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A atividade do NHE3 em túbulo proximal é inibida pela sinalização enviesada do receptor de angiotensina II tipo 1/beta-arrestina

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Proximal tubule NHE3 activity is inhibited by beta-arrestin-biased angiotensin II type 1 receptor signaling

by

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Abbreviations

AC – adenylyl cyclase

ACE – Angiotensin-I-converting enzyme

Ang – Angiotensin

AGT – Angiotensinogen

Akt – protein kinase B

Akti - Akt inhibitor or protein kinase B inhibitor

AP – aminopeptidase

AQP – aquaporin

ARB – Angiotensin II receptor blocker

AT1 receptor – Angiotensin II type 1 receptor

AT2 receptor – Angiotensin II type 2 receptor

β-arr – beta-arrestin

β2-AR - β2-adrenergic receptor

BCECF-AM – 2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxymethyl Ester

CA I – carbonic anhydrase isoform I

CA II – carbonic anhydrase isoform I

CaM – calcium-calmodulin

cAMP - 3'-5'-cyclic adenosine monophosphate

CaM kinase II - calmodulin-dependent protein kinases II

Ctrl – control

cDNA – complementary deoxyribonucleic acid

CHP – calcineurin

CP – carboxypeptidase

cGMP – 3'-5'-cyclic adenosine monophosphate

CHO – Chinese hamster ovary

CHP – calcineurin homologous protein

DAG – diacylglycerol

dd NTPs – di-deoxynucleotides

DEPC – diethylpyrocarbonate

DMEM - dulbecco's Modified Eagle's medium

DNA – deoxyribonucleic acid

dNTPs – deoxynucleotides

DPP IV – dipeptidyl peptidase IV

EDTA – Ethylenediaminetetraacetic acid

EL – extracellular loop

ELISA – enzyme-linked immunosorbent assay

EP – endopeptidase

ERK1/2 – extracellular signal-regulated kinase 1 and 2

Forsk – forskolin

GAPDH – glyceraldehyde 3-phosphate dehydrogenase

GDP – guanosine 5'-diphosphate

GFR – glomerular filtration rate

GPCRs - G-protein-coupled receptors

GRK - G protein-coupled receptor kinase

GTP - guanosine 5'-triphosphate

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IL – intracellular loop

IP3- 1',4',5'-triphosphate

IP3K- 1',4',5'-triphosphate kinase

IRBIT - inositol 1,4,5-triphosphate receptor-binding protein

JNK - c-Jun N-terminal kinase

KO – Knockout

mAb – monoclonal antibody

MAPK – Mitogen-activated protein kinase

Na⁺/K⁺-ATPase – sodium-potassium adenosine triphosphatase, also known as Na⁺/K⁺ pump or sodium-potassium pump.

MBP – mean blood pressure

MOPS – 3-(N-Morpholino)propanesulfonic acid, 4-Morpholinepropanesulfonic acid

NBCe – Na⁺/HCO₃⁻ co-transporter

NEP – neprilysin

NHE – Na⁺/H⁺ exchanger

NHE3 – Na⁺/H⁺ exchanger isoform 3

NHERF – Na⁺/H⁺ Exchanger Regulatory Factor

NLS – nuclear localization signal

NMDG – N-methyl-D-glucamine

OKP – parental opossum kidney cells

pAb – polyclonal antibody

PCR – polymerase chain reaction

PDE – phosphodiesterase

pHi – intracellular pH

PIP2 – phosphatidylinositol 4',5'-bisphosphate

PI3K – phosphoinositide 3-kinase

PKA – protein kinase A or cAMP dependent protein kinase

PKC – protein kinase C

PLP A– phospholipase A

PLP C_γ–phospholipase C_γ

PLP D – phospholipase D

PO – prolyl oligopeptidase

PRCP – prolylcarboxypeptidase

PT – proximal tubule

PTH - parathyroid hormone

PTHR- parathyroid hormone receptor

RAAS – renin-angiotensin-aldosterone system

RAS – renin-angiotensin system

RGS – regulator of G-protein signaling

RH - regulator homology

RNA – ribonucleic acid

RT – room temperature

SDS – sodium dodecyl sulfate

SGK1 – serum and glucocorticoid inducible kinase 1

siRNA – small interfering ribonucleic acid

SNS – sympathetic nervous system

Src – proto-oncogene tyrosine-protein kinase

T_m – melting temperature

TM – transmembrane

TMA-Cl – tetramethylammonium chloride

V2R – vasopressin receptor 2

7-TMRs – seven transmembrane receptors

β-arr – beta-arrestin

β2-AR - β2-adrenergic receptor

Resumo

Carneiro de Moraes, C.A *atividade do NHE3 do túbulo proximal é inibida pela sinalização enviesada do receptor tipo 1 de angiotensina II-beta-arrestina* [Tese]. São Paulo: Faculdade de Medicina, Universidade de São Paulo; 2016.

Os receptores medeiam a maioria das respostas fisiológicas em resposta a diversidade de estímulos. A ativação da sinalização mediada pelo receptor de angiotensina II tipo 1 é o principal responsável pelos efeitos do hormônio angiotensina II (Ang II) nos tecidos alvo. No rim concentrações fisiológicas de Ang II aumentam a atividade no túbulo proximal da isoforma 3 do trocador de Na^+/H^+ (NHE3). Este efeito é crucial para a manutenção do volume extracelular e pressão arterial. Evidências recentes mostraram que a ativação seletiva da sinalização enviesada da beta-arrestina/receptor AT1 induz diurese e natriurese independentemente da sinalização via proteína G. Neste estudo testamos a hipótese de que a sinalização enviesada do receptor AT1/beta-arrestina inibe a atividade do NHE3 no túbulo proximal, bem como investigar os possíveis mecanismos moleculares que medeio este efeito. Para tal, nós determinamos os efeitos do composto TRV120023, que se liga ao receptor AT1, bloqueando o acoplamento da proteína G e estimulando a sinalização da beta-arrestina, na função do NHE3 *in vivo* e *in vitro*. A atividade do NHE3 foi medida quer em túbulo proximal nativo, por meio de microperfusão estacionária, bem como em uma linha celular de túbulo proximal de gamba (OKP), por meio de recuperação de pH intracelular dependente de Na^+ . Os nossos resultados mostram que o TRV120023 na concentração de 10^{-7} M inibe marcadamente a atividade do NHE3 em túbulo proximal quer *in vivo* quer *in vitro*, sendo que este efeito é completamente abolido nas células silenciadas para a beta-arrestina 1 e 2 através de RNA de interferência. Adicionalmente, a estimulação do NHE3 pela Ang II é completamente suprimida pelo TRV120023 quer *in vivo* quer *in vitro*. A inibição do NHE3 pelo TRV120023 foi associada com a diminuição do NHE3 expresso na superfície da membrana plasmática em células OKP e com a redistribuição entre o corpo e a base das microvilosidades em túbulo proximal de rato. A diminuição do NHE3 na superfície da membrana plasmática em células OKP estava associado com um aumento na internalização do NHE via endocitose mediada por clatrina. A inibição do NHE3 mediada pela beta-arrestina não envolve a sinalização do receptor AT2, cAMP/ PKA, Akt e ERK1/2. Estes achados indicam que a sinalização enviesada do

receptor AT1/beta-arretina inibe a atividade do NHE3 em túbulo proximal, pelo menos em parte, devido a alterações na localização subcelular do NHE3.

Descritores: Angiotensina II, receptor de angiotensina, arrestina, antiportador de sódio hidrogênio, receptores acoplados a proteína-G, agonista.

Abstract

Carneiro de Moraes, C.[Thesis]. *Proximal tubule NHE3 activity is inhibited by beta-arrestin-biased angiotensin II type I receptor signaling*. São Paulo: School of Medicine, University of São Paulo; 2016.

Cell surface receptors mediate most of our physiological responses to an array of stimulus. The triggering of the angiotensin II type I (AT1) receptor signaling is the major control point in the regulation of the ultimate effects of the peptide hormone angiotensin II (Ang II) on its target tissue. In the kidney physiological concentrations of Ang II upregulate the activity of proximal tubule Na⁺/H⁺ exchanger isoform 3 (NHE3). This effect is crucial for maintenance of extracellular fluid volume homeostasis and blood pressure. Recent findings have shown that selective activation of the beta-arrestin-biased AT1 receptor signaling pathway induces diuresis and natriuresis independent of G-protein mediated signaling. This study tested the hypothesis that activation of this AT1 receptor/beta-arrestin signaling inhibits NHE3 activity in proximal tubule as well as investigate the underlying molecular mechanisms mediating this effect. To this end, we determined the effects of the compound TRV120023, which binds to the AT1R, blocks G protein coupling, and stimulates beta-arrestin signaling, on NHE3 function *in vivo* and *in vitro*. NHE3 activity was measured in both native proximal tubules, by stationary microperfusion, and in opossum proximal tubule (OKP) cells, by Na⁺-dependent intracellular pH recovery. Our results showed that 10⁻⁷ M TRV120023 remarkably inhibited proximal tubule NHE3 activity both *in vivo* and *in vitro*, and the effect was completely abolished in OKP cells silenced for beta-arrestin 1 and 2 by small interference RNA. Additionally, stimulation of NHE3 by Ang II was completely suppressed by TRV120023 both *in vivo* as well as *in vitro*. Inhibition of NHE3 activity by TRV120023 was associated with a decrease in NHE3 surface expression in OKP cells and with a redistribution from the body to the base of the microvilli in the rat proximal tubule. The decreased surface NHE3 in OKP cells was associated with an increase in NHE3 internalization via clathrin mediated endocytic. Beta-arrestin mediated NHE3 inhibition did not involve AT2 receptor, cAMP/ PKA, Akt and ERK1/2 signaling. These findings indicate that biased signaling of the AT1

receptor/beta-arrestin pathway inhibits NHE3 activity in the proximal tubule at least in part due to changes in NHE3 subcellular localization.

Key-words: **Angiotensin II**, angiotensin receptor, arrestin, hydrogen sodium antiporter, G-protein coupled receptors, agonist.

Chapter 1 – G-protein coupled receptors

In multicellular organisms communication cell-to-cell is essential for life. This communication allows the cells to respond to several stimuli which are important for diverse physiological functions such as proliferation, differentiation, migration, and apoptosis. The cell surface receptors and their downstream cascades are of interest since they play key roles in modulating cell physiology. The G-protein coupled receptors (GPCRs) mediate most of our physiological responses to an array of chemical stimuli, including hormones, neurotransmitters, chemoattractants, calcium ions, among others molecules as well as sensory stimuli, including light, odorants and taste molecules. G-protein-coupled receptors (GPCRs) have emerged as the most important targets for human therapeutics and are the target of about 50% of the current therapeutic agents on the market (13).

1.2 – Structure of G-protein-coupled receptors (GPCRs)

G-protein-coupled receptors, also known as seven transmembrane receptors (7-TMRs), are a large, diverse and highly conserved class of transmembrane protein superfamily of cell surface receptors in the body. They comprise 2% of the human genome (14, 15). GPCRs contain an extracellular N-terminal domain, seven transmembrane α -helice regions (TM-I to TM-VII), and an intracellular C-terminal domain. The transmembrane region is connected by three intracellular (IL1-2-3) and three extracellular loops (EL1-2-3). The intracellular domain contains several serine and threonine residues, and their hydroxyl (OH) groups can be phosphorylated. Intracellular domain phosphorylation may regulate activity, intracellular signaling and receptor desensitization. The G-proteins themselves interact with the intracellular loop in the cytoplasmic portion of the receptor (Fig. 1) (3).

Figure 1 - Diagrammatic representation of a typical G-protein coupled receptor. Red, blue, black and green spheres represent amino acids. Serpentine receptors are so-called because they pass through the plasma membrane seven times. Structural characteristics include the three extracellular loops (EL-1, EL-2, EL-3) and three intracellular loops (IL-1, IL-2, IL-3). Most GPCRs are modified by carbohydrate attachment to the extracellular portion of the protein. Shown as a typical N-linked carbohydrate attachment. The different colored spheres are involved in ligand-binding and/or association with the G-proteins as indicated in the legend (adapted from (3)).

1.3 – Simple view of GPCR signaling: the two-state model

GPCRs signal transduction begins when an extracellular agonist ligand binds and switches the receptor conformation. This conformational change of the receptors leads to the catalyze exchange of GDP for GTP on the α -subunit of heterotrimeric G protein ($G\alpha\beta\gamma$), which in turn engages conformational and/or dissociation events between the $G\alpha$ subunit and dimeric $G\beta\gamma$ subunits (16). The GPCRs can then signaling through a variety of subclasses of $G\alpha$ proteins, such as G_{as} , G_{ai} , G_{aq} and $G_{\alpha 12/13}$ initiating or suppressing the activity of effector enzymes, such as adenylyl cyclase (AC),

phosphodiesterases (PDE), phospholipases (PLP), and ion channels. These effectors in turn modulate the flow of secondary messengers such cAMP, cGMP, diacylglycerol (DAG) or inositol trisphosphate (IP3) (Fig. 2). These second messengers are involved in the regulation of multiple intracellular signaling pathways that modulates cell functions as diverse as the skeletal, endocrine, cardiovascular and nervous systems, among others (17, 18).

Figure 2 – Classical GPCRs signaling system. Ligand binding to the receptor leads to conformational changes promoting the coupling to heterotrimeric G proteins ($G\alpha\beta\gamma$) and the catalytic exchange of GDP for GTP on the α -subunit, triggering conformational and/or dissociation events between the α -subunit and $\beta\gamma$ -subunit. This event can then lead to adenylyl cyclase activation by $G\alpha_s$, leading to cAMP synthesis. Phospholipase activation by $G\alpha_q$, which cleaves phosphatidylinositol 4',5'-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol 1',4',5'-trisphosphate (IP3). Activation of $G\alpha_i$, which blocks adenylyl-cyclase-mediated cAMP synthesis. On the other hand $G\beta\gamma$ -mediated signalling can activate of G-protein-regulated inwardly rectifying potassium (GIRK) channels (adapted from (9)).

Besides the activation of the heteromeric G-proteins, switches in the conformation of the GPCRs also triggers other cellular events that lead to rapid attenuation of the receptor responsiveness, a process termed desensitization.

1.3.1 – G-protein coupled receptor desensitization and downregulation: uncoupling of the G proteins

GPCR signaling is critical for the regulation of various physiological functions, and the magnitudes of these physiological responses are intimately linked to the delicate balance between GPCR signal generation and signal termination. Almost all GPCR are tightly regulated by a common desensitizing mechanism. The process of agonist-specific homologous desensitization of receptors is characterized by an increase in the refractoriness of a receptor to signal in response to repeated or sustained exposure to its agonist, limiting both the magnitude and the temporal extend of the receptor signal, thus protecting cells from over-stimulation. The desensitization of the receptor signaling must be rapidly terminated in order to prevent uncontrolled signaling. These mechanisms involve the activities of two families of proteins: G protein-coupled receptor kinases (GRKs) and arrestins. The first step of desensitization is the phosphorylation of the receptor by the G-protein coupled receptor kinases (GRKs) (11). The second step is the binding of arrestins to the phosphorylated receptor preventing further G-protein coupling, terminating the G protein dependent signal initiated at the cell surface membrane. The coupling of arrestins to the GPCRs also leads to receptor internalization, which then can be recycled or proteolytically degraded (Fig. 3) (8, 12, 13). This is believed to be the common mechanism of all GPCRs desensitization, and is important for the maintaining of homeostasis. Our knowledge concerning the basic mechanisms underlying the phenomenon of desensitization, internalization, downregulation, and resensitization of GPCRs has been far advanced during the last few decades.

Figure 3 – GPCRs desensitization and internalization. Following receptor activation and G-protein dissociation (1) downstream signaling pathways are activated (2). Ligand-activated GPCRs are then phosphorylated by GRKs (3), resulting in the recruitment of arrestins (4). This prevents further G-protein coupling to the receptor, thereby attenuating further receptor signaling. The binding of arrestin to the receptor also promotes internalization of the receptor (5) that can result in the downregulation of receptor, but can also contribute to a second round of signaling such as activation of the MAPK cascade (6). The receptor can then be degraded by proteolysis or recycle back to the membrane, process called resensitization (7) (adapted from (8)).

1.3.1.1 – G-protein coupled receptor kinases

G-protein coupled receptor kinases (GRKs) comprise a cytosolic multigene family of serine–threonine kinases which are capable of specifically phosphorylate the ligand bounded GPCRs (19). Seven mammalian genes encoding GRKs (GRK1-GRK7) have been cloned to date (20-22). GRK 1 and 7 are specific to the visual system (23, 24), GRK 4 is selectively present in sperm cells (24). In contrast, the GRKs 2, 3, 5 and 6 are ubiquitously distributed (25-28). Structurally, all isoforms of GRK share similar

amino sequence domains. An amino-terminal domain, unique to the GRK family of kinases, the regulator of G-protein signaling (RGS) homology (RH) domain, which can regulate GPCR signaling by phosphorylation independent mechanisms, a serine/threonine protein kinase domain, and a carboxi-terminal domain (Fig. 4). Based on sequence homology, the GRK family have been divided in three subfamilies: the GRK1 subfamily, composed of GRK1 and GRK7, the GRK2 subfamily, composed of GRK2 and GRK3, and the GRK4 subfamily composed of GRK4, GRK5, and GRK6 (11, 29-31).

The amino-terminal domain of GRK2 interacts with the subunit $G\beta\gamma$, whereas amino-terminal domain GRK4, GRK5, and GRK6 interacts with phosphatidylinositol 4',5'-bisphosphate (PIP2) (24, 32-34). Divergent sequences between GRKs in the carboxyl-terminal domain have been observed. The GRK1 and GRK7 have short prenylation sequences (35), GRK2 and GRK3 have pleckstrin homology domains that interact with $G\beta\gamma$ subunits (36, 37) and PIP2 (38), and the members of the GRK4 subfamily have palmitoylation sites (39, 40) and/or positively charged lipid-binding elements (41, 42). The carboxyl-terminal of GRKs appear to be important for the localization and translocation of kinases to the membrane by means of posttranslational modifications or sites of interaction with lipids or membrane proteins (43). The GRK4 subfamily (GRK4, GRK5, and GRK6) have been found to contain a functional nuclear localization signal (NLS) (41-43), and GRK5 and GRK6 have been shown to bind to DNA (41). These properties could lead to functional diversification among GRKs. In fact, knockout mice for each GRK showed different phenotypes (11, 44).

Receptor phosphorylation by GRKs has been ultimately identified as the initial and critical step in the uncoupling of receptor from the heteromeric G-protein. GRK phosphorylation of receptors is not sufficient for desensitization, but rather serves to create high affinity sites to promote the binding of arrestin proteins which in turn guarantee desensitization by preventing further coupling to G proteins, leading to the attenuation or desensitization of GPCR signaling (45). To appropriate interaction of the GRKs with the receptor domains, the GRKs translocates from the cytosol to the plasma membrane, and it is known that free $G\beta\gamma$ subunits bind to the C-terminal domain of GRK and facilitate the translocation process. This phosphorylation of agonist-bound GPCR also leads to the translocation and binding of arrestins and beta-arrestins to the receptors, inhibiting further G-protein activation by blocking receptor-G protein

coupling (46, 47). The phosphorylation of GPCR by GRKs and the binding of arrestins ultimately promote agonist bound GPCR internalization (Fig. 3) (47, 48).

Figure 4 – Structural domains of GRKs. Then numbers above the structures indicate amino acid residue of human GRKs. All GRKs have a short N-terminal region (green), which is implicated in GPCR binding, followed by regulator of G-protein signaling (RGS) homology (RH) domain (violet). This N-terminal region is unique to the GRK family of kinases. The RH domain is interrupted by the catalytic domain shared by all kinases (dark yellow). The defining feature of the GRK2/3 subfamily is a C-terminal pleckstrin homology (PH) domain (blue) implicated in binding anionic phospholipids and G $\beta\gamma$. Members of GRK4/5/6 subfamily use alternative mechanisms for membrane targeting, which include palmitoylation and positively charged residues (amphipathic helix motifs are shown as green boxes); N-terminal basic are shown as red boxes), and, in case of visual subtypes, prenylation (C-terminal prenylation sites in GRK1 and 7 are shown as red triangles). Residues Arg106 and Asp110 in GRK2/3, among others, are important for binding G α_q , a function unique to this subfamily. The blue box shows the position of the nuclear localization signal (NLS) in GRK5 (residues 388–395) (Adapted from (11)).

1.3.1.2 – Arrestins

Arrestins consist in a small gene family of four members. All of them interact with GPCRs after these receptors have been activated by agonists and phosphorylated by GRKs. Among them are the visual arrestins 1 and arrestin 4, usually called visual

and cone arrestin, respectively, expressed almost exclusively in the retina where they regulate photoreceptor function (49). By contrast arrestin 2 and arrestin 3 are non-visual arrestins, and ubiquitously expressed in most tissues, often referred as beta-arrestin 1 and beta-arrestin 2. Beta-arrestin 1 and 2 are structurally similar, with 78% amino acid identity and play an important role in regulating signal transduction of numerous GPCRs (50, 51). Studies of GPCRs, such as the β 2-adrenergic receptor, revealed that receptor activation promotes the translocation of arrestins from the cytoplasm to the cell membrane and posterior interaction of arrestins with the activated receptor. The binding of the arrestin leads to uncoupling of the receptor from its cognate G-proteins, causing termination of the coupling of GPCR-G-protein (52-54). In addition, arrestins also facilitate the internalization of the GPCRs and act as a molecular scaffold recruiting signaling proteins to internalized GPCRs in endosomes. They also interact with proteins of the endocytic machinery, such as clathrin, promoting internalization of receptors via clathrin-coated vesicles (55, 56), regulating receptor down-regulation (57) and resensitization (58, 59). The final regulatory step influences the G-protein signal transduction, duration and sensitivity.

After internalization, the fate of GPCRs depends on both cell type and type of GPCR receptor. Typically, following agonist induced internalization the GPCRs recycle back to the cell surface membrane. However, some are trafficked to degradative pathways and proteolytically degraded in lysosome, a process called downregulation (Fig. 3) (60-62). Although most of the research regarding the desensitization process has been carried out using β 2-adrenergic receptor as a model, it is now clear that this process regulates the function of many GPCRs (63, 64).

1.4 – The "new view" of GPCR signaling: the multi-state model

Previous to the employment of molecular and cellular biological methodologies, pharmacological research was restricted, in most cases, to the observable responses and to a limited number of isolated tissue and organ systems. Then, the experimental analysis of receptor behavior was indirectly monitored by the physiological responses. As a corollary, the receptor was conceived as a fixed and rigid structure that oscillates between two alternative conformations related to the active versus inactive functional

states (65). However, recent evidences from biophysical and biochemical technologies demonstrated that receptor is rather intrinsically dynamic with structurally plastic, and the signal machinery complex and highly organized in time and space. This pointed to a “new view” of signal transduction of GPCRs. The “new view” of the proteins supported that signaling patterns and physiological responses are determine not by gene products, but rather by spatiotemporal dynamics of the same repertoire of signaling components. Whereby a single protein can adopt multiple conformations and a single receptor protein can exist as an ensemble of multiple, interconvertible, pre-existing conformations in equilibrium, before binding the ligand (66, 67). The ligand binding to the receptor forces the receptor complex to stabilize in a ligand-specific receptor conformation (67). Furthermore, evidences reveled that different ligands can act at the same receptor and stabilize distinct receptor conformation linked to diverse functional outcomes (Fig. 5). This phenomenon was termed biased agonism (also referred as stimulus bias or functional selectivity), and expresses the ability of a ligand to produce a selective response (68).

Figure 5 – Biased agonism. Each agonist promotes distinct conformational changes of GPCRs. Unbiased agonists activate both G protein signaling and beta-arrestin-dependent signaling, whereas biased agonists activate either G-protein or beta-arrestin-dependent signaling as shown in figure. Physiological responses mediated by beta-arrestin-dependent signaling are believed to be distinct from those G-protein dependent (adapted from (5)).

1.4.1 – Biased agonism

That a given GPCR can functionally couple with more than one heteromeric G-protein has been known for many years. However, it was quite surprisingly, when it was first noted in the 90s, that different ligands for a single GPCR could be “biased” or “functionally selective” toward one or another of these G-proteins. Even more surprising was the discovery, a few years later that the same GPCRs could bias toward a G-protein or beta-arrestin mediated pathways.

It has been known for a long time that beta-arrestin interaction with the GPCRs is a very important mechanism for desensitization of the G-protein dependent signaling and receptor internalization, by linking receptors to the endocytic machinery, such as clathrin and clathrin adaptor protein 2 (63, 64). However, it has also become apparent that beta-arrestin mediates signaling by its own. The beta-arrestin mediated signaling has been described to function as a complex between the receptor, the beta-arrestin and various cytosolic mitogen-activated protein kinases (MAPK). This complex is called signalosome and can produce low-level, long-lasting cellular signaling through the activation of proteins which include extracellular signal-regulated kinase (ERK1/2), p38 MAPK and c-Jun N-terminal kinase, and also function as scaffolds to connect GPCRs to tyrosine kinase, such as c-Src, phosphoinositide 3-kinase (PI3K), the protein kinase B (PKB or Akt) and the nuclear factor κ B pathways (63, 69). This beta-arrestin dependent signaling not only requires beta-arrestins but also GRKs. Among GRKs, the isoforms GRK5 and/or GRK6 have been associated with beta-arrestin dependent ERK1/2 activation by angiotensin II type 1 (AT1) receptor (70), vasopressin receptor 2 (V2R) (71), and β 2-adrenergic receptor (β 2-AR) (72).

The mechanism by which GRKs and beta-arrestins determine whether to promote GPCR desensitization or beta-arrestin dependent signaling remains unclear. However, it is possible that the different conformational states of GPCRs selectively recruit specific GRKs, leading to the activation of GRK/beta-arrestin dependent signaling pathways (73). There are significant evidences that receptor phosphorylation at different sites will direct differential activation of distinct signaling cascades. In this manner, different agonists might be expected to activate different kinases that result in the phosphorylation of the receptor at different sites, producing that way a ligand-specific “barcode” (74, 75). Those differences in receptor phosphorylation can be the

key translational modification which may serve to facilitate or perturb interactions with the neighboring scaffolds and delineating a specific signal cascade as illustrated in Fig. 6.

Figure 6 - Barcode hypothesis to explain differential functions of beta-arrestin. At the level of the receptor, biased ligands stabilize active receptor conformations structurally distinct from active conformations stabilized by balanced ligands. These unique conformations, in turn, recruit unique subsets of GRKs and as a consequence, differential phosphorylation patterns or 'barcodes' are generated on the C-terminal of the given receptor. At the level of the transducer, in this case beta-arrestin, phosphorylation on the receptor promotes its recruitment and binding to the receptor. However, different phosphorylation 'barcodes' may stabilize distinct active conformations of transducers resulting in unique functional profiles. These ligand-specific functional profiles promote activity of distinct complex intracellular signaling networks and ultimately lead to divergent physiological responses (adapted from (10)).

Over the past few years, several receptors have been added to the list of GPCRs capable of eliciting G-protein independent signaling, including metabotropic glutamate receptor, β_2 -AR, parathyroid hormone receptor (PTHr), dopamine receptor D2, and the AT1 receptor, among others (76-78). The angiotensin II type 1 (AT1) receptor was the first GPCR demonstrated to elicit beta arrestin signaling and will be the focus of our study.

Chapter 2- Angiotensin II, sodium balance and blood pressure control

2.1 – Components of the renin-angiotensin system

The renin-angiotensin system (RAS) is a major physiological regulator of body fluid volume homeostasis, electrolyte balance, and blood pressure. The first element from this system to be described was the enzyme rennin in 1898 by Tigerstedt and Bergman (79). Over 30 years later, in 1934 Harry Goldblatt and collaborators (80), associated the decrease of blood pressure in kidneys with hypertension by partially clamping dog renal arteries which result in renovascular hypertension. Using the same methodology, in 1940 Braun-Menendez and collaborators (81) isolated from the renal venous the vasoconstrictor substance responsible for this renovascular hypertension, and called it “hypertensin”. At the same time, Page and collaborators independently described a vasoconstrictor substance, which appear after renin injection into cats, and named it “angiotonin” (82). The same group also described angiotensinogen, first referred to as a “renin activator” (82). In 1958, Braun-Menendez and Page combine both terms (hypertensin and angiotonin) and agree to use the name angiotensin which derived from half of each original name. Later in 1987, the World Health Organization and the American Heart Association suggested the abbreviation Ang for Angiotensin, numbering the amino acids residues based in the angiotensin I (Ang I) as a reference for all angiotensin-derived peptides (83).

In a classical RAS, the substrate angiotensinogen (AGT), which is released into the circulation from the liver, is degraded by the enzyme renin originated in the kidney, generating the inactive peptide angiotensin I (Ang I). When this decapeptide encounter the angiotensin-I-converting enzyme (ACE), at the endothelial surface of blood vessels, the C-terminal dipeptide is cleaved, giving rise to angiotensin II (Ang II), the main effector molecule of the RAS. Although the RAS originally defined as a circulating system, recent evidences showed that many of its components are localized in several tissues, indicating the existence of a local tissue RAS as well, which have independent function and regulation. Moreover, several components have been added to the system, and are summarized in more detail in Fig. 7 (84).

Figure 7 – Renin angiotensin system. Classic view of renin-angiotensin system cascade (blue) and recent view of renin-angiotensin system cascade (black). AP: Aminopeptidase; APA: Aminopeptidase A; APN: Aminopeptidase N; CP: Carboxypeptidase; EP: Endopeptidase; ACE: Angiotensin converting enzyme; ACE2: Angiotensin converting enzyme 2; CPP: Carboxypeptidase P; PRCP: Prolylcarboxypeptidase; NEP: Neprilysin; PO: Prolyloligopeptidase; Mas: Ang-(1-7) Mas receptor (6) (adapted from (6)).

Even though, RAS was described more than a century ago, the system remains a fascinating subject of research. The functions of Ang II are mainly modulated by its actions through its receptors. Two major types were identified, the angiotensin type I (AT1) receptor and the angiotensin type II (AT2) receptor. However, its major functions have been associated to the binding of Ang II to the AT1 receptor (84).

2.1.1 – Structure of the angiotensin II type 1 receptor

The human AT1 receptor belongs to the seven-membrane superfamily of GPCRs and contains 359 amino acids (41kD) (85). The human AT1 receptor gene was mapped in the chromosome 3. Since the first description of the AT1 receptor sequence in 1991, two highly homologous isoforms were identified in rodents, the isoform A (AT1_A) in chromosome 17 and the isoform B (AT1_B) in chromosome 2. The rat and mouse AT1

receptors also contain 359 amino acids and they are well conserved among species. Human AT1 receptor is about 90-95% identical to the bovine and rat AT1 receptor, and the majority of this identity is found on the transmembrane domains. The isoforms are indistinguishable at the pharmacological and functional level, however *in vivo* experiments have been shown that AT1_A may be more relevant than AT1_B with respect to blood pressure control. The AT1_A receptor accounts for 90% of the total binding and is expressed in the kidney, vascular smooth muscle cells, heart, liver and in some areas of the brain, while AT1_B receptor is found predominantly in the pituitary and adrenal glands, placenta, lung and brain. Both of these isoforms are selectively antagonized by angiotensin receptor blockers (ARBs), such as losartan. A third isoform, the AT1_C, was isolated from rat placenta and was shown to be 90% homologous to AT1_A receptor and 82% to the AT1_B receptor. The extracellular domain of the receptor is characterized by 3 sites of glycosylation and mutation on those sites has no effect on agonist binding. Along with several residues located on the extracellular region of the receptor, 4 cysteine residues of the AT1 receptor form a disulfide bridge and are essential for angiotensin II (Ang II) binding. The coupling to the G-proteins occurs at the second and third intracellular loops. Similar to many other receptors, the AT1 receptor possesses a cytoplasmic tail that contains many serine/threonine residues, which can be phosphorylated by GRKs. Modifications on these functional sites may be responsible for the altered receptor function (Fig. 8) (12, 86).

Figure 8 –Schematic representation of the AT1 receptor. Red, blue, black and green spheres represent amino acids. The different colored spheres are involved in ligand-binding and association with G-protein as indicated in the figure. The two extracellular disulfide bonds between Cys-Cys residues and the tree sites of glycosylation are also represented (based on (7) and (12)).

2.1.2 – Structure of the angiotensin II type 2 receptor

The human AT2 receptor belongs to the seven-membrane superfamily of GPCRs and contains a sequence of 363 amino acid (41 kDa). The polypeptide sequence shows a 92% homology as well as the same pharmacological profile as the AT2 receptors isolated from mouse and rat. The AT2 receptor gene is located at the X chromosome. The AT2 receptors shares only 34% of sequence homology with the AT1 receptor, and most of the identity is found in the transmembrane domains. Like the AT1 receptor, the AT2 receptors are also well conserved among species, with 90% identity between rat and mouse and 72% identity between rat and human. The AT2 receptor is widely expressed in fetal tissues, but in early postnatal period rapidly regresses to low levels or completely disappears. The tissues where the AT2 receptor expression does not

substantially regress and disappears in adulthood include brain, uterine myometrium, adrenal glands and myocardium. The extracellular domain of the AT2 receptor contains 5 potential glycosylation sites in its extracellular N-terminal tail. Among the many differences in amino acid sequence, the AT2 receptor, but not the AT1 receptor, has a conserved lysine (LYS¹⁹⁹) which is important in ligand-receptor interactions. In addition, there is a potential protein kinase C (PKC) phosphorylation site in the second intracellular loop. Moreover, there are 3 consensus sequences for phosphorylation by PKC and 1 phosphorylation site for cyclic AMP (cAMP)-dependent protein kinase, also known as protein kinase A (PKA), in the C-terminal cytoplasmic tail of the receptor (Fig. 9) (86, 87).

Figure 9 – Schematic representation of the AT2 receptor. The spheres represent amino acids. The different colored spheres are involved in ligand-binding, association with G-protein or PKA and PKC consensus sites (based on (7) and (87)).

2.2 – Classical signaling at the AT1 receptor: G-protein mediated signal

The triggering of the AT1 receptor signaling is the major control point in the regulation of the ultimate effects of the peptide hormone Ang II on its target tissue. Once Ang II binds to the AT1 receptor, it activates a series of signaling cascades, which in turn regulate various physiological effects of the Ang II. Traditionally, the activation of the AT1 receptor leads to the uncoupling and activation of the G-protein mediated signaling, which can also cross-talk with several tyrosine kinases, like EGFR, insulin receptor and non-receptor tyrosine kinase, c-Src family kinases, among others (88).

On its targets tissues the AT1 receptor signaling elicits multiple cellular responses G-protein mediated, predominantly via $G\alpha_{q/11}$, but also via $G\alpha_{2/13}$, $G\alpha_i$ and $G\beta\gamma$ (89). The $G\alpha_{q/11}$ mediated signaling via PLC activation, producing DAG and IP3/ Ca^{2+} , the primary transduction signal initiated by Ang II on its target tissues. Conversely, IP3 binds to its receptor on the sarcoplasmic reticulum, opening the calcium channel, allowing calcium efflux and ultimately leading to contraction. In addition, the DAG also leads to the activation of PKC increasing the pH during cell contraction, and participates as an effector in the MAPK family, including the MAPKs c-Jun N-terminal kinase (JNK), p38 MAPK. These downstream effectors were associated in proliferation, differentiation, migration and fibrosis in vascular smooth muscle cells (7, 88). Another important mechanism is the activation of $G\alpha_{11/12}$ family, which signaling has been associated with the activation of PLC, L-type Ca^{2+} channels, and Rho kinase, leading to sustained muscle contraction and cell migration (89-93). The AT1 receptor can also couple to $G\alpha_i$ that inhibits the AC in some target tissues. Activation of heterotrimeric G-proteins by the AT1 receptor also releases their $G\beta\gamma$ subunits, which can further activate tyrosine kinases and PLD (88, 93). At least one tyrosine kinase (Jak2) has been shown to interact directly with a tyrosine-containing motif in the cytoplasmic tail of the AT1 receptor. The Ang II mediated AT1 receptor activation can also lead to the phosphorylation of the PLA_2 , which in turn produces arachidonic acid and metabolites. The derivatives of the arachidonic acid function in the maintenance of vascular tone (Fig. 10) (7).

Figure 10 – Schematic representation AT1 receptor signaling. Major signal transduction pathways triggered by the AT1 receptor (based on (7)).

Within the kidney Ang II plays a pivotal role in the regulation of body fluid content and blood pressure by altering sodium and water homeostasis. In the kidney, Ang II participates in vascular, tubular, and growth-promoting activities. Evidences showed that administration of exogenous Ang II decreases renal blood flow and glomerular filtration rate (GFR), and constricts afferent and efferent arterioles dose-dependently (94). The vasoconstrictive responses of the afferent arteriole to Ang II are mediated by AT1_A and AT1_B receptors, whereas efferent arteriolar vasoconstrictor responses to Ang II are mediated only by AT1_A receptors. Ang II also reduces the glomerular filtration coefficient while increasing afferent and efferent arteriolar resistances, which contributes to the decreases in GFR (95). Besides the hemodynamics effects, Ang II also exerts modulatory actions on the sensitivity of the tubuloglomerular feedback mechanism, which provides a balance between the reabsorption on the tubules and the filtered load by adjusting the GFR. Micropuncture analysis in transgenic mice showed an essential role of Ang II in tubuloglomerular feedback regulation mediated through the AT1_A receptor (96).

The regulation of renal sodium and water excretion by Ang II is not only mediated via effects on renal hemodynamics, glomerular filtration rate (GFR) and regulation of aldosterone secretion, but also via direct effects on renal tubule transport. In the kidney, Ang II has several targets and signaling pathways that regulate sodium balance. It stimulates H⁺ secretion and HCO₃⁻ reabsorption in both proximal and distal tubules and regulates H⁺-ATPase activity in the intercalated cells of the collecting

tubule (97). The activation of apical Na^+/H^+ exchange (NHE) (98), basolateral $\text{Na}^+/\text{HCO}_3^-$ cotransport (99), and basolateral Na^+ , K^+ -ATPase (100, 101) and apical H^+ -ATPase (102, 103) are implicated in Ang II induced transcellular sodium and bicarbonate reabsorption within the proximal tubule. Ang II also modulates the co-transporter $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ in the thick ascending limb of the loop of Henle, the co-transporter $\text{Na}^+/\text{2Cl}^-$ in the distal tubule and the epithelial sodium channel in the collector duct (104, 105). In this work we will only focus on the isoform 3 of the Na^+/H^+ exchanger (NHE3) in proximal tubule.

Chapter 3- NHE3 regulation and blood pressure control

3.1 – Structure of the Na^+/H^+ exchanger isoform 3

The NHE3 (SLC9A3) is a member of the Solute Carrier classification of transporters and a subgroup of the monovalent Cation Proton Antiporter (CPA) superfamily. In mammals the Na^+/H^+ exchanger (NHE) is a family of proteins that contains at least nine isoforms (NHE1-9) and is encoded by the SLC9 gene. The family can be divided in two major subdivisions: those, which are primarily found in the plasma membrane, which include the isoforms NHE1-5, and those present in intracellular organelles, the isoforms NHE6-9. Computer modeling and molecular physiology studies have attempted to relate the NHEs function with the specific part of the N and C terminal. The computer modeling of NHEs predicts a common membrane topology, with 12 relatively conserved transmembrane segments at the N-terminus, which carries out the Na^+ and H^+ exchange, (human NHE3, amino acid L456) and a more variable hydrophilic C-terminus that faces the cytoplasm, which regulates the transport rate (human NHE3 amino acids 457–834) (Fig. 11). The C-terminus contains numerous canonical sites for phosphorylation by different protein kinases and for binding of other ancillary factors, indicating a regulatory function of this segment. However, the complete structure of the NHE3 transport domains has not yet been solved (2).

Figure 11 - Transmembrane topological organization and C-terminal binding partners of NHE3. Small numbers denote amino acids on the C-terminal of NHE3 where regulation occurs. In green are represented the sites which can be phosphorylated by PKA. (Legend: **R-loop**: reentrant loop, **CHP**: calcineurin homolog protein, **CaM**: calcium-calmodulin, **DPPIV**: dipeptidyl peptidase IV, **NHERF**: NHE regulatory factor, **IRBIT**: inositol 1,4,5-triphosphate receptor-binding protein; **PLC_γ**: phospholipase C_γ; **PKA**: protein kinase A. N-terminal topology based on (1, 2).

NHE3 is most abundant in the luminal membranes of intestine and kidney segments. In addition, NHE3 is also present in the epididymis, ovary, thymus, prostate, and in some respiratory neurons in the ventrolateral medulla (1). In the kidney, NHE3 is localized at the apical membrane of epithelial cells of the proximal tubule (PT) and, to lesser extent, in the medullary thick ascending limb (106-108). NHE3 is the major apical sodium transporter of the proximal tubule, and flow-modulated NHE3 activity is one of the key mechanisms for glomerulotubular balance (106, 109).

3.2 – Mechanisms of NHE3 Regulation

NHE3 is one of the most regulated transport proteins and its activity can be both acutely and chronically modulate. The mechanisms by which NHE3 is regulated

involve transcription and translation regulation, protein phosphorylation, protein-protein interaction and trafficking (151).

The majority of the literature describes the acute regulation of NHE3, which occurs within the time span of minutes to a few hours of cellular activation. Acute regulation is rapid and reversible and often involves changes in phosphorylation, trafficking, and dynamic interaction with regulatory proteins. The cytoplasmic domain at the C-terminus of the NHE3 contains multiple putative sites of phosphorylation. In fact, it has been predicted that rabbit NHE3 present 19 putative Ser/Thr phosphorylation sites. These putative sites are believed to be phosphorylated as part of the signal transduction that modulates NHE3 activity. Phosphorylation by PKA is the best understood, and changes in NHE3 phosphorylation by PKA have been demonstrated to inhibit NHE3 activity, both *in vitro* culture cells as well as *in vivo* rat kidney (110-112). Specifically, mutation in serine 552 and serine 605 (Ser-552 and Ser-605) abolish NHE3 inhibition by PKA (111). In addition, it has also been shown that serum and glucocorticoid inducible kinase 1 (SGK1) requires NHE3 phosphorylation at Ser-663 and mutation at this residue blocks NHE3 regulation by glucocorticoids (113). Even though, the phosphorylation of NHE3 at Ser-552 and Ser-605 modulate NHE3, the mechanisms by which phosphorylation alters NHE3 activity are not known. In fact, Kocinsky and collaborators (114) showed that there is a temporal dissociation between NHE3 phosphorylation and its activity, suggesting that phosphorylation may not directly affect the transport activity of NHE3. Alternatively, phosphorylation of NHE3 may modulate NHE3 subcellular trafficking or interaction with other regulatory proteins and phosphorylation sites.

The NHE3 C-terminal is also capable of interacting with a large number of proteins. This interaction allows not only NHE3 modulation as well as link NHE3 to the cytoskeletal network. Indeed, it has been shown in rabbit ileal brush border membrane that NHE3 protein exists as part of a large complex (159). The interactions with PDZ domain (postsynaptic density 95, discs large, and zonulaoccludens- 1), like the NHERF 1 and 2 (Na⁺/H⁺ Exchanger Regulatory Factor 1 and 2), is one of the regions of the C-terminal under intense study. Other well-studied interacting proteins include megalin, dipeptidyl peptidase IV, and calcineurin homologous protein (CHP) (115-117) (Fig. 11). In addition, direct binding to ezrin and phospholipase C- γ has been reported (118, 119)

NHE3 can also be regulated by recycling between the plasma membrane and intracellular compartments by exocytic insertion or endocytosis (120, 121). There is evidence for regulated endocytosis of NHE3 in cultured cell lines. In Chinese hamster ovary (CHO) cells, a fraction of transfected NHE3 was localized in recycling endosomes (122), and the plasma membrane NHE3 is endocytosed via a clathrin-mediated pathway and cytoskeleton (123-125). Moreover, it has been shown that, in opossum kidney (OK) cells, PTH and dopamine acutely stimulates NHE3 endocytosis via clathrin-coated vesicles (125, 126). In contrast to proximal tubule derived OKP cells, native proximal tubule brush border is very complex morphologically, including tall and densely packed microvillus and well-defined intramicrovillus domain and coated pit regions. Contrarily to what is observed *in vitro*, McDonough and colleagues have shown that NHE3 can only retract in intact proximal tubules from villi to the intermicrovillar domain front to some stimuli, like acute hypertension and PTH (127, 128).

On the other hand, chronic regulation usually involves transcriptional and translational modification. It has been shown that chronic exposure to glucocorticoids, metabolic acidosis, and chronic hyperosmolality increases NHE3 mRNA abundance and activity (129-131). Moreover, proinflammatory cytokines, such as IFN- γ and TNF α , and enteropathogenic microbial products downregulate NHE3 expression (132, 133). In short, the regulation of NHE3 is complex with a myriad of cellular signals converging onto a single protein at different levels.

3.2.1 – NHE3 regulation by angiotensin II

One of the key regulators of NHE3 is Ang II. It is long been known that Ang II infusion into the kidney is associated, at high doses ($> 10^{-8}$ M) with increased sodium and water excretion, and at low doses (10^{-12} - 10^{-10} M), with sodium and fluid retention (98, 134, 135). Classically Ang II activates the AT1 receptor leading to the uncoupling of the heteromeric G-proteins (G α_q , G α_i and G $\beta\gamma$ subunits) spreading the signal. Different studies have identified several signal transduction involved in the acute stimulatory effect of Ang II on NHE3 activity. Traditionally, the signal cascade was associated with activation of protein kinase C and/or adenylyl cyclase inhibition, which in turn leads to a decrease of intracellular cAMP generation (136-140). The activation of

the non-receptor tyrosine Kinase c-Src (141) and the binding of inositol 1,4,5-triphosphate receptor-binding protein (IRBIT) to the C-terminal of the NHE3 have also been implicated in the Ang II stimulatory effect (137). Exocytic insertion of NHE3 into the plasma membrane was also implicated in the increased activity of the exchanger by Ang II. This increase in insertion was associated with changes in intracellular Ca^{2+} and Ca^{2+} /calmodulin-dependent protein kinases II (CaM kinase II), and the transduction pathways of the PI3 kinase, Akt, PLC (120, 142), and required the integrity of actin cytoskeleton (120). Moreover, unpublished data from our group also shows that Ang II can increase NHE3 activity by the activation of the AT1 receptor/Gi-protein signal, which in turn decreases intracellular cAMP/PKA-mediated NHE3 phosphorylation at serine 552 and 605. On the other hand, the acute inhibitory effect of Ang II was associated with the activation of phospholipase A2 and protein kinase G (143, 144).

The long-term regulation of NHE3 by Ang II promotes critical changes in NHE activity, and NHE3 mRNA and protein abundance by up-regulating its gene promoter activity (132, 135). The integrity of the binding site Sp1/Egr-1 on NHE3 promotor was identified was relevant for the transcriptional activation by Ang II (136).

3.3 – Physiological importance of the proximal tubule NHE3

The proximal tubule accounts for the reabsorption of approximately 2/3 of the ~180 liters of water and ~25 mEq of Na^+ that is filtered by the glomerulus on a daily basis. NHE3 is the major contributor to the bulk of sodium and fluid reabsorption in the proximal tubule (145). In adults, the associated secretion of H^+ by NHE3 into the lumen of the renal tubule, together with the basolateral $\text{Na}^+/\text{HCO}_3^-$ co-transporter (NBCe1), is essential for almost 2/3 of the renal HCO_3^- reabsorption. This exchange is driven by a concentration gradient for Na^+ , generated by basolateral sodium-potassium adenosine triphosphatase pump (Na^+/K^+ -ATPase) effluxing of the apically absorbed Na^+ (Fig. 12) (146, 147). This massive absorption of Na^+ from the proximal tubule via NHE3 plays a key role in preserving extracellular fluid volume (145). Given the importance of proximal tubular Na^+ transport, it is not surprising that NHE3^{-/-} mice presents sharply decreased HCO_3^- and fluid absorption in PT, mild acidosis, reduced blood pressure, and have an increased activation of renin-angiotensin system under normal salt diet (148).

When NHE3^{-/-} mice were submitted to a low sodium diet they were unable to control volume homeostasis and die in few days (149).

Figure 12 – Model of major mechanisms for HCO₃⁻ transport in proximal tubule. Proximal tubule reabsorbs HCO₃⁻ using the active-transport process that secretes H⁺ into the tubule lumen and the titration of HCO₃⁻ to H₂O and CO₂. Legend: **NHE3**: Na⁺/H⁺ exchanger isoform 3; **AQP1**: aquaporin isoform 1; **CA**: carbonic anhydrase II and IV; **NBCe1**: electrogenic Na⁺/HCO₃⁻ co-transporter isoform 1; **Na⁺/K⁺-ATPase**: sodium-potassium adenosine triphosphatase.

A compelling body of clinical and experimental evidences documents the importance of the kidney in the pathogenesis and maintenance of arterial hypertension. In the classic study Curtis and collaborators (150) found that essential hypertensive patients who received transplanted kidneys from normotensive donors had normal blood pressure and evidences of reversal end-organ damage. Further supporting this idea, several genetic studies also demonstrated that genes related to high or low blood pressure encode proteins that mediate or are involved in renal sodium handling (151).

Among the various regulatory systems that impact blood pressure, Ang II plays a key role. These actions are mediated primarily by AT1 receptors, which activation

increases NHE3 activity by a myriad of mechanisms. In an attempt to clarify the relative importance of the AT1 receptor in blood pressure homeostasis Crowley and collaborators performed kidney cross-transplantation between wild-type and AT1_A-knockout mice (152, 153). These studies demonstrated that in hypertension the receptors inside the kidney played the dominant role, driving elevations in blood pressure as well as the development of cardiac hypertrophy (152). It was latter demonstrated that AT1 receptor knockout only in proximal tubule was sufficient to decrease blood pressure, despite intact vascular responses. This study showed that elimination of AT1 receptors from the proximal tubule provided significant protection against Ang II dependent hypertension, identifying this epithelial compartment as critical to the pathogenesis of hypertension. Moreover, protection from hypertension was associated with a decreased NHE3 and fluid reabsorption and improved pressure natriuresis response, suggesting that modulation of sodium handling is critical for these actions (154).

Supporting the relevance of NHE3 in the development of arterial hypertension, our group also showed that NHE3 activity is 80% higher in young spontaneously hypertensive rats (SHR) before the onset of hypertension comparatively to its genetic counterpart Wistar Kyoto rat at the same age (155). The increased activity of NHE3 was associated with changes in the endogenous NHE3 expression and phosphorylation at the PKA consensus site serine 552. As such, the young prehypertensive SHR presented a ratio of NHE3 phosphorylation/total NHE3 in renal cortical membranes 60% smaller than its genetic counterpart Wistar Kyoto rat at the same age. In addition, in adults SHR rats, where the hypertensive state was already established, NHE3 activity was decreased indicating that reduced NHE3-mediated sodium reabsorption may represent an integral part of the pressure-natriuresis process.

Sodium and water retention is a common feature in the pathophysiology of heart failure, which are the most devastating clinical manifestations in heart failure. The major cause for edema is salt and water retention by the kidney, which occurs due to inadequate blood pumping activating inappropriate and persistently physiological systems that maintain extracellular volume homeostasis, like neurohumoral activation of the sympathetic nervous system (SNS) and the renin-angiotensin-aldosterone system (RAAS) (156). In the kidney, SNS and Ang II plays a stimulatory role in renal tubular reabsorption of sodium and water (156). The mechanisms of tubular sodium handling in heart failure are still incompletely understood; therefore it has been shown in

experimental models of heart failure that there is an increase in protein abundance of several sodium transport proteins, like $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ -cotransporter, NHE3 and epithelial sodium channel (157, 158). Interestingly, Inoue and collaborators (156) showed that NHE3 activity is higher in proximal tubules from rats with experimentally induced heart failure than sham. This study also demonstrated that the increase NHE3 activity was accompanied by an enhanced messenger RNA and total protein expression in the renal cortex. Moreover, NHE3 expression was also increased in the microvilli domain of the brush border in parallel with a reduction in the phosphorylation of NHE3 at the PKA consensus site serine 552. These results suggest that NHE3 upregulation in heart failure apparently occurs at transcriptional, translational and posttranslational levels and may contribute to the fluid retention characteristic of this disease. The upregulation of NHE3 may be driven, at least in part, by Ang II. In fact, it has been demonstrated that Ang II receptor blockers (ARBs), normalize NHE3 levels and sodium excretion in heart failure rats (157).

Misregulation of NHE3 was also reported in others pathophysiological conditions like diabetic kidney acute kidney injury, in which NHE3 is upregulated and downregulated, respectively. These studies highlighted the central importance of NHE3 absorptive functions that profoundly influence systemic electrolyte, acid-base and blood pressure homeostasis, and the need to improve our understanding of molecular mechanisms that modulate NHE3 in human diseases.

Chapter 4 - AT1 receptor biased agonism: state of art

4.1 – Cardioresenal effects of AT1 receptor/beta-arrestin mediated signaling

The classical activation of the AT1 receptor was thought to be mediated only by G-proteins. It is now known that AT1 receptor can also mediate signal cascades which are dependent on beta-arrestins. The discovery that a subset of AT1 receptor signal pathways can be selectively activated in detriment of the other at the level of the ligand leads to the finding of a new class of pharmacological compounds, which aim to preserve the beneficial effects while reducing the unwanted ones (60, 64). The first biased agonist described for the beta-arrestin/AT1 receptor signaling was the synthetic Ang II analog [Sar¹, Ile⁴, Ile⁸]-Angiotensin II (SII). SII was originally described as an antagonist for the AT1 receptor, that promote AT1 receptor internalization without activating the G-protein signaling (159). It was latter demonstrated that SII activate the MAPKs ERK1/2 and that beta-arrestin 2 was needed as a scaffold protein for its activation. Moreover, contrarily to the Ang II mediated ERK1/2 activation, the activated ERK1/2 by SII remained associated to the complex AT1 receptor/beta-arrestin in the cytosol, and this signalsome-bound ERK1/2 cannot translocate to the nucleus and is transcriptionally silent (160, 161). This reversal effect in which SII behaves like an antagonist for the G-protein signaling but as an agonist for the beta-arrestin signaling as latter defined as biased agonism.

More recently, three other new biased agonists for the AT1 receptor were discovery, the TRV120023 (Sar-Arg-Val-Tyr-Lys-His-Pro-Ala-OH), TRV120026 (Sar-Arg-Val-Tyr-Tyr-His-Pro-NH₂) and TRV120027 (Sar-Arg-Val-Tyr-Ile-His-Pro-D-Ala-OH), which present a more potent and higher selectivity for the AT1 receptor/beta-arrestin signaling (162). Indeed, these compounds are unable to accumulate inositol monophosphate (IP1) and diacylglycerol (DAG) in HEK-293 cells overexpressing the AT1 receptor, whereas silencing of beta-arrestin-2 eliminates the ability of TRV120027 and TRV120023 to promote late phosphorylation of ERK, Akt or endothelial NO synthase, well-established downstream effectors of beta-arrestin (163, 164).

Pointing to the pharmacological significance of TRVs, recent studies reported that besides its antagonist effect on calcium signaling, it also increased cardiomyocyte inotropy and lusitropy (165), stimulate cardiomyocyte proliferation (166) and activate

the pro-survival kinase Akt (167). Moreover, *in vivo* studies showed that TRV120027 preserve the benefits of the Ang II receptor blockers (ARBs), including lowering blood pressure and preserved kidney function, while sustaining the beneficial effects of the AT1 receptor activation like cardiac performance not observed in ARBs treatment (162).

In an attempt to test the translatability of these findings to a disease model, the effects of TRV120027 were evaluated in a canine model of heart failure. These studies showed that TRV120027 functions as ARBs decreasing the pressor effect of Ang II, but unlike ARBs unloads the heart (168). Kim and collaborators (169) also assessed the effect of TRV120023, another biased agonist from the same class. In response to ischemia reperfusion and myocardial stretch injury TRV10023 showed that: (1) increased cardiac contractility, whereas losartan decreased; (2) induce the ERK1/2 and Akt signaling which was not observed in losartan treated rats; and (3) TRV120023 treatment decreases cell mortality compared to losartan. All effects were lost the in the beta-arrestin 2 knockout (KO) mice. Furthermore, TRV120023 treatment for 3 weeks blocks Ang II-induced cardiac hypertrophy while stimulating the myosin calcium sensitivity (170). All together, these findings indicate that TRVs effects are not compound specific, but a pharmacological profile of this class of peptides.

Interestingly, TRVs also promote renal actions that are distinct from those exerted by Ang II (163, 171). In dogs with acute heart failure, TRV120027 increased urinary flow and sodium excretion associated with a decrease in fractional proximal sodium reabsorption (163, 171) (Fig. 13).

A first in human study was conducted with ascending doses of TRV027 to explore its tolerability, pharmacokinetics and pharmacodynamics in healthy volunteers (172). Consistent with preclinical findings, TRV120027 reduce blood pressure by 5- 10 mmHg in patients with activated RAS, and had no effect in the volunteers with normal RAS. The sample was small to determine dose dependence response, but the study showed that TRV120027 was safety and tolerable.

Figure 13 – Major physiological and pharmacological effects of AT1 receptor modulation. Ang II stimulates G protein- and beta-arrestin mediated signaling at the AT1 receptor, which include Ca^{2+} release via the G-protein pathway. The net effect of these signals after chronic Ang II stimulation is cardiac hypertrophy, increased Ca^{2+} sensitivity, increased maximum tension, decreased Ca^{2+} cooperativity of cardiac myofilaments and increase in Na^+ and fluid retention which culminate in increased blood pressure and cardiac hypertrophy. Angiotensin receptor blockers (ARBs), such as Losartan, antagonize both G protein and beta-arrestin pathways, blocking the cardiac hypertrophy and the increase in blood pressure caused by Ang II. Beta-arrestin biased ligands, such as TRV, block Ang II-mediated cardiac hypertrophy but preserve or enhance the cardiac inotropic effects. Brown shading indicates effects that may be adverse, whereas green shading indicates effects that may be beneficial in the setting of cardiovascular disease. **GRK**: G protein-coupled receptor kinases; **AT1R**: Angiotensin II type 1 receptor.

A randomized, double-blind, placebo-controlled, titration study was performed in patients with stable chronic heart failure to evaluate the safety and pharmacology of TRV027, using escalating doses and during a 9 hour maintenance phase, followed by a washout period [ClinicalTrials.gov Identifier: NCT01187836]. This study demonstrated that TRV120027 had a rapid response, which is quickly reversible and dose-dependently modifies hemodynamics in a beneficial way in this population. A larger

Phase 2b study with TRV120027 is now progressing in acute heart failure patients. Thus, TRV120027 may not only be an innovative pharmacological tool to help elucidate G-protein–dependent versus G-protein–independent signaling at the AT1 receptor, but it may also be a novel therapeutic agent in some diseases.

Chapter 5 – Rationale and hypothesis

In the past two decades, it has become clear that GPCRs signal is more complex than previously expected. Cell surface receptors and their downstream cascades are of interest since they play key roles in modulating cell physiology, and they are the target of almost 50% of the therapeutic agents.

Having in mind that: 1) the triggering of one AT1 receptor signaling pathway in detriment of the other can have beneficial effects; 2) AT1 receptor plays a crucial role in the regulation of proximal tubule NHE3, and evidences demonstrated that NHE3 stimulation can be associated to the development and/or pathophysiology of several diseases, such as hypertension and heart failure; 3) **recent evidences demonstrating that the biased agonism of the AT1 receptor/beta-arrestin induces diuresis and natriuresis**, our major goals were:

- 1) Test the hypothesis that the biased agonism of AT1 receptor inhibits NHE3 activity in proximal tubule *in vitro* and *in vivo*.
- 2) Test the hypothesis that the biased agonism of AT1 receptor inhibits NHE3 activity in proximal tubule *in vitro* and *in vivo* while the ARBs have no tonic effect over the exchanger activity.
- 3) Evaluate the signaling pathways by which the biased agonism of AT1 receptor inhibits NHE3 activity in proximal tubule *in vitro* and *in vivo*.

Chapter 6 – Materials and Methods

6.1 – Materials

Table 1 – General reagents and kits

General reagents	Source
Acrylamide	Sigma, St. Louis, MO, USA
β-mercaptoethanol	Sigma, St. Louis, MO, USA
Bovine serum albumin (BSA)	Sigma, St. Louis, MO, USA
Bromophenol blue	Sigma, St. Louis, MO, USA
Butanol (1-butanol) 99%	Sigma, St. Louis, MO, USA
Calcium chloride	Sigma, St. Louis, MO, USA
Dithiothreitol (DTT)	Sigma, St. Louis, MO, USA
Ethylenediaminetetraacetic acid (EDTA)	Sigma, St. Louis, MO, USA
EZ-Link Sulfo-NHS-SSBiotin	Thermo Fisher Scientific, Rockford, IL, USA
Glucose	Sigma, St. Louis, MO, USA
Glycerol 99.5%	Sigma, St. Louis, MO, USA
Glycine	Sigma, St. Louis, MO, USA
Hexamethyldisilazane	Sigma, St. Louis, MO, USA
Hydrogen chloride	Sigma, St. Louis, MO, USA
(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer	Sigma, St. Louis, MO, USA
Immunopure immobilized streptavidin	Thermo Fisher Scientific, Rockford, IL, USA
Lipofectamine 2 000	Life Technologies, Carlsbad, CA
Magnesium chloride	Sigma, St. Louis, MO
Magnesium sulfate	Sigma, St. Louis, MO
Mes buffer	Sigma, St. Louis, MO
Methanol	Merck, Billerica, MA, USA
N-methyl-d-glucamine (NMDG)	Sigma, St. Louis, MO
Nigericin	Sigma, St. Louis, MO
Non-fat dry milk	

Polyvinyl difluoride (PVDF) membranes	Millipore Immobilon-P, Millipore, Bedford, MA
Ponceau	Sigma, St. Louis, MO, USA
Potassium gluconate	Sigma, St. Louis, MO, USA
Potassium chloride	Sigma, St. Louis, MO, USA
PKA kinase activity assay	Enzo Life Science, Farmingdale, NY, USA
Protein ladder	Bio-Rad, Hercules, CA, USA
Small interfering RNA (siRNA)	Life Technologies, Carlsbad, CA
Sodium chloride	Sigma, St. Louis, MO, USA
Sodium dodecyl sulfate (SDS)	Sigma, St. Louis, MO, USA
Sodium deoxycholate	Sigma, St. Louis, MO, USA
Sodium hydroxide	Sigma, St. Louis, MO, USA
Sodium phosphate mono and dihydrated	Sigma, St. Louis, MO, USA
Tetramethylethylenediamine (TEMED)	Sigma, St. Louis, MO, USA
Tetramethylammonium chloride (TMA-Cl)	Sigma, St. Louis, MO, USA
Tris Hydrogen chloride	Sigma, St. Louis, MO
Tris Base	Sigma, St. Louis, MO
Triton X-100	Sigma, St. Louis, MO, USA
Tween 20	Sigma, St. Louis, MO, USA
TRIzol	Thermo Fisher Scientific, Rockford, IL, USA
Random primers	Life Technologies, Carlsbad, CA
Reverse transcriptase Super Script III	Life Technologies, Carlsbad, CA
RNeasy Mini Kit	Qiagen, Venlo, Limburg, Netherlands

Table 2 – Inhibitors and agonists

Inhibitor and agonists	Source
Angiotensin II	Sigma, St. Louis, MO, USA
Akt inhibitor (Akti)	Tocris Bioscience, Bristol, UK
Captopril	Sigma, St. Louis, MO, USA
Losartan	Sigma, St. Louis, MO, USA
PD123319	Tocris Bioscience, Bristol, UK
PitStop 2	Abcam, Cambridge, UK
S3226	Generously provided by J. Punter Sanofi-Aventis Deutschland GmbH, Frankfurt, Germany (173)
Telmisartan	Sigma, St. Louis, MO, USA
TRV120023	Kindly provide by Jonathan Violin, Trevena, King of Prussia, USA
U0126	Sigma, St. Louis, MO, USA
Wortmannin	Tocris Bioscience, Bristol, UK

Table 3 – Cell culture reagents

Cell culture reagents	Source
Dulbecco's Modified Eagle's medium (DMEM) High glucose	Life Technologies, Carlsbad, CA
Heat-inactivated fetal bovine serum	Life Technologies, Carlsbad, CA
L-glutamine	Life Technologies, Carlsbad, CA
Penicillin and streptomycin	Life Technologies, Carlsbad, CA
Sodium pyruvate	Life Technologies, Carlsbad, CA
0.25% Trypsin-EDTA	Life Technologies, Carlsbad, CA

Table 4 – Antibodies used in the study

Antibodies	Dilution used	Source
Monoclonal (mAb) for actin, clone JLA20	1:50 000	Merck, Billerica, MA, USA
Alexa 488-conjugated	1:500	Molecular Probes, Eugene, OR, USA
Alexa 488-conjugated	1:500	Molecular Probes, Eugene, OR, USA
mAb beta-arrestin 1/2, clone D24H9.	1:1000	Cell Signaling Technology Danvers, MA, USA
polyclonal antibody (pAb) GAPDH	1:1000	Santa Cruz Biotechnology, Dallas, TX, USA
mAb for NHE3, clone 3H3	1:1000	kindly provided by Biemesderfer and Aronson, Yale University (110)
pAb for NHE3, clone NHE3-C00	1:100	From McDonough Lab (174)
mAb for Ser 552 NHE3 clone 14D5	1:1000	Santa Cruz Biotechnology, Dallas, TX, USA
mAbvillin	1:100	Immunotech, Chicago, IL, USA

6.2 – Methods

6.2.1 – Animals

Animal procedures and protocols were followed in accordance with the ethical principles in animal research of the Brazilian College of Animal Experimentation and National Institutes of Health Guide for the Care and Use of Laboratory. Protocols were approved by the institutional animal care and use committees from University of São Paulo School of Medicine and University of Southern California Keck School of Medicine. *In vivo* microperfusion experiments were performed using male Wistar rats (220-260 g BW) and *in vivo* immunofluorescence were performed on male Sprague

Dawley rats (200-250 g BW) that were kept under diurnal light (12h) conditions, humidity of 60% and temperature of 22°C with free access to food and water.

The procedures were approved by the ethical comity of the Medical School of University of São Paulo.

6.2.2 – Evaluation of natriuretic and diuretic effects of TRV120023 by acute infusion.

Wistar male rats were anesthetized by a subcutaneous administration of ketamine–xylazine (50 and 10 mg/Kg, respectively) and placed on a heated surgical table to maintain its body temperature. The trachea was exposed and cannulated with a PE-240 catheter to allow spontaneous breathing. A PE-50 catheter was inserted into the left jugular vein for drug infusion, the bladder cannulated for urine collection and right carotid for blood pressure and sampling. The rats were subjected to a constant intravenous infusion (0.04 ml/ min). The first 30 minutes the two groups were infused with 4% of bovine serum albumin in saline solution to ensure euolemia, after which the infusion of either TRV120023 (50 µg/kg) or vehicle (4% of bovine serum albumin) was performed for 30 minutes. The urine collection of 30 minutes was used to measure urinary flow, sodium and creatine.

Urinary and plasma sodium concentrations were measured on a Radiometer ABL5 blood-gas analyzer (Radiometer, Denmark). Urinary creatinine concentration was determined with a kit (Labtest, Lagoa Santa, MG, Brazil) and plasma creatinine was measured on a Beckman Coulter Synchron CX7 Analyzer (Beckman Coulter, CA). Total urinary volume was determined by gravimetrically and normalized by the body weight.

The urinary flow (**UF**), sodium excretion (**Na excretion**) and glomerular filtration rate (**GFR**) and % of fractional sodium excretion (**FENa**) were calculated as:

$$UF (\mu L/Kg/min) = \frac{Urinaryvolume (\mu L/Kg)}{Timeofcollection (min)}$$

$$Naexcretion (meq/min/Kg) = [Na]urinary(mmol/L) * UF(mL/Kg/min)$$

$$GFR (= \frac{UF(ml/Kg)/min) * urinarycreatinine (mg/dL)}{plasmacreatinine (mg/dL)}$$

$$\% FENa = 100 * \frac{[Na]urinary(mmol/L) * plasmacreatinine(mg/dL)}{[Na]plasma(mmol/L) * urinarycreatinine(mg/dL)}$$

6.2.3 – Stationary microperfusion

Rats were anesthetized with ketamine-xylazine-acepromazine (64.9, 3.20, and 0.78 mg/kg sc, respectively) and placed on a heated surgical table to maintain body temperature. The left jugular vein was cannulated for infusion of mannitol in isotonic saline solution at a rate of 0.1 ml/min. The microperfusion procedure was performed as described previously (175). Briefly, proximal tubule was punctured using a double-barreled micropipette. One barrel was used to inject FDC-green colored Ringer perfusion solution (in mM: 80 NaCl, 5 KCl, 25 NaHCO₃, 1 CaCl₂, 1.2 MgSO₄, and raffinose to reach isotonicity, at 0 P_{CO2}) and the other to inject Sudan black colored castor oil used to block the injected fluid columns in the lumen. To measure luminal pH, proximal tubules were impaled by a double-barreled asymmetric microelectrode, one barrel containing H⁺-ion-sensitive ion-exchange resin with hexamethyldisilazane (Sigma Fluka, Buchs, Switzerland) and the other containing the reference solution (1 M KCl) with FDC-green. The voltage between the microelectrode barrels, representing the luminal H⁺ activity, was sampled every 0.5 seconds by an AD converter (Lynx, São Paulo, Brazil) in a microcomputer (Fig. 14). Net bicarbonate influx was measured from luminal pH and blood P_{CO2} by a Severinghaus electrode.

The rate of tubular acidification was expressed as the half-time of the exponential reduction of the injected HCO₃⁻ concentration at its stationary level (t_{1/2}). Net HCO₃⁻ reabsorption (J_{HCO₃⁻}) per cm² of tubule epithelium was calculated from the equation:

$$J_{HCO_3^-} = \ln_2/t_{1/2} ([HCO_3^-]_0 - [HCO_3^-]_s) * r/2$$

where $t_{1/2}$ is the half-time of bicarbonate reabsorption, r is the tubule radius measured by an ocular micrometer, and $[\text{HCO}_3^-]_0$ and $[\text{HCO}_3^-]_s$ are the concentrations of HCO_3^- injected and at the stationary level, respectively.

Figure 14 – Schematic representation of proximal tubule stationary microperfusion technique.

6.2.4 – Immunofluorescence

Rats were anesthetized intraperitoneally with Inactin (110 mg/kg) and a small dose of intramuscular ketamine (100 μl). Body temperature was maintained thermostatically at 37 °C. Polyethylene catheters (PE-50) were inserted into the carotid artery to monitor blood pressure and into the jugular vein for infusion of drugs and 4% BSA in 0.9% saline at 40 $\mu\text{l}/\text{min}$ to maintain euvolemia. Blood pressure was measured continuously and remained within the autoregulatory range (80-110 mmHg). At the completion of all surgical procedures, the animals were allowed to equilibrate for 15 min before infusion of drugs. Rats were either infused with 4% BSA in 0.9% saline (control) or infused with TRV120023 (50 $\mu\text{g}/\text{kg}/\text{min}$) in the same BSA-saline solution for 20 min. At the end of each treatment, the left kidneys were fixed *in situ* by removing the capsule and placing the isolated kidney in a small Plexiglas cup and bathing it in PLP fixative (2% paraformaldehyde, 75 mM lysine, and 10 mM Na-periodate, pH 7.4) for 5 min to avoid changes in perfusion pressure. The kidneys were then removed, cut in

half on a midsagittal plane, and post-fixed in PLP fixative for 2-4 h. The fixed tissue was rinsed with PBS, cryoprotected by overnight incubation in 30% sucrose in PBS, embedded in Tissue-Tek OCT Compound, and frozen in liquid nitrogen. Cryosections (5 μ m) of TRV120023 treated and paired control were cut and transferred to charged glass slides and air dried. For immunofluorescence labeling, the sections were rehydrated in PBS for 10 min, followed by a 10 min wash in 50 mM NH_4Cl in PBS and antigen retrieval with 1% SDS in PBS for 5 min. After two 5 min washes in PBS, the sections were then blocked with 1% BSA in PBS to reduce background. Double-labeling was performed by incubating with polyclonal antiserum NHE3-C00 and monoclonal antibody against villin, both at a dilution of 1:100 in 1% BSA in PBS for 2 h at room temperature. After three 5 min washes in PBS, the sections were incubated with a mixture of Alexa 488-conjugated goat anti-rabbit and Alexa 568-conjugated goat anti-mouse secondary antibodies diluted 1:500 in 1% BSA in PBS for 1 h, washed three times with PBS, mounted in ProLongAntifade, and dried overnight at room temperature. Slides were viewed with a Zeiss LSM 510 confocal system with differential interference contrast overlay and microscopy. Results shown are representative of results in three sets of rats assayed.

6.2.5 – Cell culture

The cell line of proximal tubule-derived from the parental opossum kidney (OKP) of *Didelphis marsupialis virginiana*, which display many characteristics of kidney proximal tubular epithelial cells (176), was used. The OKP cells were maintained in 75-cm² tissue culture flasks in DMEM high glucose medium supplemented with 10% (v/v) of heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Cultures were incubated at 37 °C in a humidified 5% CO_2 -95% air atmosphere. Cells were subculture using $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free phosphate-buffered saline and 0.25% trypsin-EDTA (ethylenediaminetetraacetic acid). The culture medium was replaced every 2 days. For experiments, cells were subculture in tissue culture plates, grown to confluence and serum starved for 24 h before performing studies.

6.2.6 – Measurement of intracellular pH (pHi) recovery by fluorescence microscopy

NHE3 activity was measured in OKP cells as the rate of Na⁺-dependent intracellular pH (pHi) recovery after an acid load with NH₄Cl (in mM: 20 NH₄Cl, 125 NaCl, 5 KCl, 1 MgCl₂, 0.83 NaH₂PO₄, 0.83 Na₂HPO₄, 1 CaCl₂, 8 HEPES, 25 mM glucose, pH 7.4) as previously described (177). Intracellular pH was monitored by dual excitation ratio 440 and 495 nm with a 150 W xenon lamp, and the fluorescence emission was monitored at 530 nm by a photomultiplier-based fluorescence system (Georgia Instruments, PMT-400) at time intervals of 1 second. Briefly, cells were grown to confluence on glass coverslips loaded with 10 μM BCECF-AM in control solution (in mM: 130 NaCl, 5 KCl, 1 MgCl₂, 0.8 NaH₂PO₄, 0.83 Na₂HPO₄, 1.0 CaCl₂, 7 HEPES, 25mM glucose, pH 7.4) for 5 min and placed in a thermoregulated chamber mounted on an inverted epifluorescence microscope (Nikon, TMD). After several washes, the BCECF-loaded cells were exposed to control solution until pHi stabilization and then prepulsed with NH₄Cl for 2 min for subsequent acid loading. After acid load cells were exposed to control solution or TRV120023, losartan, telmisartan, S3226, PD123319, Akt inhibitor and U0126 (ERK1/2 inhibitor) diluted in control solution and the rates of Na⁺-dependent pHi recovery acquired. At the end of each experiment, the high K⁺-nigericin method was used to calibrate the BCECF signal (in mM: 20NaCl, 130 KCl, 1 MgCl₂, 1 CaCl₂, 5 HEPES, containing 10 μM nigericin adjusted to pH values of 7.5, 7.1, 6.5, 6.0). For all the experiments the Na⁺-dependent pHi recovery rate was calculated from the first 2 minutes by linear regression analysis and presented as dpHi/dt (pH Units/min) (Fig. 15).

In order to examine the role of Na⁺-dependent mechanisms in regulating resting pHi, cells were equilibrated in medium in which all the Na⁺ was replaced with N-methyl-D-glucamine (134 mM NMDG, Na⁺ free solution) before the control solution or control with treatment. Confirming that pHi recovery was almost dependent on Na⁺ in this cells the mean of pHi recovery in the presence of 134 mM NMDG solution (Na⁺ free solution) was 0,006 ± 0,001 pH units/min.

Figure 15 –Schematic representation of intracellular pH recovery technique and buffering process.
(adapted from (4))

6.2.7 – Total RNA extraction from OKP cells.

Total RNA from OKP cells was extracted with TRIzol reagent. Firstly, cells were trypsinized and centrifuged at 926 g for 5 minutes. The supernatant was discarded and the pellet homogenized with 1 ml of TRIzol for each 10 cm² of cells monolayer. This suspension was kept at room temperature (RT) for 10 minutes to allow the complete dissociation of the nucleoproteins complexes. After incubation, 0.2 ml chloroform was added for each 1 ml of TRIzol reagent, samples were vigorously homogenized by inversion during 15 seconds, and incubated for 3 minutes at RT. Samples were then centrifuged at 12 000 g for 15 minutes at 4°C. The supernatant was recovery for another tube and 0.5 ml of ice-cold isopropanol for each 1 ml of TRIzol reagent added, and incubated for 10 minutes at 4°C. These samples were centrifuged at 12 000 g for 10 minutes at 4°C for RNA precipitation. The supernatant was discarded and the pellet washed three times with ice-cold 70% ethanol (each wash consisted in resuspension of the pellet with the ice-cold 70% ethanol followed by centrifugation at 20 160 g for 1 minute at 4°C, in which the supernatant was discarded). The RNA pellet was then resuspended in UltraPure DEPC Water (0.1% diethylpyrocarbonate).

To test RNA integrity was assessed by electrophoresis on a denaturing agarose gel MOPS/formaldehyde. Briefly, the 0.5 g agarose was diluted in 5 ml 200 mM MOPS buffer (Table 5) and 41,45 ml DEPC water in microwave for 2 minutes. After cooling for 15 minutes 2,55 ml of 37% formaldehyde was added and placed in the gel box well. While gel was setting, the platform was filled with running buffer (Table 5), and RNA

samples were diluted in DEPC for a total amount of 5 µg in 1 ml. After dilution 8 µl of RNA sample buffer (Table 5) and 1 µl at 10 mg/ml of ethidium bromide was added.

RNA integrity was confirmed by the presence of the two ribosomal RNAs 28S and 18s. Intact total RNA of eukaryotic samples run on a denaturing gel should have sharp and clear 28S and 18S rRNA bands. The 28S rRNA band should be approximately twice as intense as the 18S rRNA band (Supplementary Fig. 1). After integrity confirmation the extracted RNA was purified with RNeasy mini kit, and quantified in Nanodrop 1 000 to assess purity (only RNAs with absorbance ratios near 2.2 ratio were considered).

Table 5 – Buffers constituents

Buffer	Components concentrations
10x MOPS (3-(N-Morpholino)propanesulfonic acid, 4-morpholinepropanesulfonic acid)	200 mM MOPS; 10 mM EDTA; 50 mMNaAcetate; pH 7.0 KOH
Running buffer	20 mM MOPS
RNA sample buffer	20 mM MOPS; 1 mM EDTA, 5 mMNaAcetate; 50 % (v/v) formamide; 2.2 M formaldehyde.

6.2.8 – Complementary DNA (cDNA) synthesis and amplification

cDNA synthesis was performed using 5 µg of purified RNA, random primers and reverse transcriptase Super Script III according to the manufacturer's instructions. The cDNA synthesized was used for polymerase chain reaction (PCR) using the selected primers for beta-arrestin 1 and 2 for *Monodelphis domestica* present in BLAST (*Monodelphis domestica* was used because no data was available for the *Didelphis marsupialis virginiana*). Four pairs (reverse and forward) of primers were designed for beta-arrestin 1 and three pairs (reverse and forward) for beta-arrestin 2. After optimization the final PCR was performed with 400 nM of each primer was used, 1 µl of cDNA, 200 µM of deoxynucleotides (dNTPs), 3 and 2 mM of MgCl₂, for beta-arrestin 1 and beta-arrestin 2, respectively, and 0.05 U/µl de Taq Polymerase. The conditions and cycles used are summarized in Table 6.

Table 6 – Summary of PCR conditions

Step	Temperature	Time	Cycles
Initial denaturation	94°C	5 minutes	1
Denaturation	94°C	30 seconds	35
Annealing	T _m of each pair of primers	30 seconds	
Extension	72°C	60 second for each 1 Kb.	
Elongation	72°C	7 minutes	1
	4°C	Hold	

With these conditions and the pairs of primers presented in Table 7 we were able to obtain amplicons matching some isoforms of beta-arrestin 1 or 2 present in BLAST for the *Monodelphis domestica* (Supplementary Fig. 2).

Table 7 – Primers used for PCR with respective melting temperature (T_m) and length

β-arr isoform	Primer sense (5' to 3')	Primer reverse (5' to 3')	T _m	Predicted length of the amplicon (pb)
1	TCGATGGTGTGGTTCTGG TG	ACCTTAGCACTGGCTGTT CC	60°C	~2 000
1	TCGATGGTGTGGTTCTGG TG	ACCTCCCTCCTTGAGGTC AT	60°C	~2 400
1	TCGATGGTGTGGTTCTGG TG	CCACATCACTGGATGCGA GA	56°C	~900
2	TGCCTTCCGATATGGTCG TG	ATAGGGAGCTTGGTCCTG CT	64°C	~1 100
2	TGGATGTTTTGGGCCTGT CA	ATAGGGAGCTTGGTCCTG CT	62°C	~1 100

The final products of the PCR were purified by chromatography using column S-300 HR and quantified on Nanodrop 1 000. These amplicons were posteriorly used for sequencing by Sanger method.

6.2.9 – DNA sequencing by automatized Sanger method

Sanger sequencing is a method of DNA sequencing based on the selective incorporation of chain-terminating di-deoxynucleotides (ddNTPs) by DNA polymerase during in vitro DNA replication. The modified di-deoxynucleotidetriphosphates (ddNTPs), terminates DNA strand elongation by the lack a 3'-OH group required for the formation of a phosphodiester bond between two nucleotides, and presents a dye label for detection in automated sequencing machines.

The sequencing reaction was performed with 40 cycles using 20 ng cDNA template, 5 mM of both DNA primer forward and reverse, 1 mM deoxynucleotidetriphosphates (dNTPs), and with 100 mM modified di-deoxynucleotidetriphosphates (ddNTPs). Taq polymerase was used according to the manufacturer. The DNA sample was divided into four separate sequencing reactions, containing all the above mentioned components but to each reaction only one of the four ddNTPs was added. The ddNTP was added in approximately 100-fold excess of the corresponding dNTP allowing for enough fragments to be produced while still transcribing the complete sequence.

DNA sequence was carry out by capillary electrophoresis for size separation, detection and recording of dye fluorescence, and data output as fluorescent peak trace chromatograms, as the example presented in the Fig. 15 below:

Figure 16 – Schematized DNA sequencing by automatized Sanger method. Each color represents a base: blue is cytosine, green is adenosine and black is guanine and red thymine.

After analyses of the sequencing data the following sequences were obtain: for beta-arrestin 1:

AGGAGAGTGTACGTGACTTTGACCTGCGCCTTCCGCTATGGCCGGGA
GGACCTCGACNTGTTGGGCCTGACCTTCCGCAAGGACCTGTTTGTGGCCAAC
ATCCAGTCCTTCCCACCTGCCCCTGAAGACAGGCCCTCACTCGACTCCAGG
AACGGCTCATCAGGAAATTGGGAGAGCACGCCTACCCCTTTACCTTTGAGA
TCCCTCCGAATTTGCCCTGCTCCGTCACACTTCAGCCAGGGCCGGAGGACAC
AGGGAAGGCCTGTGGTGTGGACTATNAAGTCAAAGCCTTCTGTGCAGAAAA
TCTGGAGGAGAAGACTCACAAACGGAATTCTGTGCGCCTGGTTATCCGAAA
GGTCCAGTATGCCCCGGAGCGGCCCGGCCCCAGCCACGGCCGAGACCAC
TCGTCAGTTTCTGATGTCTGACAAGCCCTTGCACTTGGAGGCCTCCCTGGAC
AAGGAGATCTACTACCATGGGGAACCAATCAGTGTTAATGTCCATGTCACC
AACAACACCAACAAAACAGTGAAGAAGATAAAAATTTAGTACGCCAGTA
CGCTGACATCTGCCTGTTCAACACGGCACAGTACAAATGCCCGGTGGCTGT
GGAGGAGGCTGATGACATGGTGGCCCCAAGTTCAACATTCTGCAAAGTCTA
CACACTTACCCCATTTCTTGGCCAACAACCGTGAGAAGCGAGGCCTGGCCCT
GGATGGCAAGCTGAAGCATGAAGACACCAACTTGGCTTCCAGTACCCTGTT

GAGGGATGGCACAAATAAGGAGATCTTAGGAATCATCGTGTCTACAAAGT
CAAAGTGAAGCTGGTTGTTTC

(...)

TAGCCCTTCAAATAATTGAGTGAAGAAAACCTGTCATTCTCCTTNTGA
AATATTTTCATCCAGGTTAAAATAATTCCCAGAGTCCTTACCCAGTATTTGT
ATGGCATCCTCTCCACTCCCTTTGCCCTTCTCAAGACCTGTGAGGACAAGTT
AAGTTAGAAGGTGGGAGTGAGCAGGACTTTGTAAATTGATGCCCCTTACTC
TTGAAGCTGACAGTGAGGAATGAAGACCCTCCCAGGTCGACCAAGTATTCC
ACTATGTATCATTAATTTCCAGTCCCAGAGAATGCAGAATAAACATACCAG
AAGGTAAAACTTTTGGTTTAGGTGACTGAAGAGGCTATCATATGGATGGAA
AATGATAGTTTTGGTTCTTGCCTTGAAGCCCTCCCTTTGCAAAGATTTCAGT
GCACTGATTCTTCCTTTGATTCATTTCTAAAGTCTGAGATGTTCCAAACACA
ATAGTTCTCCATGAAAACATGGTCCTAATCCCCAGATGTATTTGGTCAACTC
GTTCACTTTCTGTCAACTTGTTCACTGTCTCCTATAAGGACCCCAACACCA
CTTTGCCAGGGGTACTGCAACTCTTGGATTAGTTGGTGCTGCTTGCCTGTA
TGCAGAGGGAACTGGGGAGGCTGAATTGGATCATTGAACTCCACACAGTT
TTGAGTACTTGTCTACCACAAGGATTTGTGCCAGGTTGTAGATGTGCAAAA
AAAGTGAAAAAGTCCCTGCACTCAAGGATCTTAGGTTCTACTGGGAAAATG
CAGTGCATAAGTAAAAGGTCATTTGAGATTCCTTGAACCTTTGAAGAAAAC
ATAAATATTCTACAAGAAGTATAGTGCATTCTAGGCATATGGGGAACAGCC
AGTGCAAAGGCCTGGAGAGGAGAGATAGCATATACAAGAACTACTAGTTT
AGTTGGAACCCAAAGTGTGGGAAGGAGAGTGACATGAAATCAGTCTTGAAA
GGTAGGTTGGTGCAAGAGCGTGAAGGGATTTAAATGCCTAAATGTATTTGC
ATTGTTATCTAGAAATAATAGAGGGGCTCTGAAGATTTTTTAAGTCTTAGTT
TTGGATATTTTGGAGCATGGATTGAAAAGGGAGTGTGGTGGTGACTGGAAG
CAGGGAGGCCAGTTAGGAAGCTGTTCCAATGGTCTAAATCAAGAGAACTGC
TACTGAGAGTATGGGATTTGGAGGCAGAGGACTCCAAAATAAGGAGATTGA
CCTCAATGGTCTNNANNAATTCCTTCCACCACAAGCCTATGAATATACAACA
AGGAANAACCTGGCCTAAATATGACCGCCATATCTCATTGTTCTAATTTGGCA
TGTGT

and for beta-arrestin 2:

AAGGACCTGTTTGCAGCCACATACCAAGCTTTTCCCCCATCCCTGAC
CCTCCCCGAGCCACCACTCGACTTCAGGAAAGGCTGCTCAGGAAGCTGGGC
CAGCACGCTCACCCCTTCTCTTTCACAATTCCACAGAACCTGCCCTGTTCTGT
TACACTGCAACCTGGACCTGAGGACACAGGGAAGGCCTGTGGGGTAGACTT
TGAAATTCGAGCCTTCTGTGCCAAAGCATTGGAAGAGAANATCCACAAGAG
GAATTCAGTACGGCTGGTAATTAGGAAGGTACAGTTTGCCCCAGAGACACC
AGGTCCCCAGCCTACTGCTGAAACTGCCCCGACACTTCCTCATGTCTGACCGA
TCCCTGCACCTTGAGGCCTCATTGGACAAAGAGCTATATTACCATGGGGAG
CCACTTAGTGTTAATGTCCATGTCACCAACAACCTCCACCAAGACCGTCAAGA
AGATCAAAGTCTCTGTGAGACAATATGCTGATATCTGCCTCTTCAGCACTGC
CCAATATAAATGTCCAGTGGCTCAGATAGAACAAGATGACCAGGTGTCTCC
CAGTTCACGTTCTGTAAAGTGTATAATCTAACCCCACTGCTCAGTGAAAAT
AGGGAGAAACGAGGACTTGCCTTGGATGGGAAGCTCAAACATGAAGATAC
CAATCTGGCCTCCAGTACTATAGTGAAGGAAGGCGCCAACAAAGAGGTACT
GGGTATCCTTGTGTCCTATAGGGTCAAAGTGAAGTTGGTTGTGTCTCGGGGA
GGGGATGTTTCTGTGGAGCTCCCCTTTGTCTTAATGCACCCCAAGCCTCACG
ACCATCCCAGCCACTCCAAACCTCAGTCAGCTGCTCCTGAAACAAATGATCC
AGTGGATACCAATCTCATCGAATTTGAGACCAACTATGGCACAGATGATGA
CATTGTGTTTGAGGACTTCGCCAGGCTTCGGCTCAAANGAATGAAGGATGA
AGACTATGATGACCAATTCTGCTAGGGAGGGAGAG

Based on these sequences three small interfering RNA (siRNA) for each gene were designed, and are presented in Table 8.

Table 8 – Small interfering RNA sequences for beta-arrestin 1 and 2

Gene	Number	Sequence (5' to 3')
Beta-arrestin 1	1	CAACAUUCUGCAAAGUCUAtt
Beta-arrestin 1	2	CAGUGAAGAAGAUAAAAAUtt
Beta-arrestin 1	3	CAAUCAGUGUUA AUGUCCAtt
Beta-arrestin 2	4	GACUUGCCUUGGAUGGGAAtt
Beta-arrestin 2	5	CCAGUACUAUAGUGAAGGAtt
Beta-arrestin 2	6	CCAGUUCCACGUUCUGUAAtt

6.2.10 – Beta-arrestin 1 and 2 silencing.

OKP cells were plated in 6 wells plaque onto slides and transfected at approximately 50% confluence with both siRNAs against the transcripts of beta-arrestin 1 and 2. For transfection the Lipofectamine 2 000 was diluted in serum free medium and incubated during 5 minutes at RT. After incubation, the Lipofectamine was added to the diluted siRNAs and incubated for 20 minutes at RT. This mix was then added to each well at final amount per well of 80 nM for each siRNAs, 80 nM beta-arrestin 1 plus 80 nM for beta-arrestin 2 or 160 nM for scramble siRNA, with 0.06 mg/ml Lipofectamine and incubated by a period of 48 hours.

The siRNA sequences used for silencing were 3 and 4 presented in Table 8. As negative control, we used scramble Silencer Select Negative Control #1 (5'-UAACGACGCGACGACGUAAtt-3'). Silencing of beta-arrestin 1 and beta-arrestin 2 proteins were determined by immunoblotting using beta-arrestin 1/2 (D24H9) antibody and GAPDH (V-18) antibody, as internal control.

6.2.11 – Cell surface biotinylation.

OKP cells were grown to confluence in six-well plates, serum starved for 24 h and incubated in a $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free phosphate-buffered saline solution for 15 min with TRV120023 or vehicle (1% of bovine serum albumin), at 37 °C in a humidified 5% CO_2 -95% air atmosphere. All of the following manipulations were performed at 4 °C: After treatment, cells were washed twice with PBS-Ca-Mg, pH 7.4 (Table 9). Cell surface membrane proteins were biotinylated by adding 2 ml of biotinylation buffer (Table 9) twice for 25 min. Cells were then washed twice for 20 min with a quenching buffer (Table 9) and then solubilized for 1 h with a modified RIPA buffer. Samples were then centrifuged at 14 000 g for 10 min and 50 µl of streptavidin-coupled agarose was added to the supernatants. After overnight incubation, the beads were centrifuged for 5 minutes at 24 000 g at 4°C followed addition of 1 ml of RIPA (Table 9) to the pellet and incubation in agitation for 15 minutes at 4°C, repeat 5 times. After the washes 60 µl of sample buffer (Table 9) was added to each sample and run in polyacrylamide gel electrophoresis and immunoblotting.

Table 9– Buffers composition used for cell surface biotinylation

Buffer	pH	Components concentrations
PBS-Ca-Mg	7.4	0.1 mM CaCl ₂ and 1.0 mM MgCl ₂
Biotinylation buffer	7.4	150 mM NaCl, 10 triethanolamine, 2 mM CaCl ₂ and 2 mg/ml EZ-Link sulfo-NHS-SSbiotin
Quenching buffer	7.4	100 mM glycine in PBS-Ca-Mg
Modified RIPA	7.4	150 mM NaCl, 50 mM Tris-HCl, 1% Triton X-100 and 5 mM EDTA
Sample buffer	7.4	400 µl of 10% SDS, 400 µl of 99% glycerol, 200 µl of dithiothreitol, 1 ml of 50 mM Tris pH 6.8 and 0.05% of bromophenol blue

6.2.12 – Polyacrylamide gel electrophoresis and immunoblottings

Protein samples were solubilized in sodium dodecyl sulfate (SDS) sample buffer (2% SDS, 10% glycerol, 10 mM β-mercaptoethanol, 0.1% bromophenol blue and 50 mM Tris pH 6.8), and separated using 7.5% polyacrylamide gel electrophoresis 7.5 mA overnight. For immunoblotting, proteins were transferred to PVDF at 350 mA for 8–10 h at 4 °C with a TE 62 transfer electrophoresis unit (GE HealthCare). PVDF membranes containing the transferred proteins were first incubated during 1 h in Blotto (5% nonfat dry milk and 0.1% Tween 20 in PBS, pH 7.4) for blockage of nonspecific binding, followed by overnight incubation in primary antibody. Primary antibodies anti-NHE3 or anti-beta-arrestin1/2 (1:1,000) and anti-actin or GAPDH (1:50 000 or 1:1000) were diluted in Blotto. The PVDF membranes were then washed 5 times in Blotto and were incubated for 1 h with an appropriate horseradish peroxidase-conjugated secondary antibody diluted 1:2000 in Blotto. After washing 5 times in Blotto and 2 times in PBS (pH 7.4) the signals on the membranes were digitized using the ImageScanner III (GE HealthCare) and quantified using the Scion Image Software (Scion, Frederick, MD).

6.2.13 – Protein kinase A activity measurement in OKP cells

OKP cells grown to confluence in 24-well plates were treated with vehicle solution (control) or 10^{-7} M TRV120023 or 10^{-4} M forskolin for 15 minutes, at 37 °C in a humidified 5% CO₂-95% air atmosphere. Protein kinase A (PKA) activity was accessed using PKA kinase activity assay according to the manufacturer.

6.2.14 – Statistical analysis

Results were evaluated using Student's t-test for comparisons between two groups and one-way ANOVA complemented by post hoc Bonferroni to detect differences between three or more groups with normal distribution. A value of $p < 0.05$ was considered significant with two-tailed probability and the results are expressed as mean $\pm$ standard error of the mean (SEM).

Chapter 7 – Results

7.1 – Effects of acute infusion of TRV120023 on blood pressure and renal function

It has been shown that the beta-arrestin biased ligands of the AT1 receptor decrease blood pressure as well as promote renal actions that lead to an increase in urinary flow and sodium excretion associated with a decrease in fractional proximal sodium reabsorption in heart failure dogs (163, 171). In order to confirm that TRV120023 exerts natriuretic and diuretic effects, Wistar males rats were acutely infused with 50 µg/Kg TRV120023 or vehicle during which urine collection and blood pressure were measured. As summarized in Table 10, mean blood pressure and body weight were similar amount groups at baseline. Confirming the pressor effect of TRV120023 infused rats presented a decrease of 10 mmHg in the MBP comparatively to the MBP at the beginning of the experiment, which does not occur in the control group. The decrease in blood pressure was accompanied by an increased urinary flow, urinary sodium excretion and fractional sodium excretion. These findings confirm the acute diuretic and natriuretic effects of TRV120023.

Table 10 – TRV120023 effects on blood pressure and renal function

Parameter	Control (n= 7)	50 µg/Kg TRV120023 (n= 10)	p value
Body weight, g	257 ± 6	241 ± 9	0.25
Initial MBP, mmHg	125 ± 4	129 ± 5	0.18
Final MBP, mmHg	129 ± 5	118 ± 3	0.12
Δ MBP	6 ± 1	-10.5 ± 5	0.04
Urinary flow, µl/min	33.4 ± 4	60.39 ± 9	0.02
GFR, ml/ min	5.3 ± 1	6.67 ± 0.8	0.60
Urinary sodium excretion, µeq/min/Kg	6.0 ± 0.7	7.4 ± 1.6	0.05
%FENa⁺	0.7 ± 0.1	1.1 ± 0.1	0.03

MBP: mean blood pressure; **Δ MBP**: mean of (initial MBP - final MBP); **GFR**: glomerular filtration rate; **FENa⁺** : fractional sodium excretion.

7.2 – Effects of TRV120023 on Na⁺ dependent pHi recovery in renal proximal tubule cells

Once confirmed the diuretic and natriuretic effects of TRV120023, we evaluate if these effects were due to the modulation of Na⁺ transport in proximal tubule cells. To address this question, Na⁺-dependent pHi recovery was measured in OKP cells in the presence or absence of the biased agonist TRV120023. Figs. 17A and 17B present typical curves of pHi recovery rates from control or TRV120023, respectively. First, we evaluated the Na⁺-dependent pHi recovery of OKP cells over a concentration range of TRV120023 to determine appropriate dose as well as to evaluate if TRV120023, like Ang II, exhibits a bimodal effect. As shown is Fig. 17C, TRV120023 significantly reduced the Na⁺-dependent pHi recovery at concentrations above 10⁻⁸ M, and was consistently inhibitory. The pHi recovery rate, in pH units/min, decreased from 0.234 ± 0.014 at baseline to 0.083 ± 0.014 and 0.094 ± 0.013 for 10⁻⁷ M and 10⁻⁵ M TRV120023, respectively. The time course of inhibition of Na⁺ dependent pHi recovery by 10⁻⁷ M TRV120023 was assessed at 2, 15 and 30 minutes of treatment. As observed in Fig. 17D, the activity decreased from a baseline of 0.216 ± 0.007 to 0.109 ± 0.013 at 2 minutes of treatment and further decreased to 0.068 ± 0.005 pH units/min at 30 minutes. These findings suggest that diuretic and natriuretic effects of TRV120023 are, at least in part, due to inhibition of Na⁺- transport in proximal tubule.

Based on these findings, all the following experiments were conducted with 10⁻⁷ M TRV120023 for 15 minutes.

Figure 17 – TRV120023 decreases Na⁺-dependent pHi recovery rates in proximal tubule OKP cells. Na⁺-dependent pHi recovery measurements in OKP cells treated with vehicle or TRV120023. Representative Na⁺-dependent pHi recovery curve from **A**) vehicle (control) and **B**) 10⁻⁷ M TRV120023. **C**) Na⁺-dependent pHi recovery of OKP cells treated with 10⁻¹¹ M, 10⁻⁹ M, 10⁻⁸ M, 10⁻⁷ M, 10⁻⁶ M and 10⁻⁵ M of TRV120023 for 15 minutes (n = 7). **D**) OKP cells treated with 10⁻⁷ M TRV120023 for 2, 15 and 30 minutes (n = 7). Data expressed as mean ± SEM (*p < 0.05 or **p < 0.01 or ***p < 0.001 vs. Ctrl).

7.3 – Essential requirement for beta-arrestins in TRV120023-mediated inhibition of Na⁺ dependent pHi recovery in OKP cells

To ascertain that beta-arrestins were essential for mediating the inhibitory effects of TRV120023 on proximal tubule beta-arrestin 1/2 were knocked down by small interference RNA (siRNA) in OKP cells. As shown in Fig. 18A, transfection of OKP cells with siRNA for beta-arrestins 1/2 (siRNA b-arr) efficiently reduced beta-arrestins expression by approximately 60%. Silencing of beta-arrestin 1/2 per se did not affect the Na⁺-dependent pHi recovery rate (0.211 ± 0.019 vs. 0.193 ± 0.025 pH units/min in OKP cells transfected with siRNA scramble (siRNAsc Ctrl)) (Fig. 18B). On the other hand, the inhibitory effect of TRV120023 on Na⁺-dependent pHi recovery rate (0.083 ± 0.017 pH units/min in siRNAsc TRV123023) was completely abolished by siRNA b-arr. In concert, these results indicate that beta-arrestins 1 and/or 2 are required for proximal tubule Na⁺ dependent pHi recovery inhibition by TRV120023.

Figure 18 – Beta-arrestins are required for proximal tubule Na^+ dependent pHi recovery inhibition by TRV123023. Efficacy of beta-arrestins knocked down by siRNA in OKP cells. Confluent OKP cells transfected with either siRNA scramble (siRNA scr) or siRNA for beta-arrestins 1/2 (siRNA b-arr) were treated with vehicle or 10^{-7} M TRV120023 for 15 minutes. **A)** Cell lysates were subjected to SDS-PAGE, transferred to a PVDF membrane and incubated with a primary antibody against beta-arrestin 1/2 (1:1000) and subsequently with an antibody against GAPDH (1:1000). **B)** The relative abundance of beta-arrestin 1/2 was quantified by densitometry and normalized to GAPDH. Data expressed as mean $\pm$ SEM (***) $P < 0.001$ vs. siRNA scr, n indicated in the bars). **C)** Na^+ -dependent pHi recovery measurements in OKP cells transfected with siRNA scr or siRNA b-arr were treated with vehicle or 10^{-7} M TRV120023. Data expressed as mean $\pm$ SEM (### $p < 0.001$ vs siRNA scr(Ctrl) and ** $p < 0.01$ or * $p < 0.05$ vs siRNA scr(TRV)).

7.4 – Beta-arrestin-biased AT1 receptor signaling inhibits NHE3 activity in native renal proximal tubule

Since Na^+ -dependent pHi recovery is an indirect measure of NHE3 activity, we evaluated if these measurements were affected by 15 minutes pretreatment with the NHE3 specific inhibitor S3226 at 10^{-6} M (177, 178). As seen in Fig. 19A, the S3226 insensitive component of Na^+ -dependent pHi recovery was not affected by TRV120023 in OKP cells leading to the conclusion that it is the S3226 sensitive component that is inhibited by TRV 120023.

To determine whether beta-arrestin-biased AT1 receptor signaling inhibits NHE3 activity *in vivo*, stationary microperfusion was performed in native rat renal proximal tubule. As shown in Fig. 19B, TRV120023 decreased net bicarbonate reabsorption ($J_{\text{HCO}_3^-}$) in 27%, from 2.001 ± 0.082 to 1.530 ± 0.108 $\text{nmol}/\text{cm}^2/\text{s}$. In agreement with the *in vitro* studies the S3226 insensitive component of NHE3 activity was also not affected by TRV120023 *in vivo* native rat proximal tubule (Fig. 19B). Taken together these results support the conclusion that TRV120023 inhibits NHE3 activity in renal proximal tubule.

Figure 19 – Beta-arrestin-biased AT1 receptor signaling inhibits NHE3 activity in native renal proximal tubule. **A)** Na^+ -dependent pHi recovery OKP cells treated with 10^{-7} M TRV120023 in the presence or absence of 10^{-6} M S3226. **B)** Rates of bicarbonate reabsorption ($J_{\text{HCO}_3^-}$, $\text{nmol}/\text{cm}^2/\text{s}$) in native rat proximal tubule perfused with 10^{-7} M TRV120023 in the presence or absence of 10^{-6} M S3226. Data are expressed as mean $\pm$ SEM (***) $p < 0.001$ vs. Ctrl; n indicated on the bars).

7.5 – TRV120023 modulation of NHE3 activity is mediated by AT1 receptor activation

Previous studies have shown that TRV120023 effects are mediated via the AT1 receptor (170, 179). To confirm that the TRV120023 inhibitory effect on proximal tubule NHE3 activity is due to AT1 receptor activation, Na⁺-dependent pH_i recovery was assessed in OKP cells pretreated with the AT1 receptor antagonist losartan (10⁻⁶ M) or the AT2 receptor antagonist PD123319 (10⁻⁶ M), in the presence or absence of TRV120023. As summarized in Fig. 20, the AT2 receptor antagonist had no effect on the inhibition of NHE3 activity by TRV120023 while the AT1 receptor antagonist prevented TRV120023 mediated inhibition of OKP cell NHE3 activity. These results show that TRV120023 mediated inhibition of NHE3 is dependent on AT1 receptor activation and independent of the AT2 receptor.

Figure 20 –NHE3 inhibition by beta-arrestin-biased AT1 receptor signaling is mediated by angiotensin II type 1 receptor. A) Na⁺-dependent pH_i recovery measurements in confluent OKP cells pretreated for 15 minutes with vehicle (control), 10⁻⁶ M PD123319 or 10⁻⁶ M losartan in the presence or absence of 10⁻⁷ M TRV120023. Data are expressed as means ± SEM (***p< 0.001 vs. Ctrl; n indicated on the bars).

7.6 – Beta-arrestin-biased AT1 receptor signaling blunts the stimulatory effect of Ang II on NHE3 activity in renal proximal tubule

Since we confirmed that the TRV120023 effects were due to AT1 receptor activation in the proximal tubule cells, we tested the hypothesis that TRV120023 blocks Ang II stimulation of NHE3 activity. To address this aim, Na⁺-dependent pHi recovery was measured in OKP cells pretreated with 10⁻¹⁰ M Ang II for 15 minutes followed the addition of TRV120023 for another 15 minutes. As seen in Fig. 21A, TRV120023 completely reverses the stimulatory effect of Ang II on NHE3 activity (0.260 ± 0.013 pH units/min) to control (0.196 ± 0.016 pH units/min). Likewise, in native rat renal proximal tubule, microperfusion of TRV120023 reverses the stimulatory effect of Ang II (Ang II + TRV) on NHE3 activity (3.001 ± 0.212 vs. 2.057 ± 0.088 nmol/cm²/s; Fig 21B). Interestingly, and unlike TRV120023, Ang II is unable to reverse TRV120023 inhibitory effect on NHE3 activity, i.e., when we first inhibit NHE3 with TRV120023 for 15 min followed by Ang II for another 15 min (TRV + Ang II). Together, these findings demonstrate that beta-arrestin-biased AT1 receptor signaling triggered by TRV120023 blunts the stimulatory effect of Ang II on proximal tubule NHE3 activity.

Figure 21 – Beta-arrestin-biased AT1 receptor signaling blocks the stimulatory effect of Ang II on NHE3 activity in proximal tubule. Na⁺-dependent pHi recovery in OKP cells **A)** OKP cells pretreated for 15 minutes with vehicle (control) or 10⁻¹⁰ M Ang II in the presence or absence of TRV120023. **B)** Native renal proximal tubule bicarbonate reabsorption (J_{HCO₃⁻}, nmol/cm²/s) rates of 10⁻¹⁰ M Ang II in the presence or absence of TRV120023 (*p < 0.05; **p < 0.001 and ***p < 0.001 vs. Ctrl; && p < 0.001 vs Ang II+TRV; n indicated on the bars).

7.7 – Comparison between the effects of TRV120023, angiotensin II receptor blockers and angiotensin I converting enzyme (ACE) inhibitor on NHE3 activity.

Previous studies suggested that TRV120023/TRV120027 could provide additional beneficial effects when compared to the gold-standard therapeutic agents the angiotensin receptor blockers (ARBs) (169, 171, 179). Thus, we investigated if the AT1 receptor antagonist losartan or the ACE inhibitor captopril were able to exert any local tonic effect on NHE3 activity like TRV120023. To address this question Na^+ -dependent pH_i recovery in OKP cells and stationary microperfusion in native proximal tubule in the presence or absence of losartan or captopril were performed. As summarized in Fig. 22, Na^+ -dependent pH_i recovery rates were unaffected by the presence of either losartan or captopril. However, a reduction in the net bicarbonate reabsorption was observed with losartan treatment (2.00 ± 0.08 to 1.74 ± 0.06 $\text{J}_{\text{HCO}_3^-}$ nmol/cm²/s), representing a decrease of 13%. These results show that TRV120023 exerts a more profound inhibitory effect on NHE3 activity when compared to angiotensin II receptor blocker and ACE inhibitor.

Figure 22 – Comparison between the effects of TRV120023 and angiotensin II receptor blocker and ACE inhibitor on NHE3 activity in renal proximal tubule. A) Na^+ -dependent pH_i recovery analysis in OKP cells exposed to 10^{-7} M TRV120023, 10^{-6} M losartan or 10^{-6} M captopril for 15 minutes. B) Native renal proximal tubule bicarbonate reabsorption ($\text{J}_{\text{HCO}_3^-}$ nmol/cm²/s) rates in the presence of 10^{-7} M TRV120023 or 10^{-6} M losartan or 10^{-6} M captopril. Data are expressed as means $\pm$ SEM. Data are expressed as means $\pm$ SEM (* P <0.05; ** P <0.01 and *** p <0.001 vs. Ctrl; # p <0.05 vs. TRV120023; n expressed in the bars).

7.8 – TRV120023 effects on subcellular distribution of proximal tubule NHE3

The distribution of NHE3 along the microvillar domains *in vivo* or decrease and increase in NHE3 expression on the surface of the membrane *in vitro* are usually associated with the activity of the exchanger (174, 180). To address if the inhibition of NHE3 activity was due to a diminished expression of the exchanger in cell surface membranes, cell surface protein biotinylation was performed in OKP cells. As seen in Fig. 23A, TRV120023 treatment reduced the expression of the NHE3 protein at the surface membrane by about 40% compared to vehicle treated cells (Fig. 23A). As expected, 15 minute-exposure of OKP cells to TRV120023 did not alter the total cellular amount of NHE3 (Fig. 23B). These results suggest that TRV120023 mediates NHE3 inhibition in OKP cells via modulation of NHE3 subcellular localization.

Figure 23 –Beta-arrestin-biased AT1 receptor signaling decreases surface membrane expression of NHE3 in OKP cells. A) Confluent OKP cells treated with or without TRV120023 for 15 minutes were subjected to protein surface biotinylation followed by SDS-PAGE and immunoblotting for NHE3 (clone 3H3; 1:500) and actin (1:50000). **B)** NHE3 cell surface expression quantification by densitometry of 7 different experiments. Data are expressed as mean $\pm$ SEM (** p <0.001 vs. Ctrl).

To investigate if acute TRV120023 treatment *in vivo* provoked redistribution of renal proximal tubule NHE3 from the body of the microvilli where it is active, to the base of the microvilli where activity is inhibited (10), *in situ* immunofluorescence was performed after an acute infusion (20 minutes) of TRV120023. The microvillar domain was labelled with a monoclonal antibody to the microvillar actin bundling protein villin (red) and NHE3 with the polyclonal anti-NHE3 antibody (in green). As seen in Fig. 24, acute TRV120023 infusion leads to a clear retraction of proximal tubule NHE3 from the body to base of the microvillar domain. These findings indicate that TRV120023 mediated inhibition of NHE3 activity may be the result of the retraction of the exchanger to the base of the microvillar domain in proximal tubule, a mechanism associated with decreased NHE3 activity (105, 110, 155, 181, 182).

Figure 24 –Effect of beta-arrestin-biased AT1 receptor signaling on microvillar domain localization of NHE3 in native proximal tubule. Indirect immunofluorescence microscopy of the NHE3 redistribution in rats infused for 20 minutes with vehicle (left) or 50 µg/Kg TRV120023 (right). Different sets of experiments were conducted using anti-NHE3 (clone NHE3-C00; 1:100) detected with the secondary antibody AlexaFluor 568 (green) and anti-villin (1:100) detected with the secondary antibody AlexaFluor 488 (red). The bar in the pictures represents 20 µm.

7.9 – TRV120023 induces NHE3 internalization via clathrin-mediated endocytosis in OKP cells.

A suggested mechanism of NHE3 trafficking in proximal tubule epithelial cells is via clathrin-mediated endocytosis (124). So here we tested the hypothesis that TRV120023 decrease NHE3 surface expression by increasing clathrin-mediated NHE3 endocytosis. To this end pH_i recovery in OKP cells were performed pretreated cells with 25 μ M of the specific clathrin inhibitor (PitStop2) for 10 minutes plus the presence or absence of 10^{-7} M TRV120023 for 15 minutes. As presented in Fig 25 the PitStop2 completely blocks the inhibitory effect of TRV120023 over NHE3 activity, and the PitStop2 per se had no effect on basal pH_i recovery. These findings suggest that TRV120023 inhibitory effect is mediated, at least in part, by increasing NHE3 internalization via clathrin-mediated endocytosis in OKP cells.

Figure 25 –Beta-arrestin-biased AT1 receptor signaling stimulates NHE3 internalization via clathrin-mediated endocytosis in OKP cells. Confluent OKP cells treated with 25 μ M PitStop 2 for 10 minutes in the presence or absence of 10^{-7} M TRV120023 for another 15 minutes were evaluated by pH_i recovery. Data are expressed as mean $\pm$ SEM (*** p <0.001 vs. Ctrl).

7.10 –TRV120023 effects does not involve PKA activation and NHE3 phosphorylation at serine 552.

Increase in intracellular cAMP levels leads to NHE3 inhibition, an effect that is partly attributed to direct phosphorylation of the exchanger at the PKA consensus sites serine 552 and 605 (110, 183). Furthermore, NHE3 phosphorylated at the PKA consensus site serine 552 is localized in the localized at the base of the brush-border membrane, where it is inactive(110). So here we tested the hypothesis that TRV120023 inhibits NHE3 activity by increasing PKA activity and, consequently, NHE3 phosphorylation at the PKA consensus site serine 552. To this end, OKP cells were treated with vehicle or 10^{-7} M TRV120023 or 10^{-4} M forskolin (Forsk; positive control) for 2, 15 and 30 minutes and the PKA activity measure on OKP cells lysates by ELISA. As seen in Fig. 26 TRV120023 was incapable of increasing PKA activity, contrarily to the observed to Forsk, an adenylyl cyclase activator.

Figure 26 – NHE3 inhibition by beta-arrestin-biased AT1 receptor signaling does not involve PKA activation in OKP cells. Confluent OKP cells treated with 10^{-7} M TRV120023 or 10^{-4} M Forskolin (Forsk) or vehicle for 15 minutes were lysate and subjected to an ELISA assay Data are expressed as mean $\pm$ SEM (***) $p < 0.001$ vs. Ctrl).

Corroborating with the previous findings, the levels of NHE3 phosphorylation at the PKA consensus site, serine 552, well-known to be phosphorylated by PKA (110), were also unchanged in OKP cells exposed for 15 minutes to TRV120023 (Fig.27). As expected, Forsk (our positive control) increased NHE3 phosphorylation at serine 552 in approximately 50%. Further supporting our previous results, Forsk increases intracellular cAMP which is unchangeable by TRV120023 (Fig. 28). All together, these finding demonstrate that the biased AT1 receptor/ beta-arrestin signal does not activate the cAMP/ PKA signal in proximal tubule cells.

Figure 27 – NHE3 inhibition by beta-arrestin-biased AT1 receptor signaling does not involve PKA-mediated phosphorylation at serine 552 in OKP cells. Confluent OKP cells treated with 10^{-7} M TRV120023 or 10^{-4} M Forskolin (Forsk) or vehicle for 15 minutes were lysate and subjected to immunoblotting for **A**) phosphorylated serine NHE3 (clone 14D5) and **B**) total NHE3 (clone 3H3; 1:500) and actin (1:50000). Total expression was quantified densitometry of 3 independent experiments. Data are expressed as mean $\pm$ SEM (** $p < 0.001$ vs. Ctrl).

Figure 28 – TRV120023 effects on cAMP levels in OKP cells. Confluent OKP cells treated with 10^{-7} M TRV120023 or 10^{-4} M Forskolin or vehicle for 15 minutes were lysate and subjected to an ELISA assay. Data are expressed as mean $\pm$ SEM (***p <0.001 vs. Ctrl).

7.12 –TRV120023 effects on NHE3 activity does not involve ERK1/2 or Akt activation.

Biased agonism at the AT1 receptor is usually associated with the activation of the protein kinases B (Akt) and the extracellular signal-regulated kinases 1 and 2 (ERK1/2). (162, 184). So next we evaluate if NHE3 inhibition by TRV120023 could be due to the activation of these kinases. To address this question, Na^+ - dependent pH_i recovery in OKP cells pre-treated with 10^{-7} M TRV120023 for 15 minutes in the presence or absence of pretreatment of 10^{-8} M Akt inhibitor (Akti) or 10^{-6} M U0126 (ERK1/2 inhibitor) for 10 minutes. As presented in Fig. 29 the Akt inhibitor per se decreases the Na^+ - dependent pH_i recovery and no additive effect was observed in the presence of TRV120023. This result indicates that Akt is not involved in the NHE3 inhibition by TRV120023. In fact, this finding suggests that Akt activity is involved in basal NHE3 activity modulation.

Figure 29 –NHE3 inhibition by beta-arrestin-biased AT1 receptor signaling does not involve Akt activation in OKP cells. Confluent OKP cells treated with 10^{-7} M TRV120023 or 10^{-8} M Akt inhibitor (Akti) or vehicle for 15 minutes and were subjected to pHi recovery. Data are expressed as mean $\pm$ SEM (* P <0.05 or *** P <0.001 vs. Ctrl).

As seen in Fig. 30, the ERK1/2 inhibitor per se was unable to induce any effect on Na^+ - dependent pHi recovery as well as it did not affect the inhibitory effect of TRV120023 on NHE3 activity. This data indicates that ERK1/2 activation is not involved in the inhibitory effect of TRV120023 on NHE3.

Figure 30 –NHE3 inhibition by beta-arrestin-biased AT1 receptor signaling does not involve ERK1/2 activation in OKP cells. Confluent OKP cells treated with 10^{-7} M TRV120023 or 10^{-6} M ERK1/2 inhibitor (U0126) or vehicle for 15 minutes and were subjected to pHi recovery. Data are expressed as mean $\pm$ SEM (*** P <0.001 vs. Ctrl).

7.13 – NHE3 and beta-arrestin does not interact after acute infusion of TRV120023.

Szabó and collaborators (185) demonstrated that beta-arrestin can interact with the isoform 5 of NHE family (NHE5) leading to a decrease of the cell surface expression of the exchanger. So here, we tested the hypothesis that the activation of the AT1 receptor/beta-arrestin signaling leads to the interaction of beta-arrestin with NHE3 and consequent retraction of the exchanger to the base of the microvillus. To address this hypothesis, *in situ* immunofluorescence was performed after an acute infusion (20 minutes) of TRV120023 or vehicle. The beta-arrestin was labelled with a monoclonal antibody anti-beta-arrestin (in green) and the NHE3 with the polyclonal anti-NHE3 antibody (NHE3-C00; in red). As seen in Fig. 31, is possible to observe a co-localization between NHE3 and beta-arrestin (indicated by the arrow) at basal condition (Ctrl). Contrarily to our hypothesis, after acute TRV120023 infusion the co-localization is no longer observed. Furthermore, the diffuse pattern observed in basal condition pass to a pattern of aggregates in the infused animal. This pattern of aggregates is characteristic of beta-arrestin translocation from the cytosol to the endocytic vesicles (186) and indicates that TRV120023, as expected, induce beta-arrestin recruitment. This result suggests that an interaction of beta-arrestin with NHE3 is not the mechanism responsible for NHE3 translocation from the top to the base of the microvillus in native proximal tubule.

Figure 31 – Effect of beta-arrestin-biased AT1 receptor signaling on beta-arrestin and NHE3 localization in native proximal tubule. Indirect immunofluorescence microscopy of the NHE3 and beta-arrestin distribution in rats infused for 20 minutes with vehicle (left) or TRV120023 (right). Different sets of experiments were conducted using anti-NHE3 (1:100) detected with the secondary antibody AlexaFluor 488 (red) and anti-beta-arrestin1/2 (1:100) detected with the secondary antibody AlexaFluor 568 (green), the circle demarks a single tubule and the arrow the co-localization).

Chapter 8 – Discussion

Besides the classical role of beta-arrestins in promoting G-protein coupled receptors internalization and desensitization, recent evidence has shown that beta-arrestins 1 and 2 can also activate specific signal pathways in a G-protein-independent manner leading to distinct cellular responses (35, 39, 43). In this study, we investigated the acute effect of beta-arrestin-biased AT1 receptor signaling on NHE3 activity in renal proximal tubule. To this end, we used the Ang II synthetic analog, TRV120023, which belongs to a new class of pharmacological agents (39). TRV120023 activation of beta-arrestin-biased AT1 receptor signaling decreases blood pressure, increases urine flow rate and sodium excretion and decreases fractional sodium reabsorption in the proximal tubule of healthy canines and those with heart failure (5, 6). Herein, we extend those findings to the cellular and molecular levels to demonstrate that TRV120023 inhibits NHE3 activity in a proximal tubule cell line as well as in the native rat proximal tubule. The results suggest that the diuretic, natriuretic and anti-hypertensive effects exerted by TRV120023 may be attributed to, at least in part, inhibition of proximal tubule NHE3.

Divergent functional actions of AT1 and AT2 receptors have been reported with respect to blood pressure and sodium transport: Ang II AT1 receptor activation increases blood pressure and sodium retention whereas AT2 activation lowers blood pressure and increases sodium excretion (8, 20). A reduction in bicarbonate reabsorption mediated by AT2-receptor activation has been reported in rabbit proximal tubule cultured cells (19), suggesting that activation of the Ang II initiated AT2 signaling cascade leads to NHE3 inhibition. Our data indicate that the inhibitory effect of TRV120023 on NHE3 activity occurs through AT1 receptor activation and does not involve the activation of AT2 receptors. These results are in line with previous studies that demonstrated that these AT1 receptor biased agonists display a remarkable specificity for the AT1 receptor (39). Moreover, our findings suggest that besides the opposing physiological effects found between the AT1 and AT2 receptors, the activation of G-protein versus beta-arrestin signaling of the AT1 receptor can also lead to opposite effects with respect to NHE3 modulation, thereby adding an additional level of complexity to the regulation of NHE3-mediated proximal tubule NaCl and NaHCO₃ reabsorption. Interestingly, our results suggest that beta-arrestin-biased AT1 receptor signaling by TRV120023 exerts only inhibitory effects on proximal tubule NHE3

activity, which contrasts with the bimodal effect observed by the full agonist Ang II (Supplementary Fig. 3).

In OKP cells, the inhibitory effect of TRV120023 on the proximal tubule NHE3 activity was accompanied by a 40% decrease in the surface expression of NHE3, which is in the same order of magnitude as the decrease in the NHE3 activity (~45%). In addition, indirect immunofluorescence in native proximal tubule showed a clear and rapid retraction of the NHE3 from the top to the base of the microvillar domain. These findings indicate that subcellular redistribution of NHE3 plays a key role in the observed inhibition of the NHE3 activity by the beta-arrestin-biased AT1 receptor signaling. In fact, the association between redistribution of NHE3 between the brush border membranes and changes on NHE3 function has been repeatedly reported in the literature (10, 17, 23, 32). In this regard, a recent mathematical model for NHE3 mediated Na^+ reabsorption predicted that NHE3 redistribution to the base of the microvillar domain creates cytosolic alkaline pH microdomains (7). The predicted effect was supported *in vivo* by demonstrating the formation of alkaline pH microdomains and that NHE3 activity was reduced by approximately 32%. These findings corroborate a previous model that suggested that NHE3 is pH sensitive and predicted that NHE3 would sharply turn off in conditions of cellular alkalosis (42).

The trafficking pathway is thought to be an important and efficient mean of rapidly shuttling functional transporters to and from the cell surface. In the present study we demonstrated, using a specific clathrin inhibitor, that it completely blocks the NHE3 inhibition by TRV120023. These findings suggest that NHE3 internalization via clathrin-mediated endocytosis plays a crucial role in NHE3 regulation by the biased agonism of the AT1 receptor in OKP cells. Accordingly, a central role of clathrin-mediated endocytosis in the internalization and recycling of NHE3 have been suggested in Chinese hamster ovary cells. Chow and collaborators (124) showed that a dominant-negative form of dynamin, DynS45N, effectively prevented the endocytosis of NHE3. Thereby confirming their association in native tissues, endogenous NHE3 of native ileal villus cells was also found to co-purify with isolated clathrin-coated vesicles. Notwithstanding, this result does not exclude the possibility of other endocytic pathways to be involved. Recent results from colleagues of our laboratory have shown that activation of beta-arrestin signal by shear stress can lead to the interaction between beta-

arrestin and caveolin. So for more conclusive results others endocytic pathways should be evaluated in the future.

The involvement of clathrin mediated endocytosis *in vivo* was not evaluated in the present study, and it is well-known that are outstanding differences in the phenotypes of renal cell lines which contrasted with native proximal tubule. The brush border of the PT is very dense and consists of two distinct microdomains, the microvillus and the intermicrovillar domain. However, cultured PT cells have sparse microvillus and the intermicrovillar microdomain of the PT is lacking in cell lines. Although there is evidence for substantial intracellular pools of NHE3 in cultured cells, evidence for a significant pool of intracellular NHE3 *in vivo* is all but lacking. Contradictory findings have been reported in literature, some demonstrated that NHE3 is present in clathrin enrichment vesicles from rabbit ileum and rat PT after acute hypertension (124, 187, 188), implying that clathrin can also be involved in NHE3 internalization in native epithelia. However, other refutes this results by showing that NHE3 is actually redistributed between top and the base of microvillus above the clathrin adaptor protein 2, that is, redistribution within the apical membrane without endocytosis (127). The discrepancies observed between studies can be due to the several differences among techniques, animal models, and kidney fixation protocols. Since there are no consensuses among studies, it would be interesting to know if clathrin-mediated endocytosis inhibition, with the specific clathrin inhibitor PitSto2, also blocks the TRV120023 effects *in vivo* by means of stationary microperfusion.

Direct phosphorylation of NHE3 is a well-established physiological phenomenon, and several reports have documented the importance of intracellular cAMP/PKA signal in the regulation of NHE3. For instance, PTH, dopamine and glucagon-like peptide 1 inhibit NHE3 activity via PKA dependent pathways (125, 177, 178, 189-191). These hormones have also been demonstrated to increase total NHE3 phosphorylation (126, 178, 191). Additionally, PKA activation was associated in NHE3 internalization and inhibition (125, 126). In OKP cells which expresses endogenous NHE3, direct PKA activation increases NHE3 phosphorylation at serines 552 and 605 compared with baseline (110), and it has been suggested to be involved in NHE3 trafficking (114). However, our results demonstrated that PKA activation, and consequently, PKA-mediated phosphorylation at serine 552 of NHE3 is not involved in proximal tubule NHE3 inhibition by the biased agonism of AT1 receptor TRV120023.

Furthermore, TRV120023 was unable to induce any changes in intracellular cAMP, further supporting the idea that intracellular cAMP increase and PKA-mediated phosphorylation are not involved in the regulation of NHE3 by the biased agonism of the AT1 receptor in OKP cells.

The specialized adaptor proteins beta-arrestin 1 and 2 interact almost exclusively with specific phospho-serine/threonine residues of the GPCRs, but they also promote internalization by interacting spatially and temporally with components of the endocytic trafficking machinery, including clathrin (51, 63, 192). Surprisingly, it has been shown that beta-arrestin 1 and 2 can interact with NHE5 and its overexpression decreased cell surface NHE5 expression (185), indicating that beta-arrestins can also regulate the trafficking of integral proteins apart from receptor–ligand complexes. Interestingly, our results showed that at baseline condition, NHE3 and beta-arrestin co-localize at some extent in the microvillus. Nevertheless, after triggering the biased agonism signal at the AT1 receptor this co-localization seems to be lost. Despite the absence of co-localization in TRV120023 treated rats between NHE3 and beta-arrestin, it is possible to observe that diffuse pattern observed in basal condition pass to a pattern of aggregates in the infused animal. This pattern of aggregates is characteristic of beta-arrestin translocation from the cytosol to the endocytic vesicles (186) and confirms that TRV120023, as expected, induce beta-arrestin recruitment. Translocation of the beta-arrestin to the endocytic vesicles may difficult the observation of co-localization in the infused animal which is a weakness of the technique used. Further studies should be performed for a more conclusive result.

Pharmacological inhibition of the renin-angiotensin system (RAS) is widely used in the treatment of patients with chronic renal failure and cardiovascular disorders, including hypertension and heart failure. Clinical studies are now underway to assess the efficacy and safety of the biased agonist of the AT1 receptor TRV120027 to treat acute heart failure (14). It remains to be established whether biased agonism of the AT1 receptor may indeed provide additional beneficial effects when compared to angiotensin receptor blockers (ARBs). Similar to ARBs, TRV120023/TRV120027 block the pressor effect of the AT1 receptor but unlike ARBs, TRV120023/TRV120027 are capable of unloading the heart while preserving renal function (33, 39). These benefits were associated with the selectivity and potency to evoke beta-arrestin recruitment, which were absent in the ARBs treatment (33, 39). In the present study, we show that the

biased agonist TRV120023 exerts a tonic inhibitory effect on NHE3 activity both *in vitro* and *in vivo*. Conversely, neither the ARBs, losartan as well as the ACE inhibitor, captopril, inhibit NHE3 activity in OKP cells whereas by *in situ* stationary microperfusion experiments, losartan caused a 13% reduction of NHE3-mediated bicarbonate reabsorption. Previous studies reported diuretic and natriuretic effects of candesartan, losartan and captopril by acute systemic infusion, which lead to NHE3 retraction from the top to the bottom of microvilli (193-195). However, an increase in sodium reabsorption was reported in a chronic treatment with enalapril (196). In addition, ACE knockout mice proximal tubular fluid reabsorption was comparable to the wild-type mice despite the almost complete absence of tissue ACE (197). The inconsistency between our study and the above mentioned effects can be due systemic versus local and/or acute versus chronic. Moreover, there are multiple technical differences between our study and earlier studies which could explain this apparent disparity in findings. The main conclusion from our experiments (under our conditions) is that proximal tubular perfusion/incubation with the biased AT1 agonist TRV120023 seems to be much more effective in inhibiting NHE3 transport activity than the others RAS inhibitors.

The beneficial effects of the biased agonism of the AT1 receptor has been associated with the activation of the kinases Akt and ERK1/2 (162, 167). In the present study the activation of both of these kinases do not seem to be involved in NHE3 modulation. In fact, Akt inhibitor per se decreases the Na⁺- dependent pHi recovery and no additive effect was observed in the presence of TRV120023. This result indicates that NHE3 inhibition by TRV120023 is not associated with Akt activation. In fact, this finding suggests that Akt activity is involved in basal NHE3 activity modulation. Accordantly, it has been reported that Akt activation is required for NHE3 activation by directly phosphorylate NHE3 C-terminal where ezrin directly binds (198). On the other hand, ERK1/2 had no effect on either basal or TRV120023 inhibit NHE3. This result was also not surprising since TRV120023 inhibits NHE3 as early as 2 minutes, and beta-arrestin dependent ERK1/2 activation has been reported to be later with a peak between 5 and 10 minutes which is quiet persistent until 90 minutes (199).

Activation of Akt and ERK1/2 are characteristic of the beta-arrestin dependent signal, and the fact that they do not modulate NHE3 does not exclude the possibility that they can be activated in proximal tubule by AT1 biased signal. In fact, it has been

reported that AT1 receptor biased signal activates both ERK1/2 and Akt in arrestin-based signalosomes, making beta-arrestin dependent signaling spatially discrete. Moreover, it has already been shown that signalosome-associated ERK1/2, unlike ERK1/2 activated by G-protein-mediated pathways, does not translocate to the cell nucleus and fails to elicit a transcriptional response or stimulate cell proliferation (160, 200). In other words, it is possible that Akt, which, as referred above, modulates NHE3, can be active but not modulating NHE3 due to differential cell signal compartmentalization.

Gurley and collaborators (18) emphasized the importance of proximal tubule sodium transport in blood pressure control by demonstrating that selectively deleting proximal tubule AT1 receptors decreases blood pressure about 10 mmHg. Those mice demonstrated improved pressure-natriuresis against Ang II-dependent hypertension associated with a significant downregulation of NHE3. The pressure natriuresis mechanism is the central feedback system for control of blood pressure, whereby increases in renal perfusion pressure lead to a decrease in renal sodium reabsorption. Interestingly, the redistribution of NHE3 between the microvillar microdomains of the apical membrane of the proximal tubule plays a crucial role in the pressure natriuresis response (10, 32). Several studies have attempted to identify the intrarenal mechanisms that could explain the interplay among hypertension, NHE3 redistribution and pressure natriuresis (25, 26). These studies suggest that high renal perfusion pressure induce the production of nitric oxide (NO) and metabolites by the endothelial cells and that diffusion of NO to the proximal tubule cells may induce a redistribution of NHE3 to the base of the microvilli, inhibiting NHE3-mediated proximal tubule sodium reabsorption and consequently increasing natriuresis. In favor of this hypothesis, systemic inhibitors of the nitric oxide synthase decrease the natriuretic effect induced by the acute increase in the blood pressure (13, 31, 36). Recent findings have shown the beta-arrestin-biased AT1 receptor signaling may be involved in the mechanotransduction of shear stress to intracellular signals and NO production by endothelial cells (35). It is therefore tempting to speculate that activation of the beta-arrestin-biased AT1 receptor signaling may play a role in pressure natriuresis by regulating NHE3 subcellular distribution in the proximal tubule.

In summary, our data provide the first evidence that activation of the AT1 receptor/beta-arrestin signaling leads to proximal tubule NHE3 inhibition associated

with subcellular redistribution of the exchanger. The modulation of NHE3 by RAS is mediated by a myriad of molecular mechanisms and numerous signaling pathways. Our results bring another player to the complexity of NHE3 regulation in renal proximal tubule and raise the question of whether biased signaling through beta-arrestin-biased AT1 receptor signaling is physiologically active in the renal proximal tubule.

Chapter 9 – Conclusion

The results from our project showed that AT1 receptor/ beta-arrestin biased signal inhibits proximal tubule NHE3 due to changes in subcellular localization both *in vitro* and *in vivo*, and it was associated with clathrin-mediated endocytosis *in vitro*. Our data also indicates that cAMP/PKA signaling, a common signal in NHE3 modulation, is not involved in NHE3 inhibition by AT1 receptor/ beta-arrestin biased signal in proximal tubule cells. The classical kinases, ERK1/2 and Akt, known to be activated by the biased AT1 receptor/ beta-arrestin signal, were also not involved in NHE3 modulation in proximal tubule.

In summary, the decrease in blood pressure caused by AT1 receptor/beta-arrestin signaling is, at least in part, due to an increase in natriuresis and diuresis as a result of NHE3 inhibition. A compromised renin-angiotensin system is characteristic of some prevalent diseases. Thus, the understanding of the AT1 receptor/ beta-arrestin signaling can be useful tool to discovery new therapeutic targets for diseases like heart failure, hypertension and some renal disorders.

References

1. Zachos NC, Tse M, Donowitz M. Molecular physiology of intestinal Na⁺/H⁺ exchange. *Annu Rev Physiol.* 2005;67:411-43.
2. Orłowski J, Grinstein S. Diversity of the mammalian sodium/proton exchanger SLC9 gene family. *Pflugers Arch.* 2004;447(5):549-65.
3. Itoh Y, Cai K, Khorana HG. Mapping of contact sites in complex formation between light-activated rhodopsin and transducin by covalent crosslinking: use of a chemically preactivated reagent. *Proc Natl Acad Sci U S A.* 2001;98(9):4883-7.
4. Frelin C, Vigne P, Ladoux A, Lazdunski M. The regulation of the intracellular pH in cells from vertebrates. *Eur J Biochem.* 1988;174(1):3-14.
5. Watari K, Nakaya M, Kurose H. Multiple functions of G protein-coupled receptor kinases. *J Mol Signal.* 2014;9(1):1.
6. Ferrão FM, Lara LS, Lowe J. Renin-angiotensin system in the kidney: What is new? *World J Nephrol.* 2014;3(3):64-76.
7. Mehta PK, Griendling KK. Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system. *Am J Physiol Cell Physiol.* 2007;292(1):C82-97.
8. de Munnik SM, Smit MJ, Leurs R, Vischer HF. Modulation of cellular signaling by herpesvirus-encoded G protein-coupled receptors. *Front Pharmacol.* 2015;6:40.
9. Vilardaga JP, Agnati LF, Fuxe K, Ciruela F. G-protein-coupled receptor heteromer dynamics. *J Cell Sci.* 2010;123(Pt 24):4215-20.
10. Wisler JW, Xiao K, Thomsen AR, Lefkowitz RJ. Recent developments in biased agonism. *Curr Opin Cell Biol.* 2014;27:18-24.
11. Gurevich EV, Tesmer JJ, Mushegian A, Gurevich VV. G protein-coupled receptor kinases: more than just kinases and not only for GPCRs. *Pharmacol Ther.* 2012;133(1):40-69.
12. Balakumar P, Jagadeesh G. Structural determinants for binding, activation, and functional selectivity of the angiotensin AT1 receptor. *J Mol Endocrinol.* 2014;53(2):R71-92.
13. Flower DR. Modelling G-protein-coupled receptors for drug design. *Biochim Biophys Acta.* 1999;1422(3):207-34.
14. Fredriksson R, Lagerström MC, Lundin LG, Schiöth HB. The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. *Mol Pharmacol.* 2003;63(6):1256-72.
15. Kochman K. Superfamily of G-protein coupled receptors (GPCRs)--extraordinary and outstanding success of evolution. *Postepy Hig Med Dosw (Online).* 2014;68:1225-37.
16. Hofmann KP, Scheerer P, Hildebrand PW, Choe HW, Park JH, Heck M, et al. A G protein-coupled receptor at work: the rhodopsin model. *Trends Biochem Sci.* 2009;34(11):540-52.

17. Cabrera-Vera TM, Vanhauwe J, Thomas TO, Medkova M, Preininger A, Mazzoni MR, et al. Insights into G protein structure, function, and regulation. *Endocr Rev.* 2003;24(6):765-81.
18. Hamm HE. The Many Faces of G Protein Signaling. *Journal of Biological Chemistry.* 1998;273(2):669-72.
19. Benovic JL, Mayor F, Somers RL, Caron MG, Lefkowitz RJ. Light-dependent phosphorylation of rhodopsin by beta-adrenergic receptor kinase. *Nature.* 1986;321(6073):869-72.
20. Pitcher JA, Freedman NJ, Lefkowitz RJ. G protein-coupled receptor kinases. *Annu Rev Biochem.* 1998;67:653-92.
21. Weiss ER, Raman D, Shirakawa S, Ducceschi MH, Bertram PT, Wong F, et al. The cloning of GRK7, a candidate cone opsin kinase, from cone- and rod-dominant mammalian retinas. *Mol Vis.* 1998;4:27.
22. Palczewski K. GTP-binding-protein-coupled receptor kinases--two mechanistic models. *Eur J Biochem.* 1997;248(2):261-9.
23. Penela P, Ribas C, Mayor F. Mechanisms of regulation of the expression and function of G protein-coupled receptor kinases. *Cell Signal.* 2003;15(11):973-81.
24. Willets JM, Challiss RA, Nahorski SR. Non-visual GRKs: are we seeing the whole picture? *Trends Pharmacol Sci.* 2003;24(12):626-33.
25. Benovic JL, DeBlasi A, Stone WC, Caron MG, Lefkowitz RJ. Beta-adrenergic receptor kinase: primary structure delineates a multigene family. *Science.* 1989;246(4927):235-40.
26. Benovic JL, Onorato JJ, Arriza JL, Stone WC, Lohse M, Jenkins NA, et al. Cloning, expression, and chromosomal localization of beta-adrenergic receptor kinase 2. A new member of the receptor kinase family. *J Biol Chem.* 1991;266(23):14939-46.
27. Kunapuli P, Benovic JL. Cloning and expression of GRK5: a member of the G protein-coupled receptor kinase family. *Proc Natl Acad Sci U S A.* 1993;90(12):5588-92.
28. Benovic JL, Gomez J. Molecular cloning and expression of GRK6. A new member of the G protein-coupled receptor kinase family. *J Biol Chem.* 1993;268(26):19521-7.
29. Carman CV, Parent JL, Day PW, Pronin AN, Sternweis PM, Wedegaertner PB, et al. Selective regulation of G α (q/11) by an RGS domain in the G protein-coupled receptor kinase, GRK2. *J Biol Chem.* 1999;274(48):34483-92.
30. Sallese M, Mariggiò S, D'Urbano E, Iacovelli L, De Blasi A. Selective regulation of Gq signaling by G protein-coupled receptor kinase 2: direct interaction of kinase N terminus with activated galphaq. *Mol Pharmacol.* 2000;57(4):826-31.
31. Dhami GK, Babwah AV, Sterne-Marr R, Ferguson SS. Phosphorylation-independent regulation of metabotropic glutamate receptor 1 signaling requires g protein-coupled receptor kinase 2 binding to the second intracellular loop. *J Biol Chem.* 2005;280(26):24420-7.

32. Premont RT, Macrae AD, Aparicio SA, Kendall HE, Welch JE, Lefkowitz RJ. The GRK4 subfamily of G protein-coupled receptor kinases. Alternative splicing, gene organization, and sequence conservation. *J Biol Chem.* 1999;274(41):29381-9.
33. Hall RA, Premont RT, Chow CW, Blitzer JT, Pitcher JA, Claing A, et al. The beta2-adrenergic receptor interacts with the Na⁺/H⁺-exchanger regulatory factor to control Na⁺/H⁺ exchange. *Nature.* 1998;392(6676):626-30.
34. Eichmann T, Lorenz K, Hoffmann M, Brockmann J, Krasel C, Lohse MJ, et al. The amino-terminal domain of G-protein-coupled receptor kinase 2 is a regulatory Gbeta gamma binding site. *J Biol Chem.* 2003;278(10):8052-7.
35. Inglese J, Koch WJ, Caron MG, Lefkowitz RJ. Isoprenylation in regulation of signal transduction by G-protein-coupled receptor kinases. *Nature.* 1992;359(6391):147-50.
36. Pitcher JA, Inglese J, Higgins JB, Arriza JL, Casey PJ, Kim C, et al. Role of beta gamma subunits of G proteins in targeting the beta-adrenergic receptor kinase to membrane-bound receptors. *Science.* 1992;257(5074):1264-7.
37. Daaka Y, Pitcher JA, Richardson M, Stoffel RH, Robishaw JD, Lefkowitz RJ. Receptor and G betagamma isoform-specific interactions with G protein-coupled receptor kinases. *Proc Natl Acad Sci U S A.* 1997;94(6):2180-5.
38. Pitcher JA, Touhara K, Payne ES, Lefkowitz RJ. Pleckstrin homology domain-mediated membrane association and activation of the beta-adrenergic receptor kinase requires coordinate interaction with G beta gamma subunits and lipid. *J Biol Chem.* 1995;270(20):11707-10.
39. Premont RT, Macrae AD, Stoffel RH, Chung N, Pitcher JA, Ambrose C, et al. Characterization of the G protein-coupled receptor kinase GRK4. Identification of four splice variants. *J Biol Chem.* 1996;271(11):6403-10.
40. Stoffel RH, Randall RR, Premont RT, Lefkowitz RJ, Inglese J. Palmitoylation of G protein-coupled receptor kinase, GRK6. Lipid modification diversity in the GRK family. *J Biol Chem.* 1994;269(45):27791-4.
41. Johnson LR, Robinson JD, Lester KN, Pitcher JA. Distinct structural features of G protein-coupled receptor kinase 5 (GRK5) regulate its nuclear localization and DNA-binding ability. *PLoS One.* 2013;8(5):e62508.
42. Johnson LR, Scott MG, Pitcher JA. G protein-coupled receptor kinase 5 contains a DNA-binding nuclear localization sequence. *Mol Cell Biol.* 2004;24(23):10169-79.
43. Jiang X, Benovic JL, Wedegaertner PB. Plasma membrane and nuclear localization of G protein coupled receptor kinase 6A. *Mol Biol Cell.* 2007;18(8):2960-9.
44. Kohout TA, Lefkowitz RJ. Regulation of G protein-coupled receptor kinases and arrestins during receptor desensitization. *Mol Pharmacol.* 2003;63(1):9-18.
45. Lefkowitz RJ, Hausdorff WP, Caron MG. Role of phosphorylation in desensitization of the beta-adrenoceptor. *Trends Pharmacol Sci.* 1990;11(5):190-4.
46. Benovic JL, Kühn H, Weyand I, Codina J, Caron MG, Lefkowitz RJ. Functional desensitization of the isolated beta-adrenergic receptor by the beta-adrenergic receptor kinase: potential role of an analog of the retinal protein arrestin (48-kDa protein). *Proc Natl Acad Sci U S A.* 1987;84(24):8879-82.

47. Bouvier M, Hausdorff WP, De Blasi A, O'Dowd BF, Kobilka BK, Caron MG, et al. Removal of phosphorylation sites from the beta 2-adrenergic receptor delays onset of agonist-promoted desensitization. *Nature*. 1988;333(6171):370-3.
48. Ferguson SS, Downey WE, Colapietro AM, Barak LS, Ménard L, Caron MG. Role of beta-arrestin in mediating agonist-promoted G protein-coupled receptor internalization. *Science*. 1996;271(5247):363-6.
49. Parruti G, Peracchia F, Sallese M, Ambrosini G, Masini M, Rotilio D, et al. Molecular analysis of human beta-arrestin-1: cloning, tissue distribution, and regulation of expression. Identification of two isoforms generated by alternative splicing. *J Biol Chem*. 1993;268(13):9753-61.
50. Attramadal H, Arriza JL, Aoki C, Dawson TM, Codina J, Kwatra MM, et al. Beta-arrestin2, a novel member of the arrestin/beta-arrestin gene family. *J Biol Chem*. 1992;267(25):17882-90.
51. Reiter E, Lefkowitz RJ. GRKs and beta-arrestins: roles in receptor silencing, trafficking and signaling. *Trends Endocrinol Metab*. 2006;17(4):159-65.
52. Lohse MJ, Benovic JL, Codina J, Caron MG, Lefkowitz RJ. beta-Arrestin: a protein that regulates beta-adrenergic receptor function. *Science*. 1990;248(4962):1547-50.
53. Lefkowitz RJ. Historical review: a brief history and personal retrospective of seven-transmembrane receptors. *Trends Pharmacol Sci*. 2004;25(8):413-22.
54. Gainetdinov RR, Premont RT, Bohn LM, Lefkowitz RJ, Caron MG. Desensitization of G protein-coupled receptors and neuronal functions. *Annu Rev Neurosci*. 2004;27:107-44.
55. Goodman OB, Krupnick JG, Santini F, Gurevich VV, Penn RB, Gagnon AW, et al. Beta-arrestin acts as a clathrin adaptor in endocytosis of the beta2-adrenergic receptor. *Nature*. 1996;383(6599):447-50.
56. Laporte SA, Oakley RH, Zhang J, Holt JA, Ferguson SS, Caron MG, et al. The beta2-adrenergic receptor/betaarrestin complex recruits the clathrin adaptor AP-2 during endocytosis. *Proc Natl Acad Sci U S A*. 1999;96(7):3712-7.
57. Gagnon AW, Kallal L, Benovic JL. Role of clathrin-mediated endocytosis in agonist-induced down-regulation of the beta2-adrenergic receptor. *J Biol Chem*. 1998;273(12):6976-81.
58. Zhang J, Barak LS, Winkler KE, Caron MG, Ferguson SS. A central role for beta-arrestins and clathrin-coated vesicle-mediated endocytosis in beta2-adrenergic receptor resensitization. Differential regulation of receptor resensitization in two distinct cell types. *J Biol Chem*. 1997;272(43):27005-14.
59. Oakley RH, Laporte SA, Holt JA, Barak LS, Caron MG. Association of beta-arrestin with G protein-coupled receptors during clathrin-mediated endocytosis dictates the profile of receptor resensitization. *J Biol Chem*. 1999;274(45):32248-57.
60. Shenoy SK, Lefkowitz RJ. β -Arrestin-mediated receptor trafficking and signal transduction. *Trends Pharmacol Sci*. 2011;32(9):521-33.

61. Walther C, Ferguson SS. Arrestins: role in the desensitization, sequestration, and vesicular trafficking of G protein-coupled receptors. *Prog Mol Biol Transl Sci*. 2013;118:93-113.
62. Marchese A, Trejo J. Ubiquitin-dependent regulation of G protein-coupled receptor trafficking and signaling. *Cell Signal*. 2013;25(3):707-16.
63. Luttrell LM, Lefkowitz RJ. The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. *J Cell Sci*. 2002;115(Pt 3):455-65.
64. Pierce KL, Lefkowitz RJ. Classical and new roles of beta-arrestins in the regulation of G-protein-coupled receptors. *Nat Rev Neurosci*. 2001;2(10):727-33.
65. Kobilka BK, Deupi X. Conformational complexity of G-protein-coupled receptors. *Trends Pharmacol Sci*. 2007;28(8):397-406.
66. Bosshard HR. Molecular recognition by induced fit: how fit is the concept? *News Physiol Sci*. 2001;16:171-3.
67. James LC, Tawfik DS. Conformational diversity and protein evolution--a 60-year-old hypothesis revisited. *Trends Biochem Sci*. 2003;28(7):361-8.
68. Kenakin T, Miller LJ. Seven transmembrane receptors as shapeshifting proteins: the impact of allosteric modulation and functional selectivity on new drug discovery. *Pharmacol Rev*. 2010;62(2):265-304.
69. Lefkowitz RJ, Shenoy SK. Transduction of receptor signals by beta-arrestins. *Science*. 2005;308(5721):512-7.
70. Kim J, Ahn S, Ren XR, Whalen EJ, Reiter E, Wei H, et al. Functional antagonism of different G protein-coupled receptor kinases for beta-arrestin-mediated angiotensin II receptor signaling. *Proc Natl Acad Sci U S A*. 2005;102(5):1442-7.
71. Ren XR, Reiter E, Ahn S, Kim J, Chen W, Lefkowitz RJ. Different G protein-coupled receptor kinases govern G protein and beta-arrestin-mediated signaling of V2 vasopressin receptor. *Proc Natl Acad Sci U S A*. 2005;102(5):1448-53.
72. Shenoy SK, Drake MT, Nelson CD, Houtz DA, Xiao K, Madabushi S, et al. beta-arrestin-dependent, G protein-independent ERK1/2 activation by the beta2 adrenergic receptor. *J Biol Chem*. 2006;281(2):1261-73.
73. Liu JJ, Horst R, Katritch V, Stevens RC, Wüthrich K. Biased signaling pathways in β 2-adrenergic receptor characterized by 19F-NMR. *Science*. 2012;335(6072):1106-10.
74. Doll C, Konietzko J, Pöll F, Koch T, Höllt V, Schulz S. Agonist-selective patterns of μ -opioid receptor phosphorylation revealed by phosphosite-specific antibodies. *Br J Pharmacol*. 2011;164(2):298-307.
75. Tobin AB, Butcher AJ, Kong KC. Location, location, location...site-specific GPCR phosphorylation offers a mechanism for cell-type-specific signalling. *Trends Pharmacol Sci*. 2008;29(8):413-20.
76. Beaulieu JM, Sotnikova TD, Marion S, Lefkowitz RJ, Gainetdinov RR, Caron MG. An Akt/beta-arrestin 2/PP2A signaling complex mediates dopaminergic neurotransmission and behavior. *Cell*. 2005;122(2):261-73.

77. Wang P, DeFea KA. Protease-activated receptor-2 simultaneously directs beta-arrestin-1-dependent inhibition and Galphaq-dependent activation of phosphatidylinositol 3-kinase. *Biochemistry*. 2006;45(31):9374-85.
78. Szidonya L, Süpeki K, Karip E, Turu G, Várnai P, Clark AJ, et al. AT1 receptor blocker-insensitive mutant AT1A angiotensin receptors reveal the presence of G protein-independent signaling in C9 cells. *Biochem Pharmacol*. 2007;73(10):1582-92.
79. Marks LS, Maxwell MH. Tigerstedt and the discovery of renin. An historical note. *Hypertension*. 1979;1(4):384-8.
80. Goldblatt H, Lynch J, Hanzal RF, Summerville WW. STUDIES ON EXPERIMENTAL HYPERTENSION : I. THE PRODUCTION OF PERSISTENT ELEVATION OF SYSTOLIC BLOOD PRESSURE BY MEANS OF RENAL ISCHEMIA. *J Exp Med*. 1934;59(3):347-79.
81. Braun-Menendez E, Fasciolo JC, Leloir LF, Muñoz JM. The substance causing renal hypertension. *J Physiol*. 1940;98(3):283-98.
82. Page IH, Helmer OM. A CRYSTALLINE PRESSOR SUBSTANCE (ANGIOTONIN) RESULTING FROM THE REACTION BETWEEN RENIN AND RENIN-ACTIVATOR. *J Exp Med*. 1940;71(1):29-42.
83. Dzau VJ, Baxter JA, Cantin M, de Bold A, Ganten D, Gross K, et al. Report of the Joint Nomenclature and Standardization Committee of the International Society of Hypertension, American Heart Association and the World Health Organization. *J Hypertens*. 1987;5(4):507-11.
84. Balakumar P, Jagadeesh G. A century old renin-angiotensin system still grows with endless possibilities: AT1 receptor signaling cascades in cardiovascular pathophysiology. *Cell Signal*. 2014;26(10):2147-60.
85. Miura S, Imaizumi S, Saku K. Recent progress in molecular mechanisms of angiotensin II type 1 and 2 receptors. *Curr Pharm Des*. 2013;19(17):2981-7.
86. Miyata N, Park F, Li XF, Cowley AW. Distribution of angiotensin AT1 and AT2 receptor subtypes in the rat kidney. *Am J Physiol*. 1999;277(3 Pt 2):F437-46.
87. Carey RM, Wang ZQ, Siragy HM. Role of the angiotensin type 2 receptor in the regulation of blood pressure and renal function. *Hypertension*. 2000;35(1 Pt 2):155-63.
88. Suzuki H, Motley ED, Frank GD, Utsunomiya H, Eguchi S. Recent progress in signal transduction research of the angiotensin II type-1 receptor: protein kinases, vascular dysfunction and structural requirement. *Curr Med Chem Cardiovasc Hematol Agents*. 2005;3(4):305-22.
89. Ushio-Fukai M, Griendling KK, Akers M, Lyons PR, Alexander RW. Temporal dispersion of activation of phospholipase C-beta1 and -gamma isoforms by angiotensin II in vascular smooth muscle cells. Role of alphaq/11, alpha12, and beta gamma G protein subunits. *J Biol Chem*. 1998;273(31):19772-7.
90. Macrez N, Morel JL, Kalkbrenner F, Viard P, Schultz G, Mironneau J. A betagamma dimer derived from G13 transduces the angiotensin AT1 receptor signal to stimulation of Ca²⁺ channels in rat portal vein myocytes. *J Biol Chem*. 1997;272(37):23180-5.

91. Ushio-Fukai M, Alexander RW, Akers M, Lyons PR, Lassègue B, Griendling KK. Angiotensin II receptor coupling to phospholipase D is mediated by the betagamma subunits of heterotrimeric G proteins in vascular smooth muscle cells. *Mol Pharmacol*. 1999;55(1):142-9.
92. Rattan S, Puri RN, Fan YP. Involvement of rho and rho-associated kinase in sphincteric smooth muscle contraction by angiotensin II. *Exp Biol Med (Maywood)*. 2003;228(8):972-81.
93. Baukal AJ, Hunyady L, Catt KJ, Balla T. Evidence for participation of calcineurin in potentiation of agonist-stimulated cyclic AMP formation by the calcium-mobilizing hormone, angiotensin II. *J Biol Chem*. 1994;269(40):24546-9.
94. Yamamoto T, Hayashi K, Matsuda H, Kubota E, Tanaka H, Ogasawara Y, et al. In vivo visualization of angiotensin II- and tubuloglomerular feedback-mediated renal vasoconstriction. *Kidney Int*. 2001;60(1):364-9.
95. Harrison-Bernard LM, Cook AK, Oliverio MI, Coffman TM. Renal segmental microvascular responses to ANG II in AT1A receptor null mice. *Am J Physiol Renal Physiol*. 2003;284(3):F538-45.
96. Valles P, Wysocki J, Batlle D. Angiotensin II and renal tubular ion transport. *ScientificWorldJournal*. 2005;5:680-90.
97. Oliverio MI, Delnomdedieu M, Best CF, Li P, Morris M, Callahan MF, et al. Abnormal water metabolism in mice lacking the type 1A receptor for ANG II. *Am J Physiol Renal Physiol*. 2000;278(1):F75-82.
98. Reilly AM, Harris PJ, Williams DA. Biphasic effect of angiotensin II on intracellular sodium concentration in rat proximal tubules. *Am J Physiol*. 1995;269(3 Pt 2):F374-80.
99. Horita S, Zheng Y, Hara C, Yamada H, Kunimi M, Taniguchi S, et al. Biphasic regulation of Na⁺-HCO₃⁻ cotransporter by angiotensin II type 1A receptor. *Hypertension*. 2002;40(5):707-12.
100. Gonzalez-Villalobos RA, Billet S, Kim C, Satou R, Fuchs S, Bernstein KE, et al. Intrarenal angiotensin-converting enzyme induces hypertension in response to angiotensin I infusion. *J Am Soc Nephrol*. 2011;22(3):449-59.
101. Zhang L, Guo F, Guo H, Wang H, Zhang Z, Liu X, et al. The paradox of dopamine and angiotensin II-mediated Na⁺, K⁺-ATPase regulation in renal proximal tubules. *Clin Exp Hypertens*. 2010;32(7):464-8.
102. Tojo A, Tisher CC, Madsen KM. Angiotensin II regulates H⁺-ATPase activity in rat cortical collecting duct. *Am J Physiol*. 1994;267(6 Pt 2):F1045-51.
103. Levine DZ, Iacovitti M, Buckman S, Hincke MT, Luck B, Fryer JN. ANG II-dependent HCO₃⁻ reabsorption in surviving rat distal tubules: expression/activation of H⁺-ATPase. *Am J Physiol*. 1997;272(6 Pt 2):F799-808.
104. Nguyen MT, Lee DH, Delpire E, McDonough AA. Differential regulation of Na⁺ transporters along nephron during ANG II-dependent hypertension: distal stimulation counteracted by proximal inhibition. *Am J Physiol Renal Physiol*. 2013;305(4):F510-9.

105. McDonough AA. Mechanisms of proximal tubule sodium transport regulation that link extracellular fluid volume and blood pressure. *Am J Physiol Regul Integr Comp Physiol*. 2010;298(4):R851-61.
106. Kim GH, Ecelbarger C, Knepper MA, Packer RK. Regulation of thick ascending limb ion transporter abundance in response to altered acid/base intake. *J Am Soc Nephrol*. 1999;10(5):935-42.
107. Blanchard A, Eladari D, Leviel F, Tsimaratos M, Paillard M, Podevin RA. NH_4^+ as a substrate for apical and basolateral Na^+ - H^+ exchangers of thick ascending limbs of rat kidney: evidence from isolated membranes. *J Physiol*. 1998;506 (Pt 3):689-98.
108. Aronson PS. Ion exchangers mediating Na^+ , HCO_3^- and Cl^- transport in the renal proximal tubule. *J Nephrol*. 2006;19 Suppl 9:S3-S10.
109. Wang T. Flow-activated transport events along the nephron. *Curr Opin Nephrol Hypertens*. 2006;15(5):530-6.
110. Kocinsky HS, Girardi AC, Biemesderfer D, Nguyen T, Mentone S, Orłowski J, et al. Use of phospho-specific antibodies to determine the phosphorylation of endogenous Na^+/H^+ exchanger NHE3 at PKA consensus sites. *Am J Physiol Renal Physiol*. 2005;289(2):F249-58.
111. Zhao H, Wiederkehr MR, Fan L, Collazo RL, Crowder LA, Moe OW. Acute inhibition of Na/H exchanger NHE-3 by cAMP. Role of protein kinase a and NHE-3 phosphoserines 552 and 605. *J Biol Chem*. 1999;274(7):3978-87.
112. Kurashima K, Yu FH, Cabado AG, Szabó EZ, Grinstein S, Orłowski J. Identification of sites required for down-regulation of Na^+/H^+ exchanger NHE3 activity by cAMP-dependent protein kinase. phosphorylation-dependent and -independent mechanisms. *J Biol Chem*. 1997;272(45):28672-9.
113. Wang D, Sun H, Lang F, Yun CC. Activation of NHE3 by dexamethasone requires phosphorylation of NHE3 at Ser663 by SGK1. *Am J Physiol Cell Physiol*. 2005;289(4):C802-10.
114. Kocinsky HS, Dynia DW, Wang T, Aronson PS. NHE3 phosphorylation at serines 552 and 605 does not directly affect NHE3 activity. *Am J Physiol Renal Physiol*. 2007;293(1):F212-8.
115. Di Sole F, Cerull R, Babich V, Quiñones H, Gisler SM, Biber J, et al. Acute regulation of Na/H exchanger NHE3 by adenosine $\text{A}(1)$ receptors is mediated by calcineurin homologous protein. *J Biol Chem*. 2004;279(4):2962-74.
116. Biemesderfer D, Nagy T, DeGray B, Aronson PS. Specific association of megalin and the Na^+/H^+ exchanger isoform NHE3 in the proximal tubule. *J Biol Chem*. 1999;274(25):17518-24.
117. Girardi AC, DeGray BC, Nagy T, Biemesderfer D, Aronson PS. Association of Na^+/H^+ exchanger isoform NHE3 and dipeptidyl peptidase IV in the renal proximal tubule. *J Biol Chem*. 2001;276(49):46671-7.
118. Cha B, Tse M, Yun C, Kovbasnjuk O, Mohan S, Hubbard A, et al. The NHE3 juxtamembrane cytoplasmic domain directly binds ezrin: dual role in NHE3 trafficking and mobility in the brush border. *Mol Biol Cell*. 2006;17(6):2661-73.

119. Zachos NC, van Rossum DB, Li X, Caraveo G, Sarker R, Cha B, et al. Phospholipase C-gamma binds directly to the Na⁺/H⁺ exchanger 3 and is required for calcium regulation of exchange activity. *J Biol Chem.* 2009;284(29):19437-44.
120. du Cheyron D, Chalumeau C, Defontaine N, Klein C, Kellermann O, Paillard M, et al. Angiotensin II stimulates NHE3 activity by exocytic insertion of the transporter: role of PI 3-kinase. *Kidney Int.* 2003;64(3):939-49.
121. Peng Y, Amemiya M, Yang X, Fan L, Moe OW, Yin H, et al. ET(B) receptor activation causes exocytic insertion of NHE3 in OKP cells. *Am J Physiol Renal Physiol.* 2001;280(1):F34-42.
122. D'Souza S, Garcia-Cabado A, Yu F, Teter K, Lukacs G, Skorecki K, et al. The epithelial sodium-hydrogen antiporter Na⁺/H⁺ exchanger 3 accumulates and is functional in recycling endosomes. *J Biol Chem.* 1998;273(4):2035-43.
123. Szászi K, Grinstein S, Orlowski J, Kapus A. Regulation of the epithelial Na⁽⁺⁾/H⁽⁺⁾ exchanger isoform by the cytoskeleton. *Cell Physiol Biochem.* 2000;10(5-6):265-72.
124. Chow CW, Khurana S, Woodside M, Grinstein S, Orlowski J. The epithelial Na⁽⁺⁾/H⁽⁺⁾ exchanger, NHE3, is internalized through a clathrin-mediated pathway. *J Biol Chem.* 1999;274(53):37551-8.
125. Hu MC, Fan L, Crowder LA, Karim-Jimenez Z, Murer H, Moe OW. Dopamine acutely stimulates Na⁺/H⁺ exchanger (NHE3) endocytosis via clathrin-coated vesicles: dependence on protein kinase A-mediated NHE3 phosphorylation. *J Biol Chem.* 2001;276(29):26906-15.
126. Collazo R, Fan L, Hu MC, Zhao H, Wiederkehr MR, Moe OW. Acute regulation of Na⁺/H⁺ exchanger NHE3 by parathyroid hormone via NHE3 phosphorylation and dynamin-dependent endocytosis. *J Biol Chem.* 2000;275(41):31601-8.
127. Yang LE, Maunsbach AB, Leong PK, McDonough AA. Differential traffic of proximal tubule Na⁺ transporters during hypertension or PTH: NHE3 to base of microvilli vs. NaPi2 to endosomes. *Am J Physiol Renal Physiol.* 2004;287(5):F896-906.
128. Zhang Y, Norian JM, Magyar CE, Holstein-Rathlou NH, Mircheff AK, McDonough AA. In vivo PTH provokes apical NHE3 and NaPi2 redistribution and Na-K-ATPase inhibition. *Am J Physiol.* 1999;276(5 Pt 2):F711-9.
129. Ambühl P, Amemiya M, Preisig PA, Moe OW, Alpern RJ. Chronic hyperosmolality increases NHE3 activity in OKP cells. *J Clin Invest.* 1998;101(1):170-7.
130. Ambühl PM, Amemiya M, Danczkay M, Lötscher M, Kaissling B, Moe OW, et al. Chronic metabolic acidosis increases NHE3 protein abundance in rat kidney. *Am J Physiol.* 1996;271(4 Pt 2):F917-25.
131. Ambühl PM, Yang X, Peng Y, Preisig PA, Moe OW, Alpern RJ. Glucocorticoids enhance acid activation of the Na⁺/H⁺ exchanger 3 (NHE3). *J Clin Invest.* 1999;103(3):429-35.
132. Amin MR, Malakooti J, Sandoval R, Dudeja PK, Ramaswamy K. IFN-gamma and TNF-alpha regulate human NHE3 gene expression by modulating the Sp family

transcription factors in human intestinal epithelial cell line C2BBel. *Am J Physiol Cell Physiol*. 2006;291(5):C887-96.

133. Woo AL, Gildea LA, Tack LM, Miller ML, Spicer Z, Millhorn DE, et al. In vivo evidence for interferon-gamma-mediated homeostatic mechanisms in small intestine of the NHE3 Na⁺/H⁺ exchanger knockout model of congenital diarrhea. *J Biol Chem*. 2002;277(50):49036-46.

134. Houillier P, Chambrey R, Achard JM, Froissart M, Poggioli J, Paillard M. Signaling pathways in the biphasic effect of angiotensin II on apical Na/H antiport activity in proximal tubule. *Kidney Int*. 1996;50(5):1496-505.

135. Hecht G, Hodges K, Gill RK, Kear F, Tyagi S, Malakooti J, et al. Differential regulation of Na⁺/H⁺ exchange isoform activities by enteropathogenic *E. coli* in human intestinal epithelial cells. *Am J Physiol Gastrointest Liver Physiol*. 2004;287(2):G370-8.

136. Cano A, Miller RT, Alpern RJ, Preisig PA. Angiotensin II stimulation of Na-H antiporter activity is cAMP independent in OKP cells. *The American journal of physiology*. 1994;266(6 Pt 1):C1603-8.

137. He P, Klein J, Yun CC. Activation of Na⁺/H⁺ exchanger NHE3 by angiotensin II is mediated by inositol 1,4,5-triphosphate (IP₃) receptor-binding protein released with IP₃ (IRBIT) and Ca²⁺/calmodulin-dependent protein kinase II. *The Journal of biological chemistry*. 285(36):27869-78.

138. Liu FY, Cogan MG. Angiotensin II stimulates early proximal bicarbonate absorption in the rat by decreasing cyclic adenosine monophosphate. *The Journal of clinical investigation*. 1989;84(1):83-91.

139. Liu FY, Cogan MG. Role of protein kinase C in proximal bicarbonate absorption and angiotensin signaling. *The American journal of physiology*. 1990;258(4 Pt 2):F927-33.

140. Wang T, Chan YL. The role of phosphoinositide turnover in mediating the biphasic effect of angiotensin II on renal tubular transport. *The Journal of pharmacology and experimental therapeutics*. 1991;256(1):309-17.

141. Tsuganezawa H, Preisig PA, Alpern RJ. Dominant negative c-Src inhibits angiotensin II induced activation of NHE3 in OKP cells. *Kidney Int*. 1998;54(2):394-8.

142. Musch MW, Li YC, Chang EB. Angiotensin II directly regulates intestinal epithelial NHE3 in Caco2BBE cells. *BMC Physiol*. 2009;9:5.

143. Banday AA, Lokhandwala MF. Angiotensin II-mediated biphasic regulation of proximal tubular Na⁺/H⁺ exchanger 3 is impaired during oxidative stress. *Am J Physiol Renal Physiol*. 301(2):F364-70.

144. Zheng Y, Horita S, Hara C, Kunimi M, Yamada H, Sugaya T, et al. Biphasic regulation of renal proximal bicarbonate absorption by luminal AT(1A) receptor. *J Am Soc Nephrol*. 2003;14(5):1116-22.

145. Girardi AC, Di Sole F. Deciphering the mechanisms of the Na⁺/H⁺ exchanger-3 regulation in organ dysfunction. *Am J Physiol Cell Physiol*. 2012;302(11):C1569-87.

146. Soleimani M, Singh G. Physiologic and molecular aspects of the Na⁺/H⁺ exchangers in health and disease processes. *J Investig Med*. 1995;43(5):419-30.

147. Soleimani M, Burnham CE. Physiologic and molecular aspects of the Na⁺:HCO₃⁻ cotransporter in health and disease processes. *Kidney Int.* 2000;57(2):371-84.
148. Schultheis PJ, Clarke LL, Meneton P, Miller ML, Soleimani M, Gawenis LR, et al. Renal and intestinal absorptive defects in mice lacking the NHE3 Na⁺/H⁺ exchanger. *Nat Genet.* 1998;19(3):282-5.
149. Ledoussal C, Lorenz JN, Nieman ML, Soleimani M, Schultheis PJ, Shull GE. Renal salt wasting in mice lacking NHE3 Na⁺/H⁺ exchanger but not in mice lacking NHE2. *Am J Physiol Renal Physiol.* 2001;281(4):F718-27.
150. Curtis JJ, Luke RG, Dustan HP, Kashgarian M, Whelchel JD, Jones P, et al. Remission of essential hypertension after renal transplantation. 1983. *J Am Soc Nephrol.* 2000;11(12):2404-12.
151. Lifton RP, Gharavi AG, Geller DS. Molecular mechanisms of human hypertension. *Cell.* 2001;104(4):545-56.
152. Crowley SD, Gurley SB, Herrera MJ, Ruiz P, Griffiths R, Kumar AP, et al. Angiotensin II causes hypertension and cardiac hypertrophy through its receptors in the kidney. *Proc Natl Acad Sci U S A.* 2006;103(47):17985-90.
153. Crowley SD, Gurley SB, Oliverio MI, Pazmino AK, Griffiths R, Flannery PJ, et al. Distinct roles for the kidney and systemic tissues in blood pressure regulation by the renin-angiotensin system. *J Clin Invest.* 2005;115(4):1092-9.
154. Gurley SB, Riquier-Brisson AD, Schnermann J, Sparks MA, Allen AM, Haase VH, et al. AT1A angiotensin receptors in the renal proximal tubule regulate blood pressure. *Cell Metab.* 2011;13(4):469-75.
155. Crajoinas RO, Lessa LM, Carraro-Lacroix LR, Davel AP, Pacheco BP, Rossoni LV, et al. Posttranslational mechanisms associated with reduced NHE3 activity in adult vs. young prehypertensive SHR. *Am J Physiol Renal Physiol.* 2010;299(4):F872-81.
156. Bae EH, Ma SK. Water and sodium regulation in heart failure. *Electrolyte Blood Press.* 2009;7(2):38-41.
157. Staahltoft D, Nielsen S, Janjua NR, Christensen S, Skøtt O, Marcussen N, et al. Losartan treatment normalizes renal sodium and water handling in rats with mild congestive heart failure. *Am J Physiol Renal Physiol.* 2002;282(2):F307-15.
158. Lütken SC, Kim SW, Jonassen T, Marples D, Knepper MA, Kwon TH, et al. Changes of renal AQP2, ENaC, and NHE3 in experimentally induced heart failure: response to angiotensin II AT1 receptor blockade. *Am J Physiol Renal Physiol.* 2009;297(6):F1678-88.
159. Holloway AC, Qian H, Pipolo L, Ziogas J, Miura S, Karnik S, et al. Side-chain substitutions within angiotensin II reveal different requirements for signaling, internalization, and phosphorylation of type 1A angiotensin receptors. *Mol Pharmacol.* 2002;61(4):768-77.
160. Tohgo A, Pierce KL, Choy EW, Lefkowitz RJ, Luttrell LM. beta-Arrestin scaffolding of the ERK cascade enhances cytosolic ERK activity but inhibits ERK-mediated transcription following angiotensin AT1a receptor stimulation. *J Biol Chem.* 2002;277(11):9429-36.

161. Lee MH, El-Shewy HM, Luttrell DK, Luttrell LM. Role of beta-arrestin-mediated desensitization and signaling in the control of angiotensin AT1a receptor-stimulated transcription. *J Biol Chem*. 2008;283(4):2088-97.
162. Violin JD, DeWire SM, Yamashita D, Rominger DH, Nguyen L, Schiller K, et al. Selectively engaging β -arrestins at the angiotensin II type 1 receptor reduces blood pressure and increases cardiac performance. *J Pharmacol Exp Ther*. 2010;335(3):572-9.
163. Boerrigter G, Soergel DG, Violin JD, Lark MW, Burnett JC, Jr. TRV120027, a novel beta-arrestin biased ligand at the angiotensin II type I receptor, unloads the heart and maintains renal function when added to furosemide in experimental heart failure. *Circ Heart Fail*. 2012;5(5):627-34.
164. Wei H, Ahn S, Shenoy SK, Karnik SS, Hunyady L, Luttrell LM, et al. Independent beta-arrestin 2 and G protein-mediated pathways for angiotensin II activation of extracellular signal-regulated kinases 1 and 2. *Proc Natl Acad Sci U S A*. 2003;100(19):10782-7.
165. Rajagopal K, Whalen EJ, Violin JD, Stiber JA, Rosenberg PB, Premont RT, et al. Beta-arrestin2-mediated inotropic effects of the angiotensin II type 1A receptor in isolated cardiac myocytes. *Proc Natl Acad Sci U S A*. 2006;103(44):16284-9.
166. Aplin M, Christensen GL, Schneider M, Heydorn A, Gammeltoft S, Kjølbye AL, et al. The angiotensin type 1 receptor activates extracellular signal-regulated kinases 1 and 2 by G protein-dependent and -independent pathways in cardiac myocytes and langendorff-perfused hearts. *Basic Clin Pharmacol Toxicol*. 2007;100(5):289-95.
167. Ahn S, Kim J, Hara MR, Ren XR, Lefkowitz RJ. β -Arrestin-2 Mediates Anti-apoptotic Signaling through Regulation of BAD Phosphorylation. *J Biol Chem*. 2009;284(13):8855-65.
168. Boerrigter G, Lark MW, Whalen EJ, Soergel DG, Violin JD, Burnett JC. Cardiorenal actions of TRV120027, a novel β -arrestin-biased ligand at the angiotensin II type I receptor, in healthy and heart failure canines: a novel therapeutic strategy for acute heart failure. *Circ Heart Fail*. 2011;4(6):770-8.
169. Kim KS, Abraham D, Williams B, Violin JD, Mao L, Rockman HA. β -Arrestin-biased AT1R stimulation promotes cell survival during acute cardiac injury. *Am J Physiol Heart Circ Physiol*. 2012;303(8):H1001-10.
170. Monasky MM, Taglieri DM, Henze M, Warren CM, Utter MS, Soergel DG, et al. The beta-arrestin-biased ligand TRV120023 inhibits angiotensin II-induced cardiac hypertrophy while preserving enhanced myofilament response to calcium. *Am J Physiol Heart Circ Physiol*. 2013;305(6):H856-66.
171. Boerrigter G, Lark MW, Whalen EJ, Soergel DG, Violin JD, Burnett JC, Jr. Cardiorenal actions of TRV120027, a novel β -arrestin-biased ligand at the angiotensin II type I receptor, in healthy and heart failure canines: a novel therapeutic strategy for acute heart failure. *Circ Heart Fail*. 2011;4(6):770-8.
172. Soergel DG, Subach RA, Sadler B, Connell J, Marion AS, Cowan CL, et al. First clinical experience with TRV130: pharmacokinetics and pharmacodynamics in healthy volunteers. *J Clin Pharmacol*. 2014;54(3):351-7.

173. Schwark JR, Jansen HW, Lang HJ, Krick W, Burckhardt G, Hropot M. S3226, a novel inhibitor of Na⁺/H⁺ exchanger subtype 3 in various cell types. *Pflügers Arch.* 1998;436(5):797-800.
174. Yang LE, Leong PK, Ye S, Campese VM, McDonough AA. Responses of proximal tubule sodium transporters to acute injury-induced hypertension. *Am J Physiol Renal Physiol.* 2003;284(2):F313-22.
175. Gil FZ, Malnic G. Effect of amphotericin B on renal tubular acidification in the rat. *Pflügers Arch.* 1989;413(3):280-6.
176. Cole JA, Forte LR, Krause WJ, Thorne PK. Clonal sublines that are morphologically and functionally distinct from parental OK cells. *Am J Physiol.* 1989;256(4 Pt 2):F672-9.
177. Carraro-Lacroix LR, Malnic G, Girardi AC. Regulation of Na⁺/H⁺ exchanger NHE3 by glucagon-like peptide 1 receptor agonist exendin-4 in renal proximal tubule cells. *Am J Physiol Renal Physiol.* 2009;297(6):F1647-55.
178. Crajoinas RO, Oricchio FT, Pessoa TD, Pacheco BP, Lessa LM, Malnic G, et al. Mechanisms mediating the diuretic and natriuretic actions of the incretin hormone glucagon-like peptide-1. *Am J Physiol Renal Physiol.* 2011;301(2):F355-63.
179. Violin JD, DeWire SM, Yamashita D, Rominger DH, Nguyen L, Schiller K, et al. Selectively engaging beta-arrestins at the angiotensin II type 1 receptor reduces blood pressure and increases cardiac performance. *J Pharmacol Exp Ther.* 2010;335(3):572-9.
180. Biemesderfer D, DeGray B, Aronson PS. Active (9.6 s) and inactive (21 s) oligomers of NHE3 in microdomains of the renal brush border. *The Journal of biological chemistry.* 2001;276(13):10161-7.
181. Brasen JC, Burford JL, McDonough AA, Holstein-Rathlou NH, Peti-Peterdi J. Local pH domains regulate NHE3-mediated Na(+) reabsorption in the renal proximal tubule. *Am J Physiol Renal Physiol.* 2014;307(11):F1249-62.
182. Girardi AC, Fukuda LE, Rossoni LV, Malnic G, Reboucas NA. Dipeptidyl peptidase IV inhibition downregulates Na⁺ - H⁺ exchanger NHE3 in rat renal proximal tubule. *Am J Physiol Renal Physiol.* 2008;294(2):F414-22.
183. Cabado AG, Yu FH, Kapus A, Lukacs G, Grinstein S, Orlowski J. Distinct structural domains confer cAMP sensitivity and ATP dependence to the Na⁺/H⁺ exchanger NHE3 isoform. *J Biol Chem.* 1996;271(7):3590-9.
184. Kendall RT, Strungs EG, Rachidi SM, Lee MH, El-Shewy HM, Luttrell DK, et al. The beta-arrestin pathway-selective type 1A angiotensin receptor (AT1A) agonist [Sar1,Ile4,Ile8]angiotensin II regulates a robust G protein-independent signaling network. *J Biol Chem.* 2011;286(22):19880-91.
185. Szabó EZ, Numata M, Lukashova V, Iannuzzi P, Orlowski J. beta-Arrestins bind and decrease cell-surface abundance of the Na⁺/H⁺ exchanger NHE5 isoform. *Proc Natl Acad Sci U S A.* 2005;102(8):2790-5.
186. Wei H, Ahn S, Shenoy SK, Karnik SS, Hunyady L, Luttrell LM, et al. Independent β-arrestin 2 and G protein-mediated pathways for angiotensin II activation

of extracellular signal-regulated kinases 1 and 2. *Proceedings of the National Academy of Sciences*. 2003;100(19):10782-7.

187. Yang L, Leong PK, Chen JO, Patel N, Hamm-Alvarez SF, McDonough AA. Acute hypertension provokes internalization of proximal tubule NHE3 without inhibition of transport activity. *Am J Physiol Renal Physiol*. 2002;282(4):F730-40.

188. Leong PK, Yang LE, Holstein-Rathlou NH, McDonough AA. Angiotensin II clamp prevents the second step in renal apical NHE3 internalization during acute hypertension. *Am J Physiol Renal Physiol*. 2002;283(5):F1142-50.

189. Azarani A, Goltzman D, Orlowski J. Parathyroid hormone and parathyroid hormone-related peptide inhibit the apical Na⁺/H⁺ exchanger NHE-3 isoform in renal cells (OK) via a dual signaling cascade involving protein kinase A and C. *J Biol Chem*. 1995;270(34):20004-10.

190. Azarani A, Goltzman D, Orlowski J. Structurally diverse N-terminal peptides of parathyroid hormone (PTH) and PTH-related peptide (PTHrP) inhibit the Na⁺/H⁺ exchanger NHE3 isoform by binding to the PTH/PTHrP receptor type I and activating distinct signaling pathways. *J Biol Chem*. 1996;271(25):14931-6.

191. Wiederkehr MR, Di Sole F, Collazo R, Quiñones H, Fan L, Murer H, et al. Characterization of acute inhibition of Na/H exchanger NHE-3 by dopamine in opossum kidney cells. *Kidney Int*. 2001;59(1):197-209.

192. Tian X, Kang DS, Benovic JL. β -arrestins and G protein-coupled receptor trafficking. *Handb Exp Pharmacol*. 2014;219:173-86.

193. Riquier-Brison AD, Leong PK, Pihakaski-Maunsbach K, McDonough AA. Angiotensin II stimulates trafficking of NHE3, NaPi2, and associated proteins into the proximal tubule microvilli. *Am J Physiol Renal Physiol*. 2000;278(1):F177-86.

194. Cervenka L, Wang CT, Navar LG. Effects of acute AT1 receptor blockade by candesartan on arterial pressure and renal function in rats. *The American journal of physiology*. 1998;274(5 Pt 2):F940-5.

195. Du Z, Wan L, Yan Q, Weinbaum S, Weinstein AM, Wang T. Regulation of glomerulotubular balance: II: impact of angiotensin II on flow-dependent transport. *Am J Physiol Renal Physiol*. 2000;279(1):F1507-16.

196. Diaz-Sylvester PL, Fiori MC, Dieguez SM, Muller AC, Lopardo ML, Amorena CE. Effect of chronic inhibition of converting enzyme on proximal tubule acidification. *Am J Physiol Regul Integr Comp Physiol*. 2008;294(6):R2014-20.

197. Hashimoto S, Adams JW, Bernstein KE, Schnermann J. Micropuncture determination of nephron function in mice without tissue angiotensin-converting enzyme. *Am J Physiol Renal Physiol*. 2005;288(3):F445-52.

198. Singh V, Lin R, Yang J, Cha B, Sarker R, Tse CM, et al. AKT and GSK-3 are necessary for direct ezrin binding to NHE3 as part of a C-terminal stimulatory complex: role of a novel Ser-rich NHE3 C-terminal motif in NHE3 activity and trafficking. *J Biol Chem*. 2014;289(9):5449-61.

199. Ahn S, Shenoy SK, Wei H, Lefkowitz RJ. Differential kinetic and spatial patterns of beta-arrestin and G protein-mediated ERK activation by the angiotensin II receptor. *J Biol Chem*. 2004;279(34):35518-25.

200. DeFea KA, Zalevsky J, Thoma MS, Déry O, Mullins RD, Bunnett NW. beta-arrestin-dependent endocytosis of proteinase-activated receptor 2 is required for intracellular targeting of activated ERK1/2. *J Cell Biol.* 2000;148(6):1267-81.

Attachments

Attachment 1 – Confirmation of total RNA integrity.

To RNA integrity was confirmed by the presence of the two ribosomal RNAs 28S and 18s by denaturing gel as presented in Supplementary Fig. 1. Moreover, as expected the 28S rRNA was approximately twice as intense as the 18S rRNA band,

Figure 1 – RNA integrity confirmed by the presence of the two ribosomal RNAs 28 s and 18s.

Attachment 2 – DNA sequences amplified.

The different DNA sequences that we were able to amplify are presented in Supplementary Fig. 2. These sequences were sequenced and confirmed to match the isoforms of beta-arrestin 1 and 2 from *Monodelphis domestica* presented in BLAST.

Figure 2 – The different sequences of DNA amplified.

Attachment 3 – Confirmation of the bimodal effect of angiotensin II.

It is long been known that Ang II infusion into the kidney is associated, at high doses ($> 10^{-8}$ M) with increased sodium and water excretion, and at low doses (10^{-12} - 10^{-10} M), with sodium and fluid retention (98, 134, 135). To confirm that our experiments were indeed given reliable results, we evaluated the effects of 15 minutes exposure to Ang II at 10^{-10} M and 10^{-7} M. As expected and presented in Supplementary Fig. 3, low doses of Ang II increases NHE3 activity whereas low doses inhibits NHE3.

Figure 3 – Bimodal effect of angiotensin II on NHE3 activity.