups. We suggest that AT-1R was responsible for AngII-mediated HO-1 induction. Several other studies have corroborated this observation\textsuperscript{272,274,319}.

Mice with AT-1R PTKO had demonstrated diminished hypertensive response to systemic AngII infusion\textsuperscript{261}, and the question arises as to the effect of hypertension in stimulating HO-1 upregulation. We believe that upregulation of HO-1 was AngII-specific, and not linked to its hypertensive effect. First, AngII was capable of inducing HO-1 in our PTECs \textit{in vitro}, arguing against the effect of hypertension. Second, in previous experimental models of non-AngII mediated hypertension such as DOCA-salt sensitive rats and rats infused with norepinephrine, hypertension failed to induce renal HO-1 expression\textsuperscript{270,272}. It thus appears that the ability of AngII to induce renal HO-1 is not related to the hypertensive effect of this peptide.

Our final aim was to relate differences in tissue expression to changes in urine excretion. We demonstrated that HO-1 protein was measurable in urine of mice during AngII infusion, and displayed a trend toward lower excretion in animals with AT-1R PTKO compared to WT mice, which was paralleled by the findings in kidneys of these same animals. HO-1 protein excretion in urine is low or undetectable in normal humans and mice, but was increased in humans and animals with AKI\textsuperscript{320}, another setting where RAS activation may contribute to kidney injury\textsuperscript{321}. HO-1 protein expression was also increased in tubular cells.
isolated from urine of patients with tubulointerstitial disorders. Finally, we found a direct linear relationship between kidney HO-1 protein expression and urine HO-1 excretion, suggesting that HO-1 excreted in urine is determined by its renal production. These findings suggest that novel markers of RAS activity in PTECs of the kidney can be measured in urine.

Literature addressing markers of RAS activity in the kidney has focused primarily on measuring urine excretion rates of angiotensinogen. Angiotensinogen is a precursor protein that is enzymatically cleaved by renin to generate angiotensin I, which is subsequently cleaved by angiotensin converting enzyme to generate AngII. Several studies have reported increased urinary angiotensinogen excretion in patients with CKD, and in a few studies angiotensinogen excretion correlated with CKD progression. The rationale for measuring substrate levels is that plasma angiotensinogen level is poised near the Km for cleavage by renin, with the consequence that alteration of angiotensinogen levels also affects the formation of AngII. Nonetheless, intra-renal RAS is complex, and substrate measurements likely do not represent the true degree of RAS bioactivity. We provide the first evidence of “AngII signature proteins” differentially expressed by PTECs, which could prove useful as markers of AngII activity in the kidney.
CHAPTER 6

SUMMARY AND FUTURE DIRECTIONS
6.1. Summary

CKD is prevalent worldwide and gives rise to major morbidity and mortality. RAS has been implicated in the progression of CKD. Experimental evidence demonstrated the existence of intra-renal RAS, and the importance of the local RAS activity in the progression of CKD. The main effector of RAS is AngII, which exerts adverse hemodynamic and non-hemodynamic effects on renal cells by acting on AT-1Rs, and has been implicated in renal hypertrophy, inflammation and fibrosis. RAS blockade has emerged as the principal class of agents to halt or slow down progression of CKD. However, the response to RAS blockade is not uniform, and has been linked to significant side-effects and increased morbidity in subgroups of patients. It would thus be of great clinical interest to develop a footprint of AngII bioactivity in order to guide treatment and inform progression.

PTECs are specialized renal cells that are critically important for the generation of intra-renal AngII, and that contain AT-1Rs allowing them to respond to both local and systemically-formed AngII. The rationale for this study was thus to understand PTEC responses to AngII, in order to further our understanding of the effect of AngII on the kidney. Better understanding of AngII-regulated proteins in PTECs will help gain insight into the mechanisms of RAS-mediated renal damage, and could further help define novel markers of CKD progression, and responsiveness to RAS blockade.

There were four main goals of this study: 1) to define the proteome of AngII-stimulated primary human PTECs; 2) to confirm/verify AngII-regulated proteins; 3) to use bioinformatics-based approaches to determine the key
biological processes mediated by AngII-regulated proteins; and 4) to extend *in vitro* observations in PTECs to *in vivo* observations in the kidney.

The principal techniques utilized and key findings from the three parts of the study are summarized below:

1) **Defining AngII Proteome**

- SILAC-based quantitative proteomics strategy involving PTEC labeling with heavy and light arginine and lysine and extensive in-solution fractionation followed by LC-MS/MS using high accuracy and high sensitivity instrument LTQ-Orbitrap was utilized.
- Five experimental replicates of AngII and control-treated PTECs were performed, and four of them focused on PTEC lysates, while one examined the PTEC secretome.
- We identified 5011 proteins and quantified 4975 in the four cell lysate experiments together with one supernatant experiment (at 1.0% FDR). This represents the most extensive in-depth study of renal cell proteome.
- In four lysate replicates, 83 proteins were differentially regulated in response to AngII. Out of these, 53 were upregulated, and 30 were downregulated.
- The top candidate protein upregulated with AngII treatment in all 4 cell lysates and the supernatant was HO-1. HO-1 is a transcription factor activated in response to oxidative stress. Three other proteins upregulated with AngII stimulation were Nrf2 targets.
• Apoptosis was a significantly enriched biological process among the AngII-regulated proteins, and the most enriched cell compartment was endoplasmic reticulum.

2) Confirmation and Verification of AngII-regulated proteins

• We utilized SRM-based assays to confirm differential regulation of AngII proteome in PTECs.
• We monitored peptides of 18 AngII-regulated proteins in PTEC lysates. The expression of all 18 proteins in response to AngII was concordant with SRM and SILAC approaches.
• Increased HO-1 protein expression in response to AngII was verified independently using ELISA in primary human PTECs.
• Furthermore, HO-1 and TXNIP were demonstrated to be differentially expressed at the level of mRNA by using qRT-PCR in primary PTECs. The direction of their expression supported Nrf2-mediated regulation and was concordant with our SILAC data.

3) Systems Biology Approaches to Understanding of AngII-induced processes and pathways

• BiNGO plugin of Cytoscape software was utilized to determine statistical enrichment of GO terms among AngII-regulated proteins. EM plugin of Cytoscape was used to visualize the network of significant GO terms.
• Regulation of immune response, cell proliferation, and response to stress were enriched processes among proteins upregulated by AngII, while wound healing and regulation of cell migration were enriched among both up and downregulated proteins.

• Regulation of lipoprotein clearance was enriched among proteins downregulated in response to AngII.

• EM plugin of Cytoscape was next used to validate our findings from SILAC-derived dataset. It was also applied to define the key functional protein groups.

• Overlaying our map of AngII-mediated effects on PTECs with a geneset of differentially regulated kidney genes between AngII-treated WT mice and mice with global AT-1R deletion demonstrated five functional enriched sets. These sets included: regulation of response to stimulus (p=0.027), regulation of immune response (p=0.011), response to stress (p=0.011), organelle lumen (p=0.013), and membrane enclosed lumen (p=0.011).

• HO-1 was represented in each of the five enriched functional groups, thus suggesting that this protein was important in the whole kidney response to AngII in vivo. Five Nrf2 target proteins determined the significance of GO term response to stress, suggesting that Nrf2 activation plays a key functional role in renal response to AngII.

• IPA was used to determine the overrepresented canonical pathways and interaction networks of AngII regulated proteins.
• mTOR/ p70S6K was the top canonical pathway enriched among AngII regulated proteins, suggesting that cell growth and fibrosis represent early AngII-related signals in PTECs.

• Nrf2 was again demonstrated to be significantly enriched canonical pathway among AngII-regulated proteins.

• Network that included profibrotic growth factors such as TGF-β and PDGF was significantly overrepresented, providing further evidence that our in vitro model was able to replicate well-described and chronic effects of AngII on the kidney.

• NFκB-related network of proteins was overrepresented, thus demonstrating that inflammatory responses of PTECs were important.

• Ubiquitin-related proteins are known to participate in biological processes that involve apoptosis, cell division, response to stress, immune response and inflammation.

4) **PTEC Responses to AngII in vivo**

• We examined kidneys of mice with PTEC-specific AT-1R deletion and WT mice infused with AngII.

• AngII treatment increased HO-1 expression predominantly in PTECs of WT mice.

• There was a clear trend toward higher HO-1 renal expression levels in WT compared to AT-1R PTKO mice in response to AngII-treatment.
• There was also a clear trend toward higher urine HO-1 excretion in AngII-treated WT mice compared to AngII treated AT-1R PTKO mice.

• There was a significant linear correlation between kidney HO-1 expression and urine HO-1 excretion in the experimental mice.

Our findings thus suggest that PTECs respond in a consistent manner to AngII, and that HO-1 protein and Nrf2 pathway may be critically important in PTEC responses to AngII in vivo. HO-1 can be measured in urine of mice and appears to reflect renal response to AngII.

There are several strengths of our study. First, the strengths of our SILAC-based approach include: excellent proteome coverage using extensive fractionation, instruments with high sensitivity and accuracy, multiple biological replicates and use of reverse labeling, and control experiments to minimize false positive hits. Additionally, primary human PTECs were used in this study. AngII-regulated proteome thus represents PTEC responses from 3 distinct individuals, thus signifying true biological variability, not typical of transformed cell culture models. To the best of our knowledge, this is the first effort to date to characterize proteomic responses of human kidney cells to AngII stimulation.

Second, our discovery proteomic study was followed up by SRM-based confirmation of a large number of candidates, as well as ELISA-based verification of HO-1, and qRT-PCR-based verification of three Nrf2 target proteins.

Third, we performed extensive bioinformatic analyses of AngII-regulated proteins to define the enriched processes, functions and cell compartments.
Furthermore, we used an innovative approach to validate our findings using systems biology and to determine the key functional protein sets, by statistically combining our data and data from the independent experiment performed in mice.

Lastly, we validated our main observations related to HO-1 in a mouse model *in vivo*.

Despite our novel findings, our study has several limitations. Most significantly, we studied PTEC responses to AngII at a single time point, while the expression levels of proteins are likely to be dynamic rather than static. Although we studied a single time point, the variety of processes detected have been implicated in AngII-related and CKD processes. The secretome analysis was limited to a single replicate, and thus could contribute only marginally to the overall findings. Some proteins were not amenable to SRM quantification due to their low expression levels in PTECs. Finally, our study was limited by the availability of kidney tissue and urine samples from mice with AT-1R PTKO.

In conclusion, we present the first study of human primary PTEC responses to AngII. We identified and confirmed 18 AngII signature proteins, which revealed biological processes of AngII activity. HO-1 emerged as the top AngII-regulated candidate in our *in vitro* and systems biology approaches. HO-1 was also regulated by AngII *in vivo*, and this was reflected in urinary measurements. These findings suggest that AngII signature proteins measured in urine may represent markers of AngII bioactivity in the kidney in patients and in experimental models.
6.2. Future directions

The work from this thesis describes novel insights into biology of AngII, and its effects on renal PTECs. Our study has generated information that can further be extended in several directions: 1) *in vitro* studies of AngII signature, 2) animal studies of AngII signature proteins, and 3) human translational studies of AngII signature.

Importantly, while the link between AngII and HO-1 was established previously, several interesting questions remain unanswered. For example, the dynamic nature of HO-1 expression has not been established in response to AngII, nor is it clear what happens to HO-1 in a setting of CKD, and why and at what point it becomes depleted. It would thus be interesting to study HO-1 and other AngII signature proteins in PTECs isolated from patients with CKD, and also to measure HO-1 longitudinally after exposure to AngII.

HO-1 upregulation was shown to represent a protective response in the kidney. However, it is unclear whether early HO-1 upregulation in PTECs and in mouse kidneys represents a protective response to AngII. Thus it would be interesting to use siRNA specific for HO-1 in PTECs, and then treat these cells with AngII to delineate any changes in expression of AngII-regulated proteins. Also, agonists of HO-1 could be used in cells prior to treatment with AngII. Any changes in expression of AngII-regulated proteins and processes discovered to be important in PTEC response to this agent could be measured. Finally, we discovered that proteins involved in immune response were important in both *in
vitro and in vivo response to AngII. Given the importance of inflammation and complement system in the progression of kidney disease, it would be interesting to study whether these proteins are adequately suppressed in a setting of AT-1R blockade in PTEC.

A similar approach can be used to determine the usefulness of AngII signature proteins as markers of AngII activity in experimental models. For example, the effect of AT-1R blockade on our AngII-regulated proteins in the relevant mouse models, such as a model of diabetic nephropathy or Alport’s disease should be studied. Both of these diseases are known to result in intra-renal activation of RAS, and RAS blockade has been linked to delayed disease progression, particularly in models of diabetic nephropathy. We have already demonstrated (manuscript in preparation) that many AngII signature proteins including HO-1 are dysregulated at mRNA level in the kidneys of 7-weeks old mice with Alport’s disease. We can next correlate kidney tissue expression and urine excretion of HO-1 and other AngII signature proteins with direct measures of intra-renal RAS components. We hope to demonstrate that AngII induces HO-1 and differentially regulates the proteins that we discovered in relevant animal models of renal disease.

Furthermore, HO-1 agonists have therapeutic potential. In several models of experimental renal disease HO-1 agonists diminished renal injury. The agonist response of HO-1 on development and progression of renal disease should be addressed. Also, future studies should address how HO-1 agonists affect AngII signature in the kidney and/or urine of experimental animals. Similar studies and
considerations apply to other Nrf2 target proteins. Better understanding of Nrf2 responses to AngII and other types of renal injury are necessary to delineate how and when this pathway is protective, and when the protection could be lost.

Third, our study has generated potential markers of AngII bioactivity, which can be tested further in patients with CKD. We have demonstrated that HO-1 can be measured in urine of mice, and that it reflects renal response to AngII. Next we intend to test HO-1 and other AngII signature proteins as biomarkers of AngII activity in the relevant patient populations. We aim to develop and optimize SRM-based assays for monitoring of these proteins in human urine. Several SRM-based studies of urine have been reported\textsuperscript{323,324}, and these studies can be used to inform development and optimization of our methods. We have access to two relevant patient populations with archived urine samples. The first population consists of low-risk renal transplant recipients who had protocol biopsies, and regular collection of biospecimens including urine. These patients demonstrated a dramatic increase in tubulointerstitial fibrosis two years post-transplantation, and this increase in fibrosis was significantly negatively correlated with the use of RAS blockers. HO-1 and other AngII signature proteins could be measured in urine of these transplant patients by a combination of SRM and ELISA, to determine whether urine excretion of our AngII-signature proteins predicts development of fibrosis and correlates with the use of RAS blockers. The second patient population consists of longitudinally followed patients with autosomal dominant polycystic kidney disease who had prospective MRI-based kidney and cyst size measurements as predictors of prognosis. Patients with this disease are
particularly prone to intra-renal and intra-cystic RAS upregulation and increased AngII activity, which has been proposed as one of the main mechanisms mediating disease progression. Furthermore, microarray analysis of cystic and normal renal tissues was performed to determine differentially expressed genes. A significant number of our AngII signature proteins (60%) are regulated at the level of mRNA in cystic vs normal renal tissue. Subsequent studies could entail measurement of urine excretion rates of our AngII signature proteins by using SRM in patients with autosomal dominant polycystic kidney disease to determine whether these proteins predict disease progression better than traditional markers of progression, and whether they can be used to guide therapy with RAS inhibitors, or with novel agents such as vasopressin receptor antagonists. In this way, we can study AngII signature proteins as a footprint of AngII renal bioactivity in patients with CKD.
REFERENCES


65. Ushio-Fukai M, Griendling KK, Akers M, Lyons PR, Alexander RW. Temporal dispersion of activation of phospholipase C-beta1 and -gamma isoforms


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206. Huang XR, Chen WY, Truong LD, Lan HY. Chymase is upregulated in diabetic nephropathy: implications for an alternative pathway of angiotensin II-


Kim HJ, Sato T, Rodriguez-Iturbe B, Vaziri ND. Role of intrarenal angiotensin system activation, oxidative stress, inflammation, and impaired nuclear factor-erythroid-2-related factor 2 activity in the progression of focal glomerulosclerosis. J Pharmacol Exp Ther 2011;337:583-90.


