AMERICAN UNIVERSITY OF BEIRUT

# FTY720P UPREGULATES $Na^{+}/K^{+}$ ATPASE IN LLC-PK1 CELLS: RHO KINASE, PI3K AND NITRIC OXIDE ARE ALONG THE PATHWAY

by
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# AN ABSTRACT OF THE THESIS OF

Christine Charbel Khalil for Master of Science
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Title: FTY720P upregulates $Na^+/K^+$ ATPASE in LLC-PK1 cells: Rho kinase, PI3K and Nitric oxide are along the pathway

The kidneys play a pivotal role in the regulation of blood composition and osmolarity. This regulatory role is dependent heavily on the activity of the $Na^+/K^+$ ATPase, a pump that provides the driving force for the movement of various electrolytes and solutes. Renal ischemia reperfusion injury (IRI) was shown to be associated with a decrease in the expression of the ATPase and was reduced by sphingosine-1-phosphate (S1P). Because the sphingolipid and the $Na^+/K^+$ ATPase are both implicated in renal IRI, a cause effect relationship may exist. This work aims at investigating and determining the effect of S1P, through its analogue FTY720P, on the $Na^+/K^+$ ATPase activity, and at unraveling the signaling pathway involved, using the proximal tubule cells LLC-PK1 as a model. The activity of the $Na^+/K^+$ ATPase was assayed by measuring the amount of inorganic phosphate liberated in presence and absence of ouabain, a specific inhibitor of the enzyme. The expression of S1P receptors (S1PR) in LLC-PK1 cells was studied using Western Blot analysis and revealed that all S1PRs were expressed.

FTY720P increased the activity of the ATPase in a dose and time dependent manner, with a highest effect observed at 15 minutes and at a dose of 80 nM. The activation of the $Na^+/K^+$ ATPase completely disappeared in presence of JTE-013, a specific blocker of S1PR2, as well as in presence of Y-27632, a Rho kinase inhibitor, BAPTA-AM, a $Ca^{2+}$ chelator, wortmannin, a PI3K inhibitor, carboxy-PTIO, a scavenger for nitric oxide (NO) and KT 5823, a PKG inhibitor. The involvement of S1PR2 was confirmed by treating the cells with CYM 5520, a S1PR2 agonist that mimicked FTY720P's activation. FTY720P increased the expression of p-Akt, a direct effector of PI3K, however, this increase disappeared when Rho kinase was inhibited, revealing that Rho kinase acts upstream PI3K. Glyco-SNAP-1, a NO donor, activated the pump both in presence and absence of wortmannin, an inhibitor of PI3K, indicating that PI3K is upstream NO. However, glyco-SNAP-1 and 8-bromo-cGMP, a PKG activator, exerted no effect on the $Na^+/K^+$ ATPase in absence of free $Ca^{2+}$ revealing that NO, known from the literature to activate PKG, leads to an increase in intracellular $Ca^{2+}$. RpcAMP, a PKA inhibitor and dbcAMP, a PKA activator had no effect on the pump both in presence and absence of FTY720P showing that PKA is not a mediator in the pathway. In addition, neither calphostin C, a PKC inhibitor nor indomethacin, an inhibitor of

PGE2 synthesis could eliminate the activation induced by FTY720P inferring that they are not involved in the effect of FTY720P on the ATPase.

The results suggest that FTY720P exerts its stimulatory effect on the ATPase via S1PR2 that activates Rho kinase, then PI3K inducing NO production followed by an increase in cGMP levels that stimulate PKG resulting in an elevated concentration of intracellular $Ca^{2+}$ and activation of the $Na^{+}/K^{+}$ ATPase in LLC-PK1 cells.

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# ABBREVIATIONS

| | |
|---|---|
| AKI | Acute kidney injury |
| ABC | ATP-binding cassette |
| AC | Adenylyl cyclase |
| ADCY3 | Adenylate cyclase 3 |
| ADCY8 | Adenylate cyclase 8 |
| Akt/PKB | Protein kinase B |
| AMPK | AMP-activated protein kinase |
| $AT_2R$ | Angiotensin type-2 receptor |
| ATCC | American Type Culture Collection |
| ATP | Adenosine Triphosphate |
| BSA | Bovine serum albumin |
| BUN | Blood urea nitrogen |
| CaM | Calmodulin |
| CaMK | $Ca^{2+}$/calmodulin –dependent protein kinase |
| CaMKKβ | $Ca^{2+}$/calmodulin –dependent protein kinase kinase β |
| cAMP | Cyclic adenosine monophosphate |
| CD31 | Cluster of differentiation 31 |
| cGMP | Cyclic guanosine 3’,5’ monophosphate |
| COX | Cyclooxygenase |
| CRAC | Calcium release-activated calcium channel |
| CTGF | Connective tissue growth factor |

| | |
|---|---|
| D1 receptor | Dopamine receptor 1 |
| DAG | Diacylglycerol |
| dbcAMP | dibutyryl-cAMP |
| DMEM | Dulbecco's modified eagle media |
| DMSO | Dimethyl sulfoxide |
| EDG | Endothelial differentiation gene |
| EDRF | Endothelial-derived relaxant factor |
| EMT | Epithelial to mesenchymal transition |
| eNOS | Endothelial nitric oxide synthase |
| EP receptor | Prostaglandin $E_2$ receptor |
| ER | Endoplasmic reticulum |
| ERK | Extracellular signal-regulated kinase |
| ET-1 | Endothelin-1 |
| FBS | Fetal bovine serum |
| FDA | Food and drug administration of USA |
| FTY720 | Fingolimod |
| G6PD | Glucose-6-phosphate dehydrogenase |
| GAP | GTPase activating protein |
| GC | Guanylyl cyclase |
| GDP | Guanosine diphosphate |
| GEF | Guanine nucleotide exchange factor |
| GPCR | G-protein coupled receptors |
| GTP | Guanosine triphosphate |
| HDAC | Histone deacetylases |

| | |
|---|---|
| HDL | High-density lipoprotein |
| HRP | Horseradish peroxidase |
| HUVEC | Primary human umbilical vein endothelial cells |
| iNOS | Inducible nitric oxide synthase |
| InsP3R | Inositol 1,4,5-triphosphate receptor |
| IRI | Ischemia reperfusion injury |
| LPP | Lipid-specific phosphatase |
| MAPK | Mitogen –activated protein kinase |
| MCP1 | Monocyte chemoattractant protein 1 |
| MDCK | Madin-Darby canine kidney |
| MLC | Myosin light chains |
| MLCP | Myosin light chain phosphatase |
| mTOR | Mammalian target of rapamycin |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NCKX | $Na^+/Ca^{2+}$-$K^+$ exchanger |
| NCX | $Na^+/Ca^{2+}$ exchanger |
| NF-κB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| NHE3 | $Na^+/H^+$ antiporter |
| nNOS | Neuronal nitric oxide synthase |
| NO | Nitric oxide |
| $NO_2$ | Nitrogen dioxide |
| $NO_3$ | Nitrate |
| NOS | Nitric oxide synthase |

| | |
|---|---|
| $ONOO^-$ | Peroxynitrite |
| ONOOH | Peroxynitrous acid |
| $p27^{kip1}$ | Cyclin-dependent kinase inhibitor |
| PAMP | Pathogen-associated molecular pattern |
| PBS | Phosphate-buffered-saline |
| PDK1 | Phosphoinositide-dependent kinase 1 |
| PGE2 | Prostaglandin $E_2$ |
| PH | Pleckstrin homology domain |
| PI3K | Phosphoinositide-3-kinase |
| PIP2 | Phosphatidylinositol (4,5)P2 |
| PIP3 | Phosphatidylinositol (3,4,5)P3 |
| PKA | Protein kinase A |
| PKC | Protein kinase C |
| PKG | Protein kinase G/ c-GMP-dependent protein kinase |
| PLB | Phospholipase B |
| PLC | Phospholipase C |
| PMA | Phorbol 12-myristate 13-acetate |
| PMCA | $Ca^{2+}$ ATPase |
| PS | Phosphatidylserine |
| PSC | Pluripotent stem cells |
| PtdIns | Phosphatidylinositol |
| PTEN | Phosphatase and tensin homolog |
| PTH | Parathyroid hormone |
| QFTL | Qufengtongluo |

| | |
|---|---|
| Rac | Ras-related C3 botulinum toxin substrate |
| Ras | Retrovirus-associated DNA sequences |
| RBD | Rho-binding domain |
| RGS | Regulator of G-protein signaling |
| RhoA | Ras homolog gene family A |
| RNS | Reactive nitrogen species |
| ROCC | Receptor-operated calcium channel |
| ROCK | Rho-associated protein kinase |
| ROS | Reactive oxygen species |
| RpcAMP | Rp-Adenosine 3', 5'-cyclic monophosphorothioate triethylammonium |
| RYR | Ryanodine receptor |
| S1P | Sphingosine-1-phosphate |
| S1PR | Sphingosine-1-phosphate receptor |
| SDS | Sodium dodecyl sulfate |
| SERCA | ER/SR $Ca^{2+}$ ATPase pump |
| sGC | Soluble guanylyl cyclase |
| sGC | Soluble guanylyl cyclase |
| SH3 | Src homology 3 |
| SHP1 | Src homology 2 domain-containing protein tyrosine phosphatase 1 |
| SHR | Spontaneously hypertensive rats |
| SPHK1 | Sphingosine kinase 1 |
| SPL | Sphingosine-1-phosphate lyase |

| | |
|---|---|
| SPP | S1P-specific phosphatase |
| SPSN2 | Sphingolipid transporter Spinster 2 |
| TGF | Transforming growth factor |
| TNF-α | Tumor necrosis factor-α |
| TRPV4 | Transient receptor potential cation channel subfamily V member 4 |
| UUO | Unilateral ureteral obstruction |
| VEGF | Vascular endothelial growth factor |
| VOCC | Voltage-operated calcium channel |
| W146 | (R)-3-Amino-(3-heylphenylamino)-4-oxobutylphosphonic |

# CHAPTER I

# INTRODUCTION

The kidney is a vital organ that regulates blood composition and consequently contributes to the organism's homeostasis. However, kidney functions are impaired in ischemia/reperfusion injury (IRI) when the blood flow is restored to an organ after ischemia (Malek et al., 2015) and may lead to total kidney failure (Kristensen et al., 2016; Malek et al., 2015). Sphingosine-1-phosphate (S1P), which acts via 5 different types of G-protein coupled receptors, was shown to play a protective role against renal IRI (Awad et al., 2006; Huwiler et al., 2018; Park et al., 2012; Troncoso et al., 2001). In fact, FTY720P an analogue of S1P, reduced renal injury in diabetic nephropathy by reducing inflammation, limiting leukocyte infiltration and vascular permeability and inducing lymphopenia (Awad et al., 2011). FTY720 (fingolimod) is a fungal derivative known for its immunosuppressive properties and was approved recently for the treatment of multiple sclerosis (Mendelson et al., 2014). FTY720 becomes a structural analogue of S1P upon phosphorylation, (Huwiler et al., 2018; Sobel et al., 2015) Agonists of sphingosine-1-phosphate receptor 1 (S1PR1) in particular, were shown in renal IRI, to decrease apoptosis and enhance kidney cell survival by activating protein kinase B (Akt) and the extracellular signal-regulated kinase1/2 (ERK1/2) pathway ,limiting thus the kidney injury (Bajwa et al., 2010).

Renal IRI was found to be accompanied by a reduced expression of the $Na^+/K^+$ ATPase, a ubiquitous P-type ATPase present abundantly in the kidneys (Kristensen et al., 2016). The $Na^+/K^+$ ATPase is located at the basolateral side of kidney proximal tubule cells. It establishes a sodium electrochemical gradient across the cell membrane that drives all secondary active transport like the reabsorption of glucose, amino acids, protons, chloride citrate, succinate, calcium and phosphate across renal tubular cells (Jorgensen, 1986). Any alteration in the activity of the ATPase is expected to impair kidney functions leading to kidney disease.

Because $Na^+/K^+$ ATPase and S1P were both implicated in renal IRI, the reduced expression of the ATPase may be induced by S1P. Therefore, this work was undertaken to determine the effect of FTY720P, an analogue of S1P, on the activity of the $Na^+/K^+$ ATPase in LLC-PK1 cells, a porcine proximal tubule cell line. An attempt will be made also to determine the signaling pathway involved. Identifying the different mediators would help in finding a possible treatment for renal IRI and preventing any undesirable effect of FTY720P on the kidney.

This work aims at

1. Determining the effect of FTY720P on renal $Na^+/K^+$ ATPase activity and its optimal concentration and time course.
2. Determining the type of S1PRs expressed in LLC-PK1 cells.
3. Identifying the type of S1PR involved in FTY720P's effect on renal $Na^+/K^+$ ATPase.
4. Discerning key mediators implicated in the signaling pathway.

# CHAPTER II

# LITERATURE REVIEW

## A. $Na^+/K^+$ ATPase

### *1. Structure*

The $Na^+/K^+$ ATPase, also known as the $Na^+/K^+$ pump, is a ubiquitous P-type ATPase that is implicated in many important cellular mechanisms. It belongs to the subgroup 2C of the cation-translocating ATPases that also includes the $H^+/K^+$ ATPase (Cechovaet al., 2016). It uses the energy generated from the ATP hydrolysis to transport sodium and potassium ions against their concentration gradient (Xie et al., 2013). The $Na^+/K^+$ ATPase alternates between a sodium bound state (E1) and a potassium bound state (E2) (Xie et al., 2013), and consumes approximately one-third of the total amount of ATP produced in the human body (Cechova et al., 2016) and 80% of the ATP produced in the kidney (Clausen et al., 1991).

The $Na^+/K^+$ ATPase is made of 2 sub-units: α and β that are noncovalently linked. A FXYD protein known to be the γ sub-unit (6-8 kDa) is also expressed in a tissue-specific manner. The α sub-unit (around 112 kDa) is made of 10 trans-membrane helices (Geering, 2001). It contains the binding sites for ATP and other ligands and is responsible for coupling ion movement to ATP hydrolysis. Humans have 4 α isoforms: α 1 is ubiquitously expressed; α 2 and α 3 are found in neuronal tissue, skeletal muscle and cardiac myocytes; α 4 is expressed in the testis and modulates sperm motility. Three functional domains are found within the α sub-unit: the actuator (A) which regulates the binding sites of the ions, the phosphorylation (P) domain which is highly conserved (Xie et al., 2013) and the nucleotide (N) binding domain. The β sub-unit is a glycosylated protein made of 1 trans-membrane helix (Geering, 2001) and an extracellular large domain (Cechova et al., 2016). Its molecular weight is around 55 kDa. Three β isoforms have been identified: β 1 is ubiquitously expressed, β 2 is found in the heart and skeletal muscle and β 3 is expressed in the central nervous system and the testis (Suhail, 2010). The β sub-unit is required for the correct assembly of the pump (Xie et al., 2013) and the proper folding of the α sub-unit when the pump is inserted in the basolateral membrane which is important to prevent its degradation and provide trypsin resistance. Seven γ sub-unit isoforms are known and they function as modulators of the ATPase activity (Clausen et al., 2017; Geering, 2001).

The α and β sub-units are assembled in the endoplasmic reticulum (Geering, 2001). The α 1-β 1 combination is the most widely expressed. The isoforms differ by their affinities and kinetic properties providing each cell with the best functional characteristics (Clausen et al., 2017).

A unique isoform of the $Na^+/K^+$ ATPase is expressed in sperm cells where it has a fundamental role in male fertility since it regulates ions and membrane potential. The $Na^+/K^+$ ATPase has also an important role in the nervous system. In the gray matter, it consumes three quarters of the total energy. It is needed to trigger an action potential in neurons and for neurotransmitter reuptake in astrocytes (Clausen et al., 2017).

Kidneys are responsible for the regulation of electrolyte concentration in the body (Rocafull et al., 2012). The reabsorption of filtered substances in the nephron occurs mainly in the kidney proximal tubule where 70% of the solutes and water are reabsorbed. The basolateral and apical membranes of proximal tubule cells are enriched with sodium transporters. Sodium/glucose cotransporter 2, sodium/hydrogen exchanger 3 and sodium/phosphate cotransporter type 2A are examples of the important sodium transporters on the apical side. Electrogenic sodium/bicarbonate cotransporter 1A and the $Na^+/K^+$ ATPase are located on the basolateral surface (Horita et al., 2017) (figure 1).

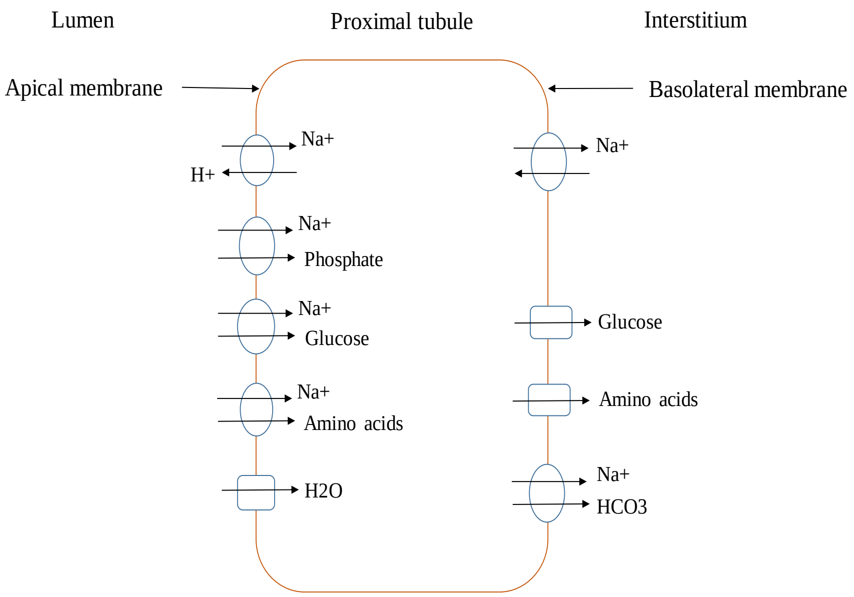


Figure 1: Different transporters present in the kidney proximal convoluted tubule.

The $Na^+/K^+$ ATPase is abundant in the kidney (Jorgensen, 1986). The α 1 isoform is responsible for the regulation of blood $Na^+$ levels and maintenance of extracellular volume (Manunta et al., 2010), and consequently, blood pressure (Xie et al., 2013). Thirty three percent of solute reabsorption in kidney proximal tubule is driven by the sodium gradient established by the $Na^+/K^+$ ATPase. The transport of glucose, amino acids, citrate, succinate, protons, calcium, phosphate and chloride across renal tubular cells (Jorgensen, 1986) are all sodium dependent processes. The proper functioning of this ATPase is needed for all the main functions of the kidney such as regulation of blood electrolyte levels, reabsorption of solutes and maintenance of blood pH (Clausen et al., 2017).

When renal epithelial cells are energy-deprived, the $Na^+/K^+$ ATPase located at the basolateral membrane translocates to intracellular compartments (Mandel et al., 1994). Renal ischemia is known to be associated with energy depletion and is a main cause of acute kidney injury (AKI) that leads to high levels of morbidity and mortality (Brodie et al., 2005; Schrier et al., 2004). During AKI, the $Na^+/K^+$ ATPase detaches from the subcortical cytoskeleton of the plasma membrane. As a result, the cells lose their polarity and the kidney's functions are impaired (Gaudio et al., 1989; Molitoris et al., 1991; Riordan et al., 2005). Regain of cell polarity and relocalization of the $Na^+/K^+$ ATPase in the plasma membrane again are signs of recovery from renal ischemia (Why et al., 1999). Ischemia reperfusion injury was also associated with a decrease in the expression of the $Na^+/K^+$ ATPase (Gong et al., 2004).

### *2. Regulation*

The literature reports a regulatory role of hormones and intracellular second messengers of the $Na^+/K^+$ ATPase activity. Kinases and phosphatases phosphorylate/ dephosphorylate the pump, thus directly affect its activity or its trafficking to and from the plasma membrane (Pedemont et al., 2001; Suhail, 2010). The pump is also subject to long term regulation through de novo synthesis or degradation (Suhail, 2010).

The $Na^+/K^+$ ATPase also acts as a receptor for cardiac glycosides like digoxin, digitoxin, and ouabain. Their binding site is highly conserved and serves as a way to modulate blood pressure, treat cardiac arrhythmias and congestive heart failure (Suhail, 2010).

Renal $Na^+/K^+$ ATPase is controlled by hormones such as atrial natriuretic peptide, aldosterone and norepinephrine (Pedemont et al., 2001). Alpha-1 adrenergic receptors and angiotensin II stimulate the pump in kidney proximal tubules via activation of PKC. PKC's effect on the pump is mediated through phosphorylation of the N-terminal domain of the α sub-unit on several serine residues. In opossum kidney cells (used as a model to study the physiological functions of the kidney proximal tubules because it shows high similarity to humans in the expression of transporters and receptors (Handler, 1986; Nakai et al., 1988)), dopamine acting through its G-protein coupled receptor, activates PKC ζ which phosphorylates the $Na^+/K^+$ ATPase located in the plasma membrane on Ser-18 revealing a polyproline domain in the α sub-unit. This domain interacts with the SH3 domain of PI3K thus activating PI3K. As a result, adaptor protein-2 will bind to phosphorylated $Na^+/K^+$ ATPase molecules leading to their translocation to intracellular compartments via clathrin vesicles (Pedemont et al., 2001).

Phorbol 12-myristate 13-acetate (PMA) treatment activates another isoform of PKC, PKC β, which is able to activate the pump by phosphorylating Ser-11 and Ser-18 (Efendiev et al., 1999). PMA treatment increased the number of the $Na^+/K^+$ ATPase at the plasma membrane by inducing an interaction between adaptor protein-1 and the $Na^+/K^+$ ATPase leading to its translocation to the cellular membrane by clathrin-coated vesicles (Pedemont et al., 2001).

Different G-protein coupled receptors were shown to modulate the activity of the pump by affecting its translocation to the plasma membrane. β- 2 adrenergic receptor activation in alveolar epithelial cells activates Gs/cAMP/PKA in addition to Gi. This pathway stimulates RhoA/ROCK responsible for actin cytoskeleton organization leading to $Na^+/K^+$ ATPase exocytosis and increased activity (Lecuona et al., 2003).

Nitric oxide is an important modulator of the kidney functions. It regulates the fluid and solute homeostasis in normal conditions, and any disturbance in NO production and actions leads to pathophysiological conditions. Different studies showed an effect of NO on the activity of the $Na^+/K^+$ ATPase in many tissues (Liang et al., 1999). In rabbit aorta, a decrease in the release of NO inhibited the $Na^+/K^+$ ATPase (Gupta et al., 1992). In porcine cerebral cortex, different NO donors were able to decrease the activity of the $Na^+/K^+$ ATPase (Sato et al., 1997).

PKG is a well-established downstream effector of NO. In fact, NO stimulates guanylyl cyclase leading to cGMP production and activation of PKG. This pathway affects many intercellular messengers responsible for the regulation of the $Na^+/K^+$ ATPase in kidney tubules (McKee et al., 1994) and other tissues . PKG was shown to activate the $Na^+/K^+$ ATPase in striatum and cerebellum slices of rats and in proximal trachea (Munhoz et al., 2005; Scavone et al., 2000; Scavone et al., 2005).

Intracellular concentrations of calcium were shown to modulate the effect of PKA and PKC on the $Na^+/K^+$ ATPase. Hormones inducing an increase in intracellular calcium in addition to activation of PKA/PKC may lead to $Na^+/K^+$ ATPase stimulation, while others, activating PKA/PKC without increasing intracellular calcium might lead to an inhibition. This effect is thought to be mediated through calcium-dependent-intracellular pathways (Cheng et al., 1999). Calcium was involved also in the norepinephrine-induced stimulation of the $Na^+/K^+$ ATPase in rat kidney proximal tubule cells which resulted from a stimulation of calcineurin, a calcium/calmodulin dependent protein phosphatase (Aperia et al., 1992).

PGE2 is a known modulator of the $Na^+/K^+$ ATPase. It was shown to be the mediator in the interleukin-1- induced inhibition of the $Na^+/K^+$ ATPase in collecting duct cells (Zeidel et al., 1991). PGE2 is also a downstream effector of FTY720P and epinephrine which both had an inhibitory effect on the $Na^+/K^+$ ATPase in Caco-2 cells (El Moussawi et al., 2018; Rida et al., 2018).

**B. Sphingosine-1-phosphate**

Eukaryotic cells contain sphingolipids in their plasma membrane. Many sphingolipid metabolites such as ceramide, ceramide-1-phosphate, sphingosine and sphingosine-1-phosphate, function as signaling molecules (Chalfant et al., 2005; Cuvillier, 2002; Kihara et al., 2007; Pettus et al., 2002; Pyne et al., 2000). The involvement of sphingosine-1-phospate in signaling processes was well studied and revealed a key role in the vascular and immune system. Sphingosine kinases (SPHK1 and SPHK2) phosphorylate sphingosine to sphingosine-1-phosphate (S1P) (Kohama et al., 1998; Liu et al., 2000) and it can be dephosphorylated back into sphingosine by S1P-specific phosphatases or lipid-specific phosphatases (SPP1, SPP2, LPP1, LPP2 or LPP3). Another enzyme, a S1P lyase (SPL) can degrade S1P producing hexadecenal and phosphoethanolamine (Kihara et al., 2008). SPHK1 and

2 are found in the cytosol and in the plasma membrane. In addition, SPHK2 localizes in internal membranes and in the nucleus (Igarashi et al., 2003; Maceyka et al., 2005). SPL and SPP are localized in the endoplasmic reticulum enabling them to degrade intracellular S1P only (Aoki et al., 2005). S1P levels in the plasma are controlled by LPP since they are located in the plasma membrane and their active site faces the extracellular medium (Sigal et al., 2005).

S1P is produced by erythrocytes and platelets (Ito et al., 2007; Yatomi et al., 2000). Degradation of S1P occurs only in platelets due to a S1P phosphatase while no degradation occurs in erythrocytes (Ito et al., 2007; Pappu et al., 2007; Yatomi et al., 2000). S1P is transported outside the cell by two types of transporters: a stimuli-dependent and a stimuli-independent transporter. Erythrocytes possess only the stimuli –independent transporter allowing constitutive release of S1P (Hanel et al., 2007; Ito et al., 2007;Yang et al., 1999). The stimuli-dependent transporter present in platelets requires ATP and releases S1P upon PKC activation or stimulation by thrombin (Anada et al., 2007; Sano et al., 2002; Yatomi et al., 1995; Yatomi et al., 1997). Transporters are ATP-binding cassette (ABC) transporters such as ABCA, ABCC1 and ABCG2 (Mitra et al., 2006; Takabe et al., 2010) and sphingolipid transporter Spinster 2 (SPSN2) (Kawahara et al., 2009). Except for platelets and erythrocytes, intracellular levels of S1P are kept low due to the increased activity of the degrading enzymes compared to the synthesizing enzymes (Ito et al., 2007; Yatomi et al., 2000). In extracellular fluids, S1P levels are high; in plasma, where it is associated with lipoproteins or albumin (Aoki et al., 2005; Murata et al., 2000; Yatomi et al., 2000), its concentration ranges between 200 nM and 3μM while in lymph it reaches 500 nM (Hla, 2005; Pappu et al., 2007).

S1P has been identified as an intracellular second messenger and a receptor ligand for five cell surface receptors. Sphingosine-1-phosphate receptors (S1PR) are G-protein coupled receptors called endothelial differentiation gene (EDG) possessing very similar sequences. S1PR1 is coupled to Giα, S1PR2 and S1PR3 are coupled to G12/13α, Gqα, and Giα, while S1PR4 is coupled to Giα, and S1PR5 is coupled to Giα and G12α.

Gi/o may activate Ras, a small guanosine triphosphatase, and ERK, the extracellular signal-regulated kinase; it may also stimulate PI3K/Akt or PI3K/Rac signaling pathways; or may lead to the activation of PKC and phospholipase C (PLC) increasing the levels of free intracellular calcium; Gi/o may inhibit the activity of Adenylyl cyclase reducing the amount of cyclic adenosine monophosphate (cAMP) (Brinkmann, 2007).

Gq stimulates PLC while G12/13 activates Rho, a small GTPase and Rho-associated kinase (ROCK) (Brinkmann, 2007).

When S1P binds to its receptor, the α sub-unit of the G protein dissociates from the βγ sub-unit leading to the activation of different signaling pathways involved in the regulation of cell migration, proliferation, adherens junction assembly, actin cytoskeleton rearrangement (Kihara et al., 2008), cell survival and growth and lymphocyte trafficking (Spiegel et al., 2003). In mammals, S1PR1, S1PR2 and S1PR3 are ubiquitously expressed, S1PR4 is expressed in lungs and lymphoid tissues while S1PR5 is expressed in skin and brain (Glickman et al., 1999; Graler et al., 1998; Ishii et al., 2001).

S1P is responsible for many cellular responses in endothelial cells such as migration, expression of adhesion molecules, proliferation, NO synthesis and cytokine secretion (Kihara et al., 2007; Pyne et al., 2000; Spiegel et al., 2000; Taha et al., 2004). In addition, S1P is implicated in angiogenesis, endothelial permeability, inflammation, vascular tone and interaction with monocytes (Hla, 2004; Tolle et al., 2007). HDL's effect against atherosclerosis is suggested to be due to its linkage to S1P (Kimura et al., 2001; Nofer et al., 2004).

S1PR1 controls recirculation of lymphocytes. In fact, expression of S1PR1 on resting T and B cells is required for the egress of lymphocytes from lymph nodes. Lymphocytes lose their ability to leave the lymph nodes in case of downregulation or deletion of S1PR1 resulting in lymphopenia (Allende et al., 2004; Matloubian et al., 2004).

Acute kidney injury is a major consequence of IRI in kidney. It triggers epithelial to mesenchymal transition (EMT) in kidney canaliculus leading to chronic kidney injury because of fibrosis (Wang et al., 2018). Many kidney transplantations are followed by fibrosis and EMT (Manotham et al., 2004). S1PR1 was shown to have a protective effect on proximal canaliculus against IRI (Coelho et al., 2007) by inhibiting PI3K/Akt pathway thus limiting EMT (Wang et al., 2018).

Transforming growth factor (TGF) β activates epithelial cells of the proximal tubules in cell culture and triggers EMT (Liu, 2010). An increased expression of the fibrotic markers connective tissue growth factor (CTGF) and fibronectin was observed following down-regulation of SPHK-1 indicating a role for intracellular S1P in suppressing inflammation and fibrosis. Down-regulation of Spns2 in human renal proximal tubular epithelial cell line (HK2) increased S1P's intracellular levels. This increase reduced fibronectin and CTGF expression induced by TGF-β and enhanced aquaporin 1's expression, an important water channel in HK2 cells (Blanchard et al., 2018).

By binding to S1PR2, S1P activates Rho kinase which is known to be implicated in diabetic nephropathy, and promotes the transition of renal tubular epithelial cells into myofibroblasts (Ishizawa et al., 2014). An isoquinoline alkaloid called berberine was able to downregulate markers of fibrosis in mice models suffering from diabetic nephropathy through inhibiting SPHK-1/S1P's pathway (Lan et al., 2010). Upon treating the glomerular mesangial cells with high levels of glucose, an overexpression of SPHK-1 and S1P was observed similar to what is observed in diabetic kidney (Geoffroy et al., 2005). Many studies concerning renal fibrosis linked SPHK-1/S1P/S1PR and TGF-$\beta_1$ /Smad signaling pathways and suggested targeting S1P signaling pathway to limit chronic kidney diseases (Zhang et al., 2018).

Mice deficient in SPHK-2 showed a higher secretion of interferon-γ protecting them from kidney fibrosis (Bajwa et al., 2017). These mice were also protected from unilateral ureter-induced kidney fibrosis through up-regulation of Smad7 in kidneys (Schwalm et al., 2017). Since the levels of S1P in plasma (Zemann et al., 2006) and some cellular systems and organs (Liang et al., 2013; Schwalm et al., 2015) were reported to be elevated in case of SPHK-2 deficiency, it is suggested that intracellular and extracellular S1P is responsible for the protective effect in the kidneys. Inhibitors of histone deacetylases (HDAC) are capable of limiting renal fibrosis (Noh et al., 2009). HDACs are inhibited by S1P present in the nucleus suggesting a mechanism by which S1P protects from unilateral ureter-induced kidney fibrosis (Bajwa et al., 2017).

### C. FTY720P

A recently identified ligand for S1PRs is fingolimod (2-amino-2[2-(4-octylphenyl) ethyl]-1,3-propanediol) known also as FTY720 (Brinkmann et al., 2004; Brinkmann et al., 2002). FTY720 is derived from myriocin, a natural substance found in the fungus *Isaria sinclairii* (Fujita et al., 1994) and has a similar structure to sphingosine. In vivo, it can be phosphorylated into FTY720P by SPHK-2 while SPHK-1’s activity is very weak (Billich et al., 2003). Platelets only have the ability to produce FTY720P and release it mainly through the stimuli-independent transporter. Stimuli-dependent transporters also exist and are known as ABC transporters (Anada et al., 2007) and SPNS2 (Hisano et al., 2011).

FTY720P has a high affinity for S1PR1, S1PR4 and S1PR5 but a low affinity for S1PR3 (about 10-fold lower) (Albert et al., 2005; Brinkmann et al., 2002). Further studies demonstrated that FTY720P binds to S1PR2 (Sobel et al., 2013). Depending on the cell type and the type of S1PR, FTY720 may be a functional agonist or antagonist to S1P (Brinkmann, 2007). The effect depends on the association of the different receptors with the different lipid raft molecules in the plasma membrane (Chini et al., 2004) such as the GPCR-desensitizing arrestins (Marie et al., 2006) and the regulator of G-protein signaling (RGS) proteins known to be responsible for the suppression of Gi and Gq pathways. Depending on the cell type, different RGS are expressed yielding different cellular responses to S1P and FTY720P (Cho et al., 2003).

FTY720P is an effective drug against many auto-immune diseases (Brinkmann et al., 2002). It was approved by the Food and Drug administration (FDA) as a drug to treat relapsing-remitting multiple sclerosis (Brinkmann et al., 2010). FTY720P mainly acts by sequestering specific T-cells in the lymph nodes suppressing their ability to reach the specific tissues relevant for the disease such as disrupted areas of the blood-brain barrier (BBB), the brain and the spinal cord (Brinkmann, 2007). FTY720P, through S1PR1, was able to abolish inflammation and impairment of the neurological functions (Rausch et al., 2004) and prevent future relapses (H. Liu et al., 1999; Webb et al., 2004).

In addition to its beneficial effects on multiple sclerosis, FTY720P was shown to be protective against many kidney diseases. FTY720P decreased MCP-1 production by activating S1PR1 and attenuated the pathological signs of nephropathy in rats (Xu et al., 2014). In mice models suffering from tubular injury induced by cisplatin, FTY720P stabilized tubular mitochondrial function by binding to S1PR1 reducing thus acute kidney injury. Both effects were independent of the lymphopenia triggered by FTY720P (Bajwa et al., 2015). Furthermore, suppression of renal fibrosis was observed at the gene and protein level upon treatment of UUO-nude mice with FTY720P (Shiohira et al., 2013). Limiting endothelial dysfunction is another aspect by which FTY720P treats renal fibrosis through up-regulation of the expressions of eNOS, NO, CD31 and VEGF (Ni et al., 2013).

FTY720P is implicated in renal IRI as well. When it binds to S1PR1, FTY720P provides the proximal tubules with protection against IRI (Awad et al., 2006; Bajwa et al., 2010) by activating mitogen –activated protein kinase (MAPK/ERK) or Akt pathways that promote cell survival and inhibit apoptosis (Bajwa et al., 2010).

Activation of S1PR3 by FTY720P promotes renal regeneration following IRI by inducing the release of neutrophil gelatinase-associated lipocalin, a mediator of regeneration, from macrophages (Sola et al., 2011).

FTY720P exerts its different cellular responses via several downstream effectors such as NO, Rho kinase and PI3K. In fact, binding of FTY720P to S1PR3 expressed on endothelial cells activates eNOS/NO pathway and triggers dilation of mouse aorta pre-contracted by phenylephrine (Tolle et al., 2005). In addition, activation of S1PR2 by FTY720P in primary human lung myofibroblasts stimulated the Gα12/13/Rho/ROCK pathway leading to a notable contraction (Sobel et al., 2015). Moreover, FTY720P inhibits PI3K/Akt and down-regulates the mTOR pathway leading to inhibition of glioblastoma cells' migration and invasion (Zhang et al., 2008). FTY720P also reduces EMT in HK2 cells by down-regulating the PI3K/Akt pathway (Wang et al., 2018).

### D. Rho kinase

Rho GTPase is a downstream effector of S1PR2 and S1PR3 (Brinkmann, 2007).It is a subfamily of small GTP-binding proteins belonging to the Ras superfamily. They modulate vesicular trafficking between the cytosol and the plasma membrane by regulating the actin cytoskeleton (Croise et al., 2014; Ridley & Hall, 1992; Ridley et al., 1992). Rho is a member of the canonical group (Aspenstrom et al., 2007) that alternates between an inactive GDP-bound state and an active GTP-bound state using GEF, a guanine nucleotide exchange factor and GAP, a GTPase activating protein (Cherfils et al., 2013; Moon et al., 2003; Rossman et al., 2005).

Rho-kinase/ROCK/ROK acts downstream Rho GTPase (Leung et al., 1995). It is a serine/threonine kinase included in the ACG family comprising protein kinases. It has three structural domains: the N-terminal possessing a catalytic activity, a coiled-coil central domain and the C-terminal PH-domain including a cysteine-rich region. Rho-kinase is activated by binding of Rho to the Rho-binding domain (RBD) (Leung et al., 1995) in the C-terminal region of the coiled-coil. Two isoforms: Rho-kinaseα/ROCK2/ROKα and Rho-kinaseβ/ROCK1/ROKβ have been identified presenting 64% homology in their amino acid sequence and 83% similarity in their kinase domain (Amano et al., 2010). The activity of the kinase is modulated and increased by phosphorylation or auto-phosphorylation (Lee et al., 2010; Lowery et al., 2007). As a result, it phosphorylates downstream effectors to regulate different cellular functions (Amano et al., 2000; Fukata et al., 2001).

ROCK is mainly involved in controlling the dynamics of the actin cytoskeleton, generating actin-myosin contractility, regulating cell morphology and migration (Schofield et al., 2013), formation of stress fibers (Ridley et al., 1992), cytokinesis (Kishi et al., 1993), retraction of neurite (Jalink et al., 1994), formation of microvilli-like structures (Shaw et al., 1998), endocytosis and exocytosis (Croise et al., 2014).

It was also found to be implicated in many diseases such as cardiovascular diseases. Upon pressure overload, an increase in the cardiac ROCK1's activity down-regulates eNOS and increases the oxidative stress (Kobayashi et al., 2002; Mita et al., 2005). Inhibition of ROCK reduces cardiomyocyte's hypertrophy and apoptosis and limits fibrosis (Brown et al., 2006; Shi et al., 2010; Yang et al., 2012).

ROCK has multiple functions in the kidney: it controls the contraction of the renal arterioles, regulates the blood flow and filtration in the glomerulus. It also controls the function and the structure of the mesangial cells, the tubular cells and the podocytes (Hayashi et al., 2006). In a model of streptozotocin-induced diabetic kidney disease, a reduction in albuminuria was observed following ROCK1 deletion (Zhou et al., 2011). This protection was due to a decreased activation of TGF-β and decreased fibrosis (Kikuchi et al., 2007).

The role of the Rho pathway in renal injury was assessed using a sub-totally nephrectomized spontaneously hypertensive rats (SHR) where Rho-kinase was shown to be up-regulated. Following treatment with fasudil, a Rho-kinase inhibitor, renal morphologic damage and proteinuria were reduced. Fasudil was efficient in preventing renal injury in many models of hypertensive renal injuries through reduction of oxidative stress, expression of extracellular matrix genes, proteinuria and infiltration of macrophages (Ishikawa et al., 2006; Kanda et al., 2003; Nishikimi et al., 2004).

RhoA and ROCK play a role in transporter trafficking. They are activated by endothelin-1 (ET-1) whose levels are increased in kidney proximal tubule cells following acidosis. ET-1 induces the formation of stress fibers leading to increased trafficking of the $Na^+/H^+$ antiporter (NHE3) from a subapical pool to the apical membrane and an increase in its activity (Yang et al., 2007).

In addition to the $Na^+/H^+$ antiporter, the Rho pathway is involved in the translocation of the $Na^+/K^+$ ATPase into microvilli-like structures in renal epithelial cells (Maeda et al., 2002). The pump interacts with the cortical actin network, specifically ankyrin and spectrin stabilizing it in the basolateral membrane (Hammerton et al., 1991; Maeda et al., 2002). RhoA provides stability to the cortical actin meshwork localized at the apical and the basolateral membrane strengthening the association of the pump with it (Maeda et al., 2002).

Activation of the Rho pathway in alveolar epithelial cells leads to the translocation of the $Na^+/K^+$ ATPase to the plasma membrane and an increase in its activity (Lecuona et al., 2003).

**E. Phosphoinositide-3-kinase (PI3K)**

The PI3K family is a group of kinases capable of phosphorylating the inositol ring of phosphatidylinositol (PtdIns) at the 3'-hydroxyl group (Bilanges et al., 2019). These lipid kinases are associated with the plasma membrane; they are made up of three subunits: two regulatory subunits p85 and p55 and a catalytic subunit p110 (Donahue et al., 2012). Based on the structure and the substrate, this family is divided into three classes (Engelman et al., 2006; Katso et al., 2001). Class I PI3K are downstream effectors of cell-surface receptors such as hormonal receptors, tyrosine kinase-linked receptors and G-protein coupled receptors. They also signal downstream small GTPases such as Rac, Rho and the mutated Ras. They receive signals as well from several proteins such as Src homology 2 domain-containing protein tyrosine phosphatase 1 (SHP1), Src and PKC. Class II and III PI3K are regulators of membrane trafficking. In addition, PI3K possesses a scaffolding function where it stabilizes the proteins it is associated with (Bilanges et al., 2019; Hennessy et al., 2005). Activation of PI3K occurs in response to extracellular signals such as cytokines, hormones and growth factors (Guo et al., 2015) and results in phosphorylation of PtdIns(4,5)P2 (PIP2) producing PtdIns(3,4,5)P3 (PIP3). Acting as a second messenger, PIP3 recruits proteins containing several lipid-binding domains especially the pleckstrin-homology (PH) domain to the plasma membrane. Akt and PDK1 are known downstream effectors of PI3K, that localize to the plasma membrane upon activation and promote growth and survival pathways (Manning et al., 2007).

The PI3K/Akt/mTOR (mammalian target of rapamycin) pathway is implicated in many cellular processes, such as anabolic reactions, nutrient uptake, cell growth and survival, apoptosis and angiogenesis (Yu et al., 2016). Hyper activation of this pathway was demonstrated in most types of cancer (Thorpe et al., 2015; Vanhaesebroeck et al., 2010). Its role was also demonstrated throughout the embryonic development since it is necessary for the self-renewal of the pluripotent stem cells (PSC) and their differentiation (Yu et al., 2016).

In the kidneys, the PI3K/Akt pathway modulates mesangial cells' expansion (Kato et al., 2006; Wan-Xin et al., 2012; Zhang et al., 2013) and apoptosis of podocytes (Wang et al., 2014) inducing renal tubular injury (Hao et al., 2013; Zhao et al., 2012). It is activated by TGF-β1 and tumor necrosis factor-α (TNF-α) leading to diabetic nephropathy (Chung et al., 2015; Huang et al., 2016; Kato et al., 2006; Zhang et al., 2013). Qufengtongluo (QFTL) decoction was efficient in treating diabetic nephropathy through reduction of proteinuria and renal fibrosis. It acts by inhibiting TGF-β and activating PTEN, an inhibitor of the PI3K/Akt pathway (Huang et al., 2018).

U50448H, an agonist for the kappa-opioid receptor, was shown to have protective effects against renal IRI. Creatinine and the blood urea nitrogen (BUN) levels in the serum of renal IRI rat models were decreased upon treatment with this agonist along with a reduction in the scores of renal tubular injury and the apoptotic index. These effects were mediated through activation of eNOS and the PI3K/Akt pathway in the kidney tissues (Liu et al., 2018).

Sodium reabsorption by proximal tubule cells is affected by the concentration of albumin they are exposed to. Low levels of albumin in the filtrate, similar to the physiological conditions, activate PI3K/Akt and PKC leading to the inhibition of PKA. As a result, the expression of the α-1 subunit of the $Na^+/K^+$ ATPase increases along with an increase in its activity. Higher concentrations of albumin, seen in pathological conditions, inhibit the pathway resulting in a decreased expression of the α subunit of the pump in LLC-PK1 cells, a porcine proximal tubule cell line (Peruchetti et al., 2011).

The thyroid hormone T3 activates the $Na^+/K^+$ ATPase in alveolar epithelial cells of adult rats by increasing the expression of the α-1 and β-1 subunits. This activation involves stimulation of the Src family of tyrosine kinases and PI3K/Akt (Lei et al., 2004).

Leptin inhibits the $Na^+/K^+$ ATPase in the renal medulla through PI3K and this effect was shown to be dependent on the actin cytoskeleton's integrity (Beltowski et al., 2004).

Several studies demonstrated PI3K as a downstream effector of S1P/FTY720P. The PI3K/Akt pathway mediated the effect of S1P on endothelial progenitor cells causing an increase in proliferation and a decrease in apoptosis (Wang et al., 2018). PI3K was shown to be inhibited by FTY720P in unilateral ureteral obstruction-induced renal fibrosis model providing protection against renal fibrosis (Tian et al., 2017).

### F. Nitric oxide (NO) and Protein kinase G (PKG)

In mammals, NO is generated endogenously from L-arginine through the action of nitric oxide synthase (NOS). Three isoforms of NOS have been identified: the neuronal (nNOS), the endothelial (eNOS) and the inducible nitric oxide synthase (iNOS). The first two are constitutive, their actions are calcium-dependent and they produce low levels of NO, while iNOS is calcium-independent and is capable of generating big amounts of NO when stimulated by immunological stimuli such as inflammatory mediators and pathogen-associated molecular patterns (PAMPs) (Spiller et al., 2019).

NO activates soluble guanylyl cyclase by binding to its heme group. This enzyme converts guanosine triphosphate (GTP) into cyclic guanosine 3’,5’ monophosphate (cGMP) (Mergia et al., 2016) that in turn activates the c-GMP-dependent protein kinase (PKG) (Carvajal et al., 2000), a serine/threonine kinase (Francis et al., 1994). Activated PKG phosphorylates several downstream effectors modulating their activities (Lincoln et al., 1993).

NO has a wide variety of functions. In addition to its anti-inflammatory and antioxidant effects (Choudhari et al., 2013; Spiller et al., 2019), NO can form reactive nitrogen species (RNS) which induce chemical stress (Subapriya et al., 2002). Other products of NO such as $ONOO^-$ and ONOOH are implicated in cancer (Patel et al., 1999; Wink et al., 1998). NO regulates immune responses and mediates the cytotoxic effects of leukocytes. It promotes vasodilation by inducing smooth muscle relaxation and consequently it is known as the endothelial-derived relaxant factor (EDRF) (Bredt et al., 1992). Knowledge about its functions in the nervous system is rapidly expanding; NO acts as a neurotransmitter in the peripheral and central nervous system. In addition, it may modulate the release of other neurotransmitters, regulate morphogenesis of the nervous system, plasticity of the synapses as well as gene expression (Dawson et al., 1995).

PKG leads to vasodilation as it activates myosin light chain phosphatase (MLCP) that in turn dephosphorylates myosin light chains (MLC) (Gao et al., 2008). In fact, cGMP/PKG pathway is the main factor leading to the vasodilation of fetal and newborn lungs (Abraham et al., 2008; Gao et al., 1999; Gao et al., 2003; Gao et al., 2005). Increase in cGMP and activation of PKG in cardiomyocytes or in an intact heart reverses cardiac hypertrophy (Takimoto et al., 2005) and protects against IRI (Salloum et al., 2008; Salloum et al., 2009).

nNOS is activated upon muscle contraction (Silvagno et al., 1996) increasing NO synthesis (Balon et al., 1994). NO was reported to activate the $Na^+/K^+$ ATPase in glycolytic muscles (Juel, 2016) and cardiac myocytes (Pavlovic et al., 2013; William et al., 2005) by stimulating soluble guanylate cyclase that generates cGMP leading to PKG activation (Juel, 2016).

Studies have demonstrated the implication of iNOS in kidney damage since its up-regulation is a major factor contributing to renal injury (Gabbai et al., 2002).

However, eNOS demonstrated however, a protective role in the kidney since it prevents renal vasospasm and reduces the infiltration of inflammatory cells (Milsom et al., 2010).

Activation of the endothelin A receptor by ET-1 leads to vasoconstriction and vasospasm aggravating renal IRI. Production of NO and prostaglandin is induced by binding of ET-1 to the endothelin B receptor leading to vasodilation and reduction of renal IRI (Abraham et al., 2008). When NO production is disrupted, renal blood flow is reduced while vascular resistance increases along with ET-1 expression. High levels of ET-1 activate PKC and reduce NO synthesis by eNOS. In contrast, low levels of ET-1 protected the proximal tubules from IRI and reduced inflammation and oxidative stress indicating that high levels of NO can exert an inhibitory feedback on ET-1's expression (De Miguel et al., 2015; Li et al., 2019; Ramzy et al., 2006). Silencing receptor A using siRNA activated the PI3K/Akt pathway that increased NO synthesis by eNOS. NO ameliorated the kidney function and reduced the histological damage caused by IRI. In addition, NOS inhibition suppressed PKG activation and increased IRI renal damage by up-regulating inflammatory agents (Li et al., 2019).

Pirfenidone is an oral drug that induces NO production, and as such, it is used to treat idiopathic pulmonary fibrosis (King et al., 2014). It was also shown to be efficient in treating some models of renal injuries such as hypertension, subtotal nephrectomy and diabetic nephropathy. In fact, Pirfenidone was able to reduce interstitial fibrosis, macrophage infiltration, mitochondrial dysfunction and expansion of the mesangial matrix. Treating ischemic rats with this drug restored renal blood flow and $NO_2$/$NO_3$ excretion. The protective effect of Pirfenidone is mediated through NO since it was able to inhibit the down-regulation of eNOS (Lima-Posada et al., 2019).

NO can modulate the activity of the $Na^+/K^+$ ATPase and the $Na^+/H^+$ exchanger 3 in kidney tubules. Binding of Angiotensin-II to angiotensin type-2 receptors ($AT_2R$) recruits additional $AT_2Rs$ to the apical membrane of kidney proximal tubule cells, thus stimulating NO and cGMP production and activating PKG. As a result, the $Na^+/K^+$ ATPase and the $Na^+/H^+$ exchanger-3 are internalized and inactivated leading to vasodilation, natriuresis and lower blood pressure in hypertensive models (Carey, 2017).

Furthermore, NO mediates FTY720P's inhibition of the $Na^+/K^+$ ATPase in Caco-2 cells (Rida et al., 2018) and in HepG2 cells (Al Alam et al., 2017). PKG is a downstream effector of FTY720P in its protective role against IRI in the heart (Vessey et al., 2013).

**G. Calcium**

Calcium is a second messenger implicated in a variety of cellular responses such as gene expression, muscle contraction, cell death and survival, and metabolism regulation (Mammucari et al., 2018). Cytoplasmic $Ca^{2+}$ has two origins: extracellular and intracellular with respective concentrations of around 1mM and 100 µM. Changes in cytoplasmic [$Ca^{2+}$] is immediately detected by the cell by buffering proteins, enzymes, $Ca^{2+}$-binding signaling molecules, pumps and channels (Rizzuto et al., 2012). In fact, the cell invests a lot of energy to maintain $Ca^{2+}$ homeostasis through chelation, cell extrusion, and subcellular compartmentalization creating a $Ca^{2+}$ gradient across the intracellular stores and the plasma membrane. $Ca^{2+}$ enters the cells through various channels like voltage-operated calcium channels (VOCC), receptor-operated calcium channels (ROCC) and many others (De Stefani et al., 2016). Some channels like the inositol 1,4,5-triphosphate receptor (InsP3R) and the ryanodine receptor families (RYR) allow the release of $Ca^{2+}$ from intracellular stores such as the endoplasmic reticulum (ER) (Rizzuto et al., 2009). To terminate the signal, $Ca^{2+}$ is removed from the cytosol into the ER by the ER/SR $Ca^{2+}$ ATPase pump (SERCA) and into the extracellular medium by the plasma membrane $Ca^{2+}$ ATPase pump (PMCA). The $Na^{+}$ gradient has also a role in this process since $Ca^{2+}$ can be transported outside the cell by $Na^{+}/Ca^{2+}$ exchanger (NCX) and $Na^{+}/Ca^{2+}$-$K^{+}$ exchanger (NCKX) (De Stefani et al., 2016).

$Ca^{2+}$ is implicated in many cellular processes such as neurotransmitter exocytosis. In fact, $Ca^{2+}$ entry to neurons via VOCC allows liberation of neurotransmitters into the synapse thus contributing to the transmission of signals (Brini et al, 2014). Alterations in $Ca^{2+}$ signaling may result in diseases like cancer and cardiovascular problems. Disruption of $Ca^{2+}$ homeostasis affects the proliferation of cells and their resistance to apoptosis, tumor growth and metastasis (Marchi et al., 2016). In the cardiovascular system, a decrease in $Ca^{2+}$ transients during systole can lead to contractile dysfunction and eventually to chronic heart failure (Bers, 2006). However, $Ca^{2+}$ has cardio-protective effects when it acts along the NO/cGMP/PKG pathway. Clobenzorex’s acute application stimulates this pathway and induces vasodilation in rat aortic rings by opening $Ca^{2+}$-activated $K^{+}$ channels (Lozano-Cuenca et al., 2017).

Several studies showed a role of $Ca^{2+}$ in the modulation of the $Na^{+}/K^{+}$ ATPase. In porcine lens cells, it was behind the enhanced activity of the ATPase in response to hyposmotic stress. Exposure of cultured epithelial porcine lens cells to a hyposmotic solution, induced calcium entry via TRPV4 ion channels and connexin and pannexin hemichannels (Mandal et al., 2015; Shahidullah et al., 2012), and activation of two calcium-sensitive adenylyl cyclase, ADCY3 and ADCY8, leading to an increase in cAMP levels and PKA activity. PKA in turn activated Src family tyrosine kinase resulting in an increase in the $Na^{+}/K^{+}$ ATPase activity (Shahidullah et al., 2017).

In kidney cells, $Ca^{2+}$ was also a mediator of norepinephrine action on the $Na^{+}/K^{+}$ ATPase. Activation of the α-adrenergic receptors increases intracellular [$Ca^{2+}$] that in turn stimulates calcineurin, a calcium/calmodulin-dependent protein phosphatase. This enzyme was reported to dephosphorylate directly the $Na^{+}/K^{+}$ ATPase in vitro increasing its activity (Aperia et al., 1992; Bertorello et al., 1991) or indirectly by dephosphorylation and stimulation of protein phosphatase-1 (Abraham et al., 2008; Hemmings et al., 1984; Meister et al., 1989).

High levels of calcium may however, have deleterious effects on the kidney. An overload of $Ca^{2+}$ inside the cells activates calmodulin CaM leading to an increase in $Na^{+}/K^{+}$ ATPase activity and resulting in kidney damage (Xu et al., 2010).

The activity of the $Na^{+}/K^{+}$ ATPase may be affected by changes in the extracellular concentrations of certain ions such as $Ca^{2+}$ and $K^{+}$. In rat glomerulosa cells, elevated extracellular levels of these ions resulted in an increase in intracellular [$Ca^{2+}$] from 70 to ~300nM inducing a twofold activation of the $Na^{+}/K^{+}$

ATPase. This effect is thought to be mediated by calmodulin (CaM) and/or Ca/CaM kinase II (CaMKII) (Yingst et al., 2001).

$Ca^{2+}$ can also affect the expression of the $Na^{+}/K^{+}$ ATPase. This was observed in alveolar epithelial cells subject to hypoxia where increased [$Ca^{2+}$] induced endocytosis of the pump. Hypoxia generates an increase in intracellular [$Ca^{2+}$] by opening calcium release-activated calcium channels (CRAC) in response to mitochondrial reactive oxygen species (ROS). Subsequently, activated CaMKKβ phosphorylates the AMP-activated protein kinase (AMPK) inducing the endocytosis of the $Na^{+}/K^{+}$ ATPase by PKCζ (Gusarova et al., 2011).

An increase in the intracellular [$Ca^{2+}$] stimulates the expression of the α-1 subunit of the $Na^{+}/K^{+}$ ATPase via calcineurin and CaMK in the outer medullary kidney tubular suspensions and primary skeletal muscle of rats (Nordsborg et al., 2010; Rayson, 1991).

In addition, many studies reported $Ca^{2+}$ as a downstream effector of S1P in various cells. In rat aortic vascular smooth muscle cells and HEK293 cells, the HDL induced elevated intracellular calcium levels were mediated by S1P via S1PR1 and S1PR3 (Lee et al., 2017). In Hela cells, overexpression of S1PR2, and S1PR3 decreased the quantity of $Ca^{2+}$ stored in the ER when S1P was constantly present in cardiac myocytes of neonatal rats, binding of S1P to S1PR1 resulted in $Ca^{2+}$ overload (Nakajima et al., 2000) with expected repercussions on cardiac functions. In neonatal mice heart, S1P protected from IRI through activation of CaMKII-PLB and SERCA2 (Yan et al., 2014). S1P controls also tonic contraction of iliac lymph vessels in mice by binding to S1PR2 and subsequently releasing $Ca^{2+}$ from intracellular stores (Kimizuka et al., 2013).

**H. Protein kinase A (PKA) and protein kinase C (PKC)**

PKA and PKC are both downstream effectors of S1P. S1PRs coupled to Gi/o mainly signal by inhibiting AC thus decreasing the intracellular levels of cAMP and subsequently inhibiting PKA (Brinkmann, 2007). PKA is a serine/threonine kinase (Lefkimmiatis et al., 2014) made of two regulatory and two catalytic subunits. Binding of cAMP to the regulatory subunits induces their dissociation relieving thus the catalytic units from their inhibitory effect. Two classes of PKA have been identified: PKA I and PKA II with differences in their regulatory subunits (Cheng et al., 2008).

S1PRs that signal through Gi/o or Gq activate PKC (Brinkmann, 2007), a serine/threonine kinase made of a N-terminal regulatory domain and a C-terminal catalytic domain. PKC isoforms are divided into three groups based on their structure and the substrate: the conventional PKCs (α, βI, βII, γ) whose activation is dependent on calcium, phosphatidylserine (PS) and diacylglycerol (DAG); novel PKCs (δ, ε, η, θ) whose activation requires DAG and PS but not calcium and atypical PKCs (ζ, ι/λ) whose stimulation depends only on PS (Way et al., 2000).

PKA plays a role in the regulation of kidney functions. It is involved in the progression of renal diseases such as diabetic nephropathy. High glucose levels activate PKA which inhibits glucose-6-phosphate dehydrogenase (G6PD) and reduces NADPH's production, increasing oxidative stress in the kidneys leading to diabetic nephropathy (Xu et al., 2005).

PKA has the ability to modulate the activity of the $Na^+/K^+$ ATPase in many tissues. Activated PKA phosphorylates the α sub-unit of the ATPase and reduces its activity (Bertorello et al., 1991; Meister et al., 1989). A specific isoform of PKA, PKA Iα, induces endocytosis of the $Na^+/K^+$ ATPase following its activation during hypercapnia in alveolar epithelial cells (Lecuona et al., 2013).

However, under different conditions, PKA induces translocation of the $Na^+/K^+$ ATPase to the cell membrane in proximal tubule cells of rats increasing its activity (Carranza et al., 1998). A similar process is observed in lung epithelial cells in response to catecholamines that activate β-adrenergic receptors (Bertorello et al., 1999).

PKC is also involved in kidney diseases. It is activated during hyperglycemia and contributes to the progression of the diabetic kidney disease. PKC maintains in addition the extracellular matrix and regulates endothelial permeability, cell growth, vasoconstriction, angiogenesis, leukocyte adhesion and cytokine secretion (Noh et al., 2007). It induces the expression of TGF-β and increases the build-up of extracellular matrix protein leading to interstitial fibrosis (Koya et al., 1997).

PKC also regulates the $Na^+/K^+$ ATPase's activity. Activation of the D1 receptor in response to dopamine activates PKC β that triggers the translocation of the $Na^+/K^+$ ATPase from late endosomes into the basolateral membrane of alveolar epithelial cells thus increasing its activity (Ridge et al., 2002). However, endocytosis of the pump occurs in response to phosphorylation of the α-subunit by a different isoform of PKC, PKC ζ. This isoform is activated when dopamine acts via its G-protein coupled receptor or in response to ROS generated following hypoxia (Abraham et al., 2008; Chibalin et al., 1999; Chibalin et al., 1998; Khundmiri et al., 2004).

Both PKA and PKC mediate the inhibitory effect of the parathyroid hormone (PTH) on the $Na^+/K^+$ ATPase in opossum proximal tubule cells. PKCα and PKA are responsible respectively for the short-term and the long-term inhibition of the pump (Khundmiri et al., 2002). In fact, endocytosis of the $Na^+/K^+$ ATPase occurs in response to the phosphorylation of its α sub-unit by PKCα (Khundmiri et al., 2008).

**I. Prostaglandin E2 (PGE2)**

Prostaglandins are synthetized and secreted in almost all cell types in a constitutive way and following a specific trauma, stimulation or in response to signaling molecules (Berenbaum, 2000; Funk, 2001; Smith, 1989). PGE2, the most available prostaglandin in humans, is produced by Cyclooxygenase (COX) enzymes that transform the arachidonic acid into prostaglandins, and transported outside the cell where it acts as a ligand for four G-protein coupled receptors known as EP receptors (EP1-4) (Kawahara et al, 2015; Serhan et al., 2003). PGE2 is involved in homeostasis (Leslie, 2004) , inflammation (Davieset al., 1984) and a major contributor to arthritic diseases like rheumatoid (Bombardieri et al., 1981) and ostheoarthritis (Amin et al., 1997).

In the kidneys, PGE2 controls water and salt balance in addition to hemodynamics (Norregaard et al., 2015). In fact, an increase in baseline systolic blood pressure is observed when EP2 is deleted leading to hypertension and kidney injury (Kennedy et al., 1999). Elevated expressions of EP2 and EP4 were observed in renal fibrogenesis indicating a putative protective function of PGE2 in renal fibrosis (Nakagawa et al., 2012; Nilsson et al., 2015). Actually, butaprost, an EP2 agonist was able to reduce the expression of fibrotic markers such as fibronectin induced by TGF-β and Smad2 phosphorylation thus limiting EMT in Madin-Darby canine kidney (MDCK) cells (a model of distal tubule cells). Butaprost also demonstrated an anti-fibrotic effect on unilateral ureteral obstructed mice and on human kidney slices exposed to TGF-β (Jensen et al., 2019). However, COX-2/Prostaglandin is also known to contribute to renal injury (Nilsson et al., 2015; Norregaard et al., 2015;Yang et al., 2015). An EP1 antagonist was able to reduce the expression of fibronectin in proximal tubule cells of mice (Thibodeau et al., 2013).

Several studies identified PGE2 as a mediator of S1P and FTY720P in their effect on the $Na^+/K^+$ ATPase in different types of cells. In HepG2 cells, PGE2 mimicked FTY720P's inhibition of the pump via PKC and NF-κB (Al Alam et al., 2016). PGE2 also acted downstream FTY720P and S1PR2 and inhibited the pump in Caco-2 cells by binding to EP3 and increasing NO production (Rida et al., 2018).

In Caco-2 cells, PGE2 release was also induced by binding of epinephrine to α-2 adrenergic receptors leading to an inhibition of the $Na^+/K^+$ ATPase (El Moussawi et al., 2018). Moreover, PGE2 mediated the effect of TNF-α in HepG2 cells where it decreased the expression of the α-1 subunit of the $Na^+/K^+$ ATPase and reduced its activity by acting on EP2 (Kreydiyyeh et al., 2007). PGE2 induced a similar effect on the pump in response to TNF-α in cultured Caco-2 cells as well as in rat distal colon (Markossian et al., 2005). PGE2 was also the mediator of the Interleukin-β inhibitory effect on the the $Na^+/K^+$ ATPase in LLC-PK1 (Kreydiyyeh et al., 2004). It was reported to exert also an inhibitory on the renal $Na^+/K^+$ ATPase in the inner medullary collecting duct (Jabs et al., 1989) and in MDCK cells (Cohen-Luria et al., 1994).

# CHAPTER III

# MATERIALS AND METHODS

## A. Materials

FTY720P, anti-EDG 1,3,5,6,8 rabbit polyclonal antibodies, p-Akt1/2/3 (Ser473)-R rabbit polyclonal antibody, Akt1/2/3 (H-136) rabbit polycolonal antibody, goat anti-mouse horseradish peroxidase (HRP) conjugated IgG, anti-GAPDH mouse monoclonal antibody, KT5823, carboxy-PTIO, glycol-SNAP-1, 8-bromo-cGMP were purchased from Santa Cruz Biotechnology, CA, USA.

Goat anti-rabbit horseradish peroxidase (HRP) conjugated IgG and the protein ladder were purchased from Abcam, Cambridge, UK.

(R)-3-Amino-(3-heylphenylamino)-4-oxobutylphosphonic acid (TFA salt, W146) was purchased from Avanti Polar Lipids, Inc.,Alabaster, Alabama.

SEW 2871, CYM 5520, CYM 5541, JTE-013 and sterile dimethyl sulfoxide were procured from TOCRIS Bioscience, Bristol, UK.

CA10444 was obtained from Cayman Chemical Company, Michigan, USA.

Y-27632 was procured from Cell Signaling Technology, Danvers, USA.

Phorbol-12-myristate-13-acetate (PMA), Rp-Adenosine 3', 5'-cyclic monophosphorothioate triethylammonium salt (RpcAMP), Calphostin C, Wortmannin and BAPTA/AM were procured from Calbiochem, San Diego, USA.

Pyrophosphate and sodium fluoride were purchased from Mallinckrodt, Staines-upon-Thames, UK.

Iron (II) sulfate, trichloroacetic acid and ponceau S were procured from ACROS organics.

Biorad assay and protein reagent, nitrocellulose membranes, clarity western ECL substrate, TEMED, Tween 20, sodium dodecyl sulfate (SDS), glycine, Tris, ammonium persulfate (APS) and 30% acrylamide /Bis solution were purchased from Bio-rad, California, USA.

Potassium dihydrogen phosphate ($KH_2PO_4$) and saponin were obtained from Merck, New Jersey, USA.

Potassium chloride was purchased from Fisher Scientific Company, New Hampshire, USA.

Magnesium chloride was procured from HiMedia laboratories.

Methanol was obtained from VWR International Company, Pennsylvania, USA.

Porcine kidney cells, LLC-PK1 were purchased from American Type Culture Collection (ATCC).

All other chemicals were obtained from Sigma, Chemical Co, St Louis Missouri, USA.

## B. Methods

### 1. *Culture and treatment of LLC-PK1 cells*

LLC-PK1 cells at passages ranging between 203 and 250 were seeded in 6-well plates, grown in DMEM to which were added 10% FBS and 1% penicillin (100µg/mL) in a humidified incubator (95% $O_2$, 5% $CO_2$) at 37°C. Cells were treated with FTY720P at 85%-90% confluence and after an overnight starvation.

An equal amount of the vehicle was always added to the control group in each treatment.

### 2. *Dose response study*

After an overnight starvation, LLC-PK1 cells were treated with different concentrations of FTY720P ranging from 0 to 750 nM for 15 minutes. An equal amount of the vehicle DMSO was added to the control group. The cells were then washed with PBS buffer (pH=7.4), scraped using lysis buffer (9.9 mL of 150 mM histidine buffer pH=7.4, 400 µL protease inhibitor (1 tablet in 2 mL $H_2O$), 100 µL Triton-X (1mg/mL $H_2O$)), homogenized for 20 seconds in a PRO homogenizer at a maximal speed (30,000 rpm) and centrifuged for 30 minutes at 35000 g, 4°C. Proteins in the supernatant were quantified according to the Bradford method. The obtained samples were used to measure the activity of the $Na^+/K^+$ ATPase or for western blot analysis.

**3. *Time response study***

LLC-PK1 cells were treated after an overnight starvation with FTY720P at a concentration of 80 nM for different time periods ranging from 0 to 4 hours. The cells were then collected and treated as described above.

**4. *Type of S1PR involved in the signaling pathway of FTY720P***

To determine the type of S1PR mediating FTY720P's effect on the $Na^+/K^+$ ATPase, S1PR1, S1PR2 and S1PR3 were individually blocked with their respective antagonists: W146 (10 µM in NaOH), JTE-013 (1 µM in DMSO) and CAY-10444 (17.4 µM in DMF). The blockers were added 30 minutes before adding FTY720P (80 nM) for another 15 minutes.

To confirm the type of S1PR involved, cells were also individually treated for 15 minutes with the respective agonists of S1PR1, S1PR2 and S1PR3: SEW2871 (100 nM in DMSO), CYM5520 (2.5 µM in DMSO), CYM5541 (2 µM in DMSO.)

**5. *Signaling pathway***

a. Involvement of PKA

To investigate if S1PR is acting via Gi/o, the involvement of PKA was studied by treating the cells with a PKA inhibitor, RpcAMP (30 µM in $H_2O$), 30 minutes prior to the addition of FTY720P or with a cell permeable cAMP analogue, dibutyryl-cAMP (dbcAMP: 10 µM in $H_2O$) for 15 minutes at 37°C.

b. Involvement of PKC

The involvement of PKC was tested to determine if S1PR is acting via Gq. The cells were pre-treated for 30 minutes with a PKC inhibitor, Calphostin C (50 nM in DMSO) and then incubated with FTY720P for an additional 15 minutes. In addition, the effect of phorbol 12-myristate 13-acetate (PMA: 100 nM in DMSO), a PKC activator was investigated by adding it to the cells for 15 minutes.

c. Involvement of Rho kinase

Since S1PR may be coupled also to G12/13, Rho kinase could be a possible mediator of FTY720P. To test for its involvement, cells were treated with a Rho kinase inhibitor, Y-27632 (10 µM in DMSO) for 3 hours prior to the addition of FTY720P.

d. Involvement of PGE2

Cells were treated at 37°C with indomethacin (100 µM in DMSO), a COX enzyme inhibitor, 30 minutes before adding FTY720P. Furthermore, the effect of exogenous PGE2 (1 nM in alcohol; 15 min) on the $Na^+/K^+$ ATPase was studied.

e. Involvement of PI3K

PI3K is known to modulate the activity of the $Na^+/K^+$ ATPase. Its activation downstream resolvin D1 activated the $Na^+/K^+$ ATPase and promoted alveolar fluid clearance (Wang et al., 2014). Therefore, the involvement of PI3K was tested. Cells were incubated with a PI3K inhibitor, wortmannin (100 nM in DMSO) for 30 minutes at 37°C before adding FTY720P.

f. Position of PI3K relative to Rho kinase

To position Rho kinase and PI3K, cells were treated with Y-27632 (10 µM in DMSO), a Rho kinase inhibitor for 3 hours before adding FTY720P for an additional 15 minutes.

g. Involvement of NO

The involvement of NO was investigated by treating the cells with carboxy-PTIO, a NO scavenger (30µM in $H_2O$) for 30 minutes before the treatment with FTY720P.

h. Position of NO relative to PI3K

To locate NO relative to PI3K, the cells were treated for 15 minutes with glyco-SNAP-1 (4 µM in DMSO), a NO donor, in the presence or absence of wortmannin (100 nM in DMSO), a PI3K inhibitor added 30 minutes before glycol-SNAP-1.

i. Involvement of calcium

Cells were incubated with a $Ca^{2+}$ chelator, BAPTA-AM (20 nM in DMSO) for 30 minutes at 37°C prior to the treatment with FTY720P.

j. Position of calcium relative to NO

Positioning calcium with respect to NO was done by treating the cells with a NO donor, glyco-SNAP-1 (4 µM in DMSO) for 15 minutes in presence of a $Ca^{2+}$ chelator, BAPTA-AM (20 nM in DMSO) added 30 minutes earlier.

k. Involvement of PKG

To determine if PKG is involved downstream FTY720P, KT-5823 (2.34 µM in DMSO), a PKG inhibitor, was added to the cells 30 minutes before treating with FTY720P.

l. Position of PKG relative to calcium

To position PKG relative to calcium, cells were treated with a cell permeable cGMP analogue, 8-bromo-cGMP (0.5 mM in DMSO) in presence and absence of a $Ca^{2+}$ chelator, BAPTA-AM (20 nM in DMSO) added 30 minutes earlier.

### 6. *The $Na^+/K^+$ ATPase activity assay*

Cells were collected, washed, homogenized and spun as described before and the supernatant obtained was used to assay for the $Na^+/K^+$ ATPase activity. The protein concentration of each sample was adjusted to 0.5 µg/µL by addition of histidine buffer. 17 µL of 2% saponin were added to 65 µL of the homogenate, and the mixture was incubated for 15 minutes at room temperature, followed by another incubation for 15 minutes with 13 µL of a phosphatase inhibitor cocktail (500 µL pyrophosphate (200 mM), 500 µL glycerophosphate (200 mM) and 400 µL $H_2O$).

12 µL from each sample were added to a buffer whose composition is shown in the table below in presence or absence of ouabain.

Table 1: Composition of the buffer solution.

| Reagent | Volume | Volume |
|---|---|---|
| **NaCl (1240 mM)** | 10 µL | 10 µL |
| **KCl (200 mM)** | 10 µL | 10 µL |
| **$MgCl_2$ (40mM)** | 10 µL | 10 µL |
| **Histidine Buffer** | 42 µL | 42 µL |
| **ATP (30mM)** | 10 µL | 10 µL |
| **Ouabain (1Mm)** | 8 µL | 0 µL |
| **H2O** | 0 µL | 8 µL |

After a 15-minute incubation at 37°C, 10 µL of 50% trichloroacetic acid was added to the mixture to stop the reaction. Samples were then centrifuged for 5 minutes at a speed of 16.163 g. Ninety µL of the supernatant were added to 80 µL of ferrous sulfate-molybdate reagent (0.5 mg ferrous sulfate, 1 mL ammonium molybdate (100 g/L in 10N $H_2SO_4$), 9 mL $H_2O$) generating a blue color that indicates the amount of inorganic phosphate liberated which was quantified at a wavelength of 750 nM.

## 7. *Western Blot analysis*

Forty µg proteins of each sample were loaded and run on a 10% SDS polyacrylamide gel (109 mL of 30% polyacrylamide, 100 mL Tris-HCl (1.5 M, pH=8.8), 4 mL 10% SDS, 216 mL $H_2O$), and transferred to a nitrocellulose membrane that was next, incubated for 40 minutes with a blocking buffer (1 L of 1x PBS, 1 mL of Tween 20, 30 g skimmed milk/ 30g BSA, 10 mL 0.1% sodium azide). The membranes were incubated overnight at 4°C with a specific primary antibody to p-Akt1/2/3 (Ser473)-R or Akt1/2/3 (H-136) or to each of the various receptors: anti-EDG1 (S1PR1), anti-EDG5 (S1PR2), anti-EDG3 (S1PR3), anti-EDG6 (S1PR4), anti-EDG8 (S1PR5). The incubation was followed by 4 washes, 10 minutes each, with a washing buffer (900 mL $H_2O$, 100 mL 10x PBS, 1 mL Tween 20). The membranes were then incubated with Goat anti-rabbit HRP conjugated IgG secondary antibodies for 1 hour at room temperature followed by another 4 washes. 1 mL luminol Clarity ECL substrate was added to each membrane and the signal was detected using a ChemiDoc$^{TM}$MP. imaging system.

### 8. ***Statistical analysis***

Statistical significance of the data was tested by a one-way analysis of variance followed by a Tukey-Kramer multiple comparison test using GraphPad InStat 3. The results are reported as mean ± SEM.

# CHAPTER IV

# RESULTS

## A. Dose response study

In order to determine the effect of FTY720P on kidney proximal convoluted tubules, a porcine kidney proximal tubule cell line, LLC-PK1, was chosen because it possesses several properties similar to humans (Handler, 1986). LLC-PK1 cells treated for 15 minutes with different concentrations of FTY720P showed an increase in the activity of the $Na^+/K^+$ ATPase with the highest effect observed at 80 nM (figure 2).

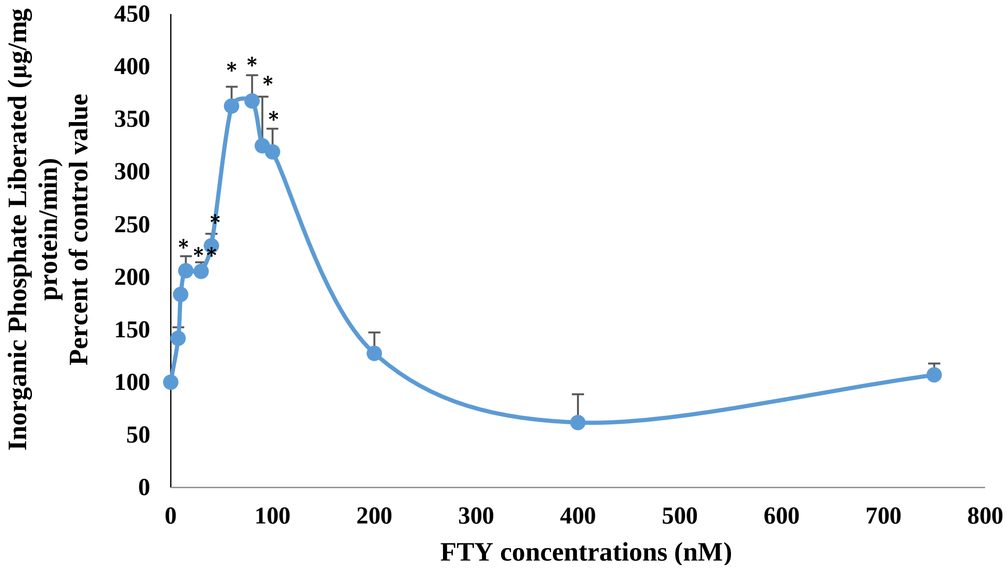


Figure 2: FTY720P applied for 15 minutes increased dose dependently the activity of the $Na^+/K^+$ ATPase in LLC-PK1. Values are means ±SEM of 3 observations. * significantly different from the control at P<0.01, ** significantly different from the control at P<0.05 as indicated by ANOVA followed by Tukey Kramer test.

**B. Time response study**

FTY720P (80nM) was applied for different time periods and exerted a maximal activation of the $Na^{+}/K^{+}$ ATPase at 15 minutes (figure 3).

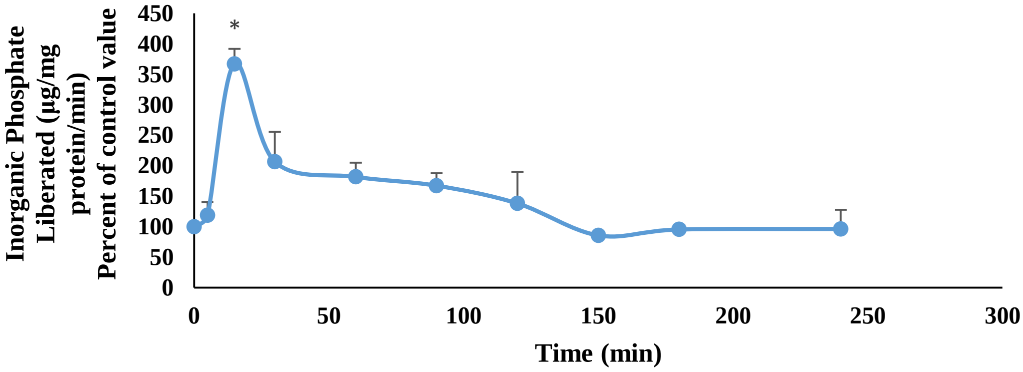


Figure 3: FTY720P (80 nM) applied for 15 minutes stimulates in a time dependent manner the $Na^{+}/K^{+}$ ATPase. Values are means ± SEM of 3 observations. * significantly different from the control at P<0.01, as indicated by ANOVA followed by Tukey Kramer test.

**C. S1P receptors expressed in LLC-PK1 cells**

Since FTY720P exerts its effects by binding to one of the S1PRs, the type of S1P receptors expressed in LLC-PK1 was determined using Western Blot analysis. All S1PRs were found to be expressed. The highest abundance was for S1PR4 (figure 4).

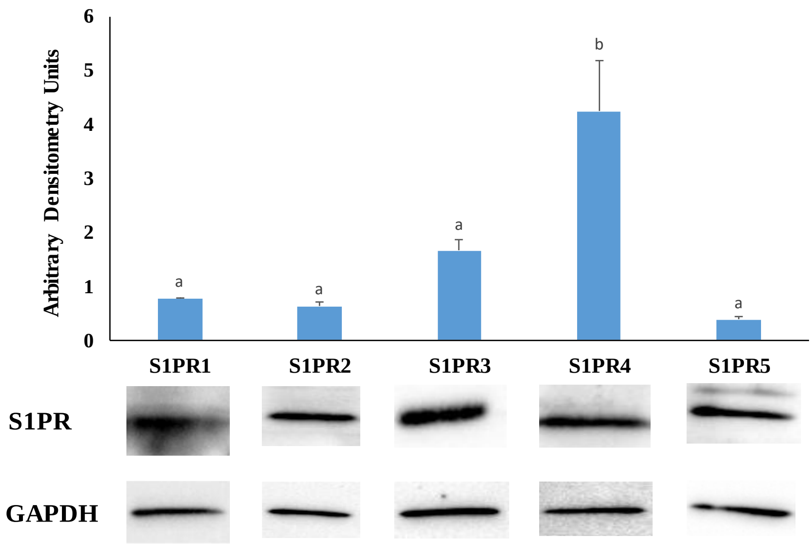


Figure 4: The expression of S1PRs in LLC-PK1 (40µg protein loaded in each lane). Values are means ± SEM of 3 observations. The expression of S1PR4 is significantly higher than that of S1PR1, S1PR2 and S1PR5 at P<0.01, while it is significantly higher than that of S1PR3 at P<0.05, as indicated by ANOVA followed by Tukey Kramer test.

**D. Type of S1PR involved in the signaling pathway of FTY720P:**

Blocking S1PR1 with W146 (figure 5), and S1PR3 with CAY-10444 (figure 6), didn't eliminate FTY720P's effect of the $Na^+/K^+$ ATPase. In addition, neither SEW2871, a S1PR1 agonist (figure 7) nor CYM5541, a S1PR3 agonist (figure 8) exerted any effect on the activity of the ATPase.

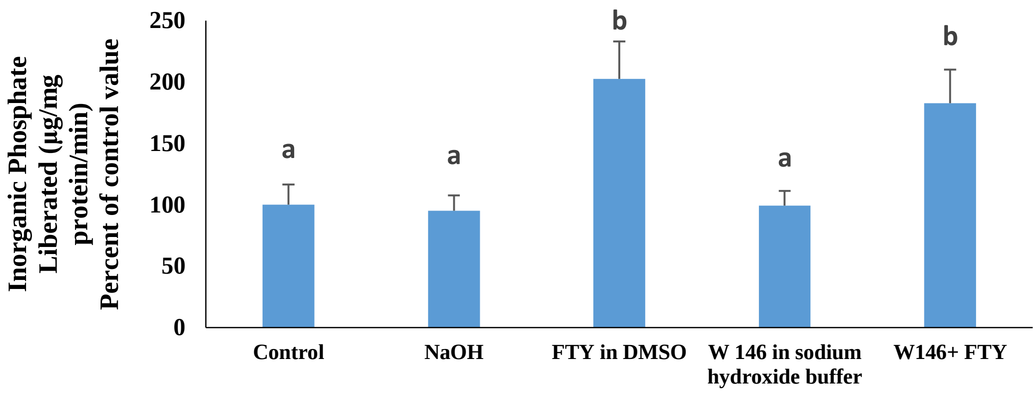


Figure 5: Effect of FTY720P (80 nM, 15 minutes) on the $Na^+/K^+$ ATPase activity in presence of W146 (10 µM in NaOH), a S1PR1 antagonist. An equal volume of the vehicle(s) was added to the control. Values are means ± SEM of 3 observations. Bars not sharing the same letter are considered significantly different from each other at P<0.01, as indicated by ANOVA followed by Tukey Kramer test. (FTY=FTY720P)

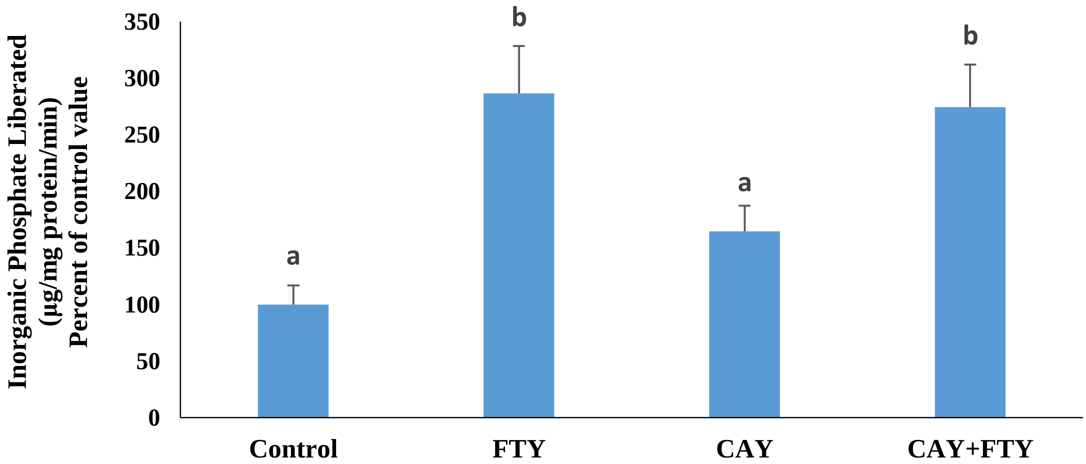


Figure 6: Effect of FTY720P (80 nM, 15 minutes) on the Na+/K+ ATPase activity in in presence of Cay-10444 (17.4 µM in DMF), a S1PR3 antagonist. An equal volume of the vehicle(s) was added to the control. Values are means ± SEM of 3 observations. Bars not sharing the same letter are considered significantly different from each other at P<0.001, as indicated by ANOVA followed by Tukey Kramer test. (FTY=FTY720P, CAY=CAY-10444)

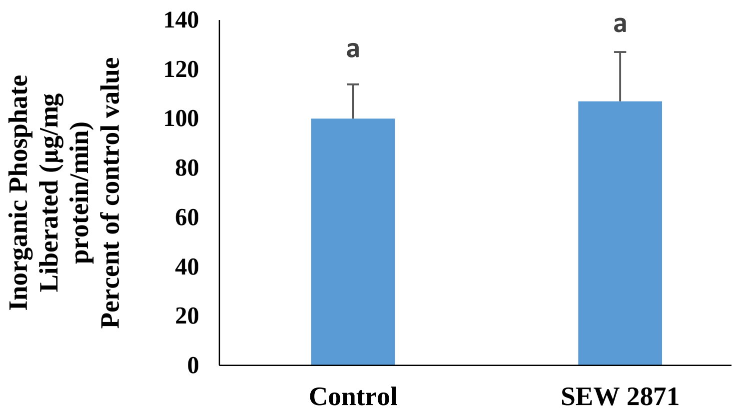


Figure 7: Effect of SEW2871 (100 nM, 15 mins), a S1PR1 agonist on the activity of $Na^{+}/K^{+}$ ATPase. An equal volume of the vehicle(s) was added to the control. Values are means ± SEM of 3 observations. Bars sharing the same letter are not considered significantly different from each other as indicated by ANOVA.

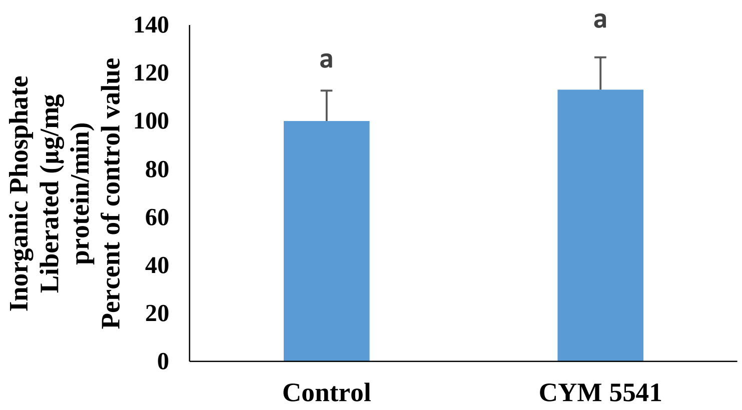


Figure 8: Effect of CYM5541 (2µM, 15 mins), a S1PR3 agonist on the activity of the$Na^{+}/K^{+}$ ATPase. An equal volume of the vehicle(s) was added to the control. Values are means ± SEM of 3 observations. Bars sharing the same letter are not considered significantly different from each other as indicated by ANOVA.

In presence of JTE-013, a S1PR2 antagonist, FTY720P's activation of the pump wasn't observed anymore (figure 9). CYM5520, a S1PR2 agonist induced a significant increase in the pump's activity (figure 10).

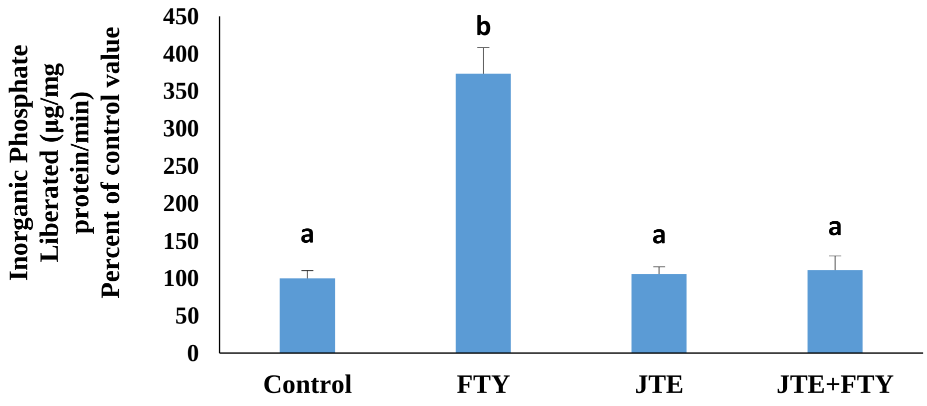


Figure 9: Effect of FTY720P (80 nM, 15 minutes) on the $Na^{+}/K^{+}$ ATPase activity in presence of JTE-013 (1 µM in DMSO), a S1PR2 antagonist. An equal volume of the vehicle(s) was added to the control. Values are means ± SEM of 3 observations. Bars not sharing the same letter are considered significantly different from each other at $P<0.001$, as indicated by ANOVA followed by Tukey Kramer test. (FTY=FTY720P, JTE=JTE-013)

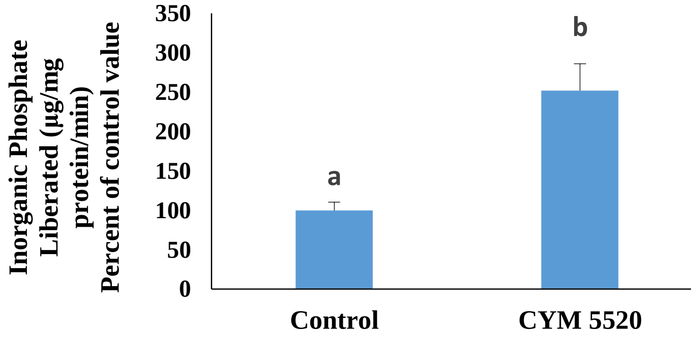


Figure 10: Effect of CYM5520 (2.5 µM, 15 mins), a S1PR2 agonist, on the activity of the $Na^{+}/K^{+}$ ATPase. An equal volume of the vehicle(s) was added to the control. Values are means ± SEM of 3 observations. Bars not sharing the same letter are considered significantly different from each other at $P<0.001$, as indicated by ANOVA followed by Tukey Kramer test.

**E. Mediators of FTY720P's effect on the $Na^+/K^+$ ATPase**

S1PR2 is coupled to Gi/o, Gq and G12/13. Gi/o inhibits adenylyl cyclase decreasing the levels of cAMP and consequently inhibiting PKA, Gq activates PKC while G12/13 acts via Rho kinase.

The involvement of PKA was studied using a PKA inhibitor, RpcAMP, and a cell permeable analogue of cAMP, dibutyryl-cAMP (dbcAMP). RpcAMP did not mimick the effect of FTY720P on the pump (figure 11). Moreover, the activation of the pump didn't disappear in presence of dbcAMP (figure 12).

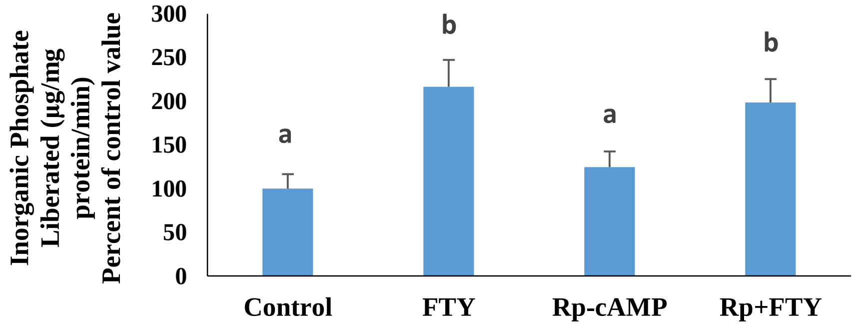


Figure 11: Effect of FTY720P (80nM, 15 minutes) on the $Na^+/K^+$ ATPase activity in presence of RpcAMP (30 µM in $H_2O$), a PKA inhibitor. An equal volume of the vehicle(s) was added to the control. Values are means ± SEM of 3 observations. Bars not sharing the same letter are considered significantly different from each other at $P<0.001$, as indicated by ANOVA followed by Tukey Kramer test. (FTY=FTY720P, Rp=RpcAMP)

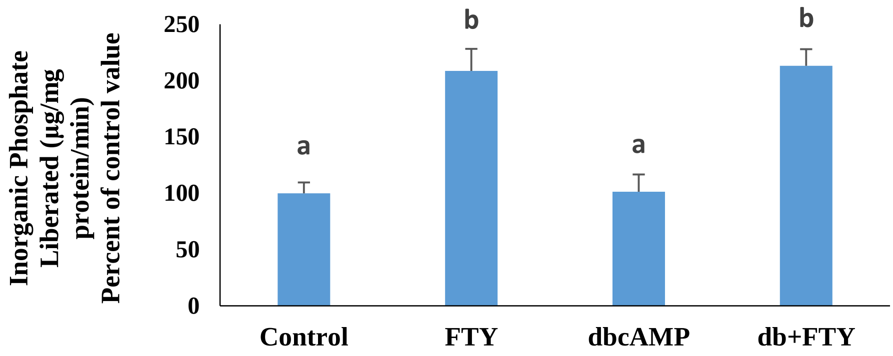


Figure 12: Effect of FTY720P on the activity of the $Na^+/K^+$ ATPase in presence of dbcAMP (10 µM in $H_2O$, 15 mins), a PKA activator. An equal volume of the vehicle(s) was added to the control. Values are means ± SEM of 3 observations. Bars not sharing the same letter are considered significantly different from each other at P<0.01, as indicated by ANOVA followed by Tukey Kramer test. (FTY=FTY720P, db=dbcAMP)

The involvement of PKC in the effect of FTY720P on the $Na^+/K^+$ ATPase was studied using a PKC inhibitor, calphostin C, and a PKC activator, phorbol 12-myristate 13-acetate (PMA). The activation of the pump was still observed in presence of calphostin C (figure 13). In addition, the activity of the pump was unchanged by the treatment with PMA (figure 14).

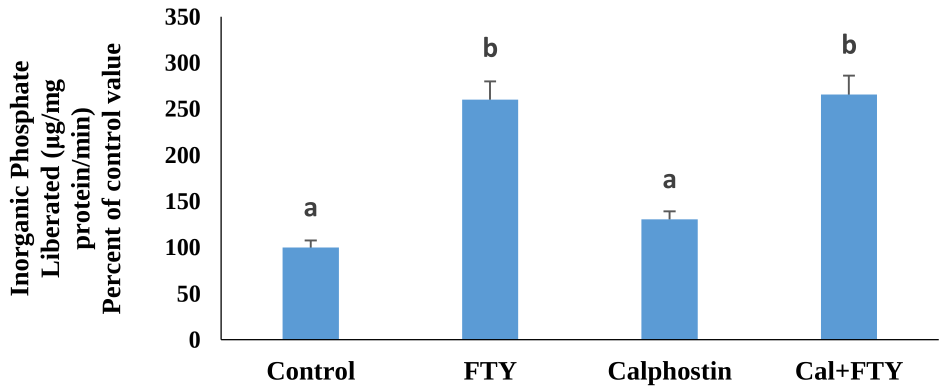


Figure 13: Effect of FTY720P (80 nM, 15 minutes) on the $Na^+/K^+$ ATPase activity in presence of Calphostin C (50 nM in DMSO), a PKC inhibitor. An equal volume of the vehicle was added to the control. Values are means ± SEM of 3 observations. Bars not sharing the same letter are considered significantly different from each other at P<0.001, as indicated by ANOVA followed by Tukey Kramer test. (FTY=FTY720P, Cal=Calphostin C)

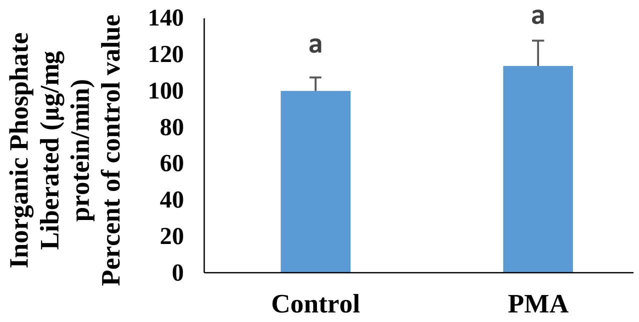


Figure 14: Effect of PMA (100 nM, 15 mins), a PKC activator on the $Na^+/K^+$ ATPase. An equal volume of the vehicle DMSO was added to the control. Values are means ± SEM of 3 observations. Bars sharing the same letter are not considered significantly different from each other as indicated by ANOVA.

To check for the involvement of G12/13, the effect of Rho kinase on the $Na^+/K^+$ ATPase was investigated. Treating the cells with a Rho kinase inhibitor, Y-27632, abolished the stimulation induced by FTY720P (figure 15).

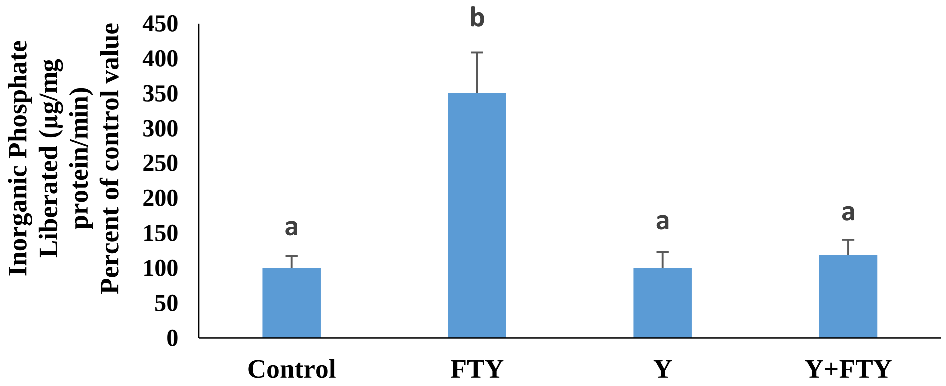


Figure 15: Effect of FTY720P (80 nM, 15 minutes) on the $Na^+/K^+$ ATPase activity in presence of Y-27632 (10 µM in DMSO), a Rho kinase inhibitor. An equal volume of the vehicle was added to the control. Values are means ± SEM of 3 observations. Bars not sharing the same letter are considered significantly different from each other at $P<0.001$, as indicated by ANOVA followed by Tukey Kramer test. (FTY=FTY720P, Y=Y-27632)

**F. PGE2 is not along the signaling pathway**

Production of PGE2 was inhibited using a COX enzyme's inhibitor, indomethacin. The activation of the $Na^+/K^+$ ATPase by FTY720P wasn't affected by indomethacin (figure 16). Furthermore, exogenous PGE2 didn't induce any change in the activity of the pump compared to the control (figure 17).

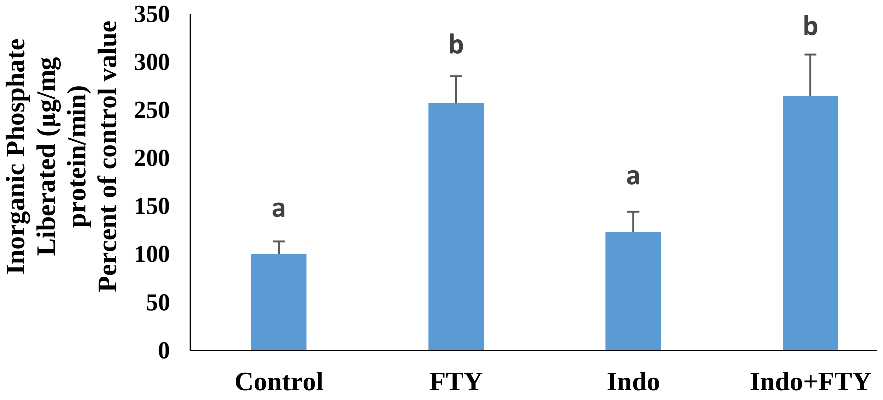


Figure 16: Effect of FTY720P (80 nM, 15 minutes) on the $Na^+/K^+$ ATPase activity in presence of indomethacin (100 µM in DMSO), a COX enzyme inhibitor. An equal volume of the vehicle was added to the control. Values are means ± SEM of 3 observations. Bars not sharing the same letter are considered significantly different from each other at $P<0.001$, as indicated by ANOVA followed by Tukey Kramer test. (FTY=FTY720P, Indo=Indomethacin)

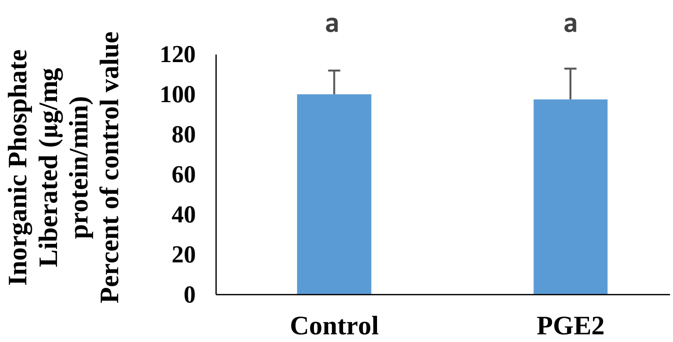


Figure 17: Effect of exogenous PGE2 (1 nM, 15 mins) on the $Na^+/K^+$ ATPase activity. An equal volume of the vehicle was added to the control. Values are means ± SEM of 3 observations. Bars sharing the same letter are not considered significantly different from each other as indicated by ANOVA.

## G. PI3K mediates the effect of FTY720P on the $Na^+/K^+$ ATPase

Inhibiting PI3K using wortmannin eliminated the activation induced by FTY720P on the $Na^+/K^+$ ATPase (figure 18).

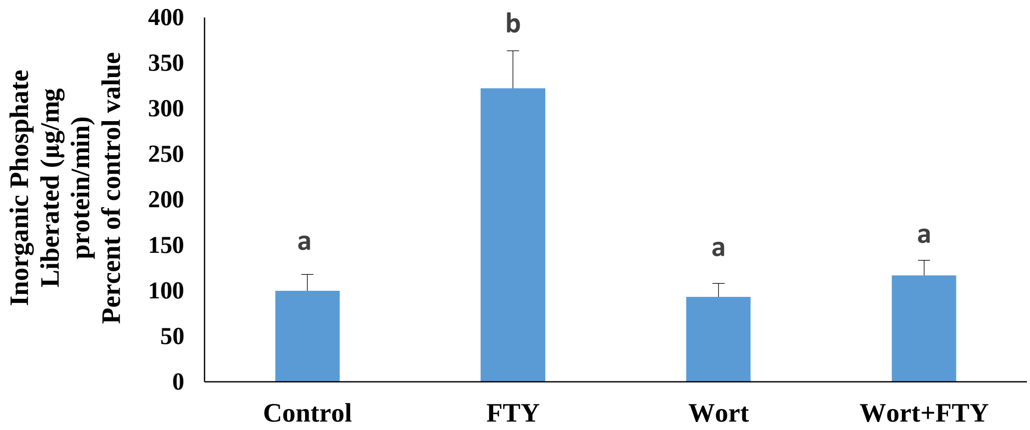


Figure 18: Effect of FTY720P (80 nM, 15 minutes) on the $Na^+/K^+$ ATPase activity in presence of wortmannin (100 nM in DMSO), a PI3K inhibitor. An equal volume of the vehicle was added to the control. Values are means ± SEM of 3 observations. Bars not sharing the same letter are considered significantly different from each other at $P<0.001$, as indicated by ANOVA followed by Tukey Kramer test. (FTY=FTY720P, Wort=Wortmannin)

## H. Rho kinase is upstream PI3K

The expression of p-Akt, a mediator activated downstream PI3K, increased following treatment with FTY720P. However, it came back to the control value when Rho kinase was inhibited with Y-27632 (figure 19). On the other hand, the expression of total Akt wasn't affected by any of the treatments (figure 20).

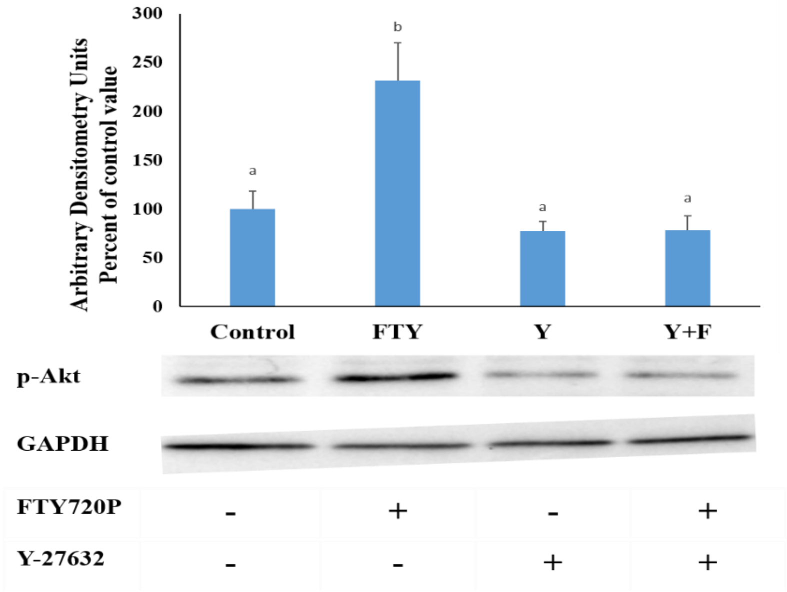


Figure 19: Effect of FTY720P (80 nM, 15 minutes) on the expression of p-Akt in presence and absence of Y-27632 (10 µM in DMSO), a Rho kinase inhibitor. Values are means ± SEM of 2 observations. The expression of p-Akt in presence of FTY is significantly different from that of the control at $P<0.05$, while it is significantly different from its expression in presence of Y and Y+F at $P<0.01$, as indicated by ANOVA followed by Tukey Kramer test. (FTY=FTY720P, Y=Y-27632, Y+F=Y-27632 + FTY720P)

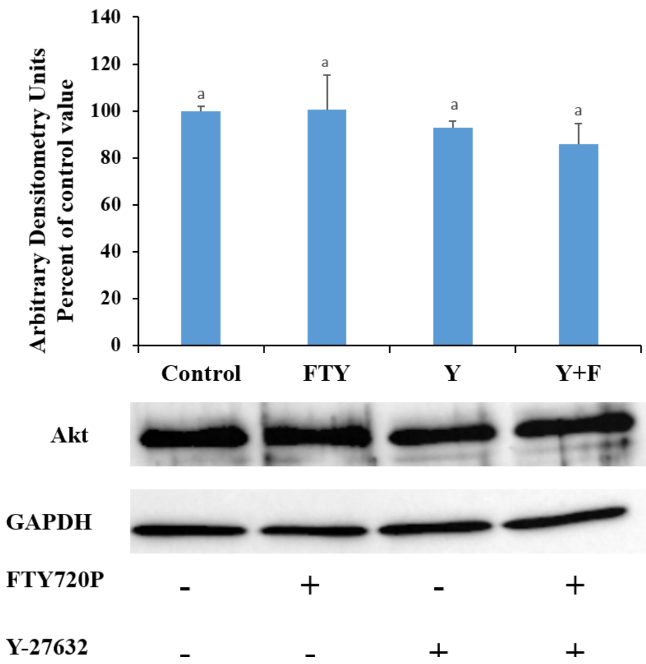


Figure 20: Effect of FTY720P (80 nM, 15 minutes) on the expression of Akt in presence and absence of Y-27632 (10 µM in DMSO), a Rho kinase inhibitor. Values are means ± SEM of 2 observations. Bars sharing the same letter are not considered significantly different from each as indicated by ANOVA. (FTY=FTY720P, Y=Y-27632, Y+F=Y-27632 + FTY720P)

## I. Nitric oxide is along the signaling pathway

The NO scavenger, carboxy-PTIO abolished FTY720P's activation of the $Na^+/K^+$ ATPase (figure 21).

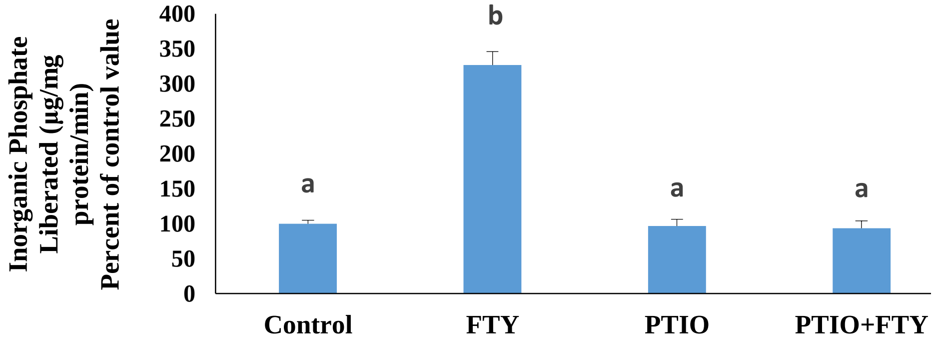


Figure 21: Effect of FTY720P (80 nM, 15 minutes) on the $Na^+/K^+$ ATPase activity in presence of carboxy-PTIO (30 nM in $H_2O$), a NO scavenger. Values are means ± SEM of 3 observations. Bars not sharing the same letter are considered significantly different from each other at $P<0.001$, as indicated by ANOVA followed by Tukey Kramer test. (FTY=FTY720P, PTIO= carboxy-PTIO)

## J. PI3K is upstream NO

Glyco-SNAP-1, a NO donor, mimicked the effect of FTY720P on the $Na^+/K^+$ ATPase and this effect did not disappear in presence of wortmannin the PI3K inhibitor (figure 22).

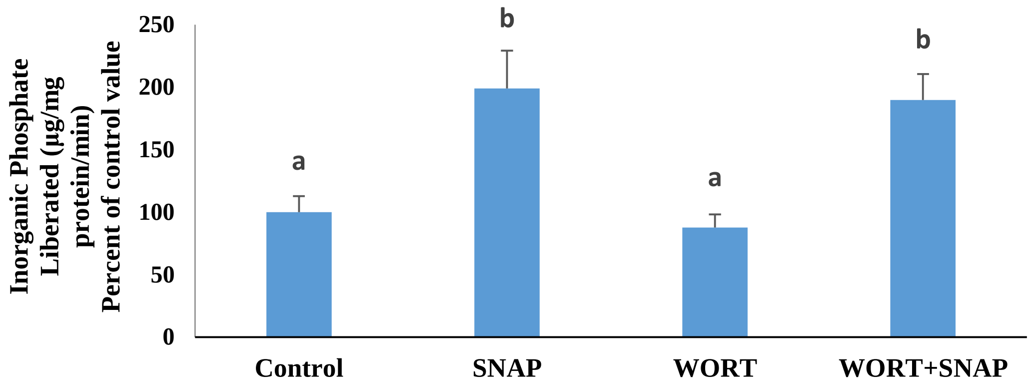


Figure 22: Effect of Glyco-SNAP-1 (4 µM, 15 mins) on the $Na^{+}/K^{+}$ ATPase in presence or absence of wortmannin (100 nM in DMSO), a PI3K inhibitor. An equal volume of the vehicle was added to the control. Values are means ± SEM of 3 observations. Bars not sharing the same letter are considered significantly different from each other at $P<0.01$, as indicated by ANOVA followed by Tukey Kramer test. (FTY=FTY720P, SNAP=Glyco-SNAP-1, WORT=wortmannin)

## K. Involvement of calcium in the signaling pathway

The activation of the $Na^{+}/K^{+}$ ATPase induced by FTY720P disappeared in presence of BAPTA-AM, a $Ca^{2+}$ chelator (figure 23).

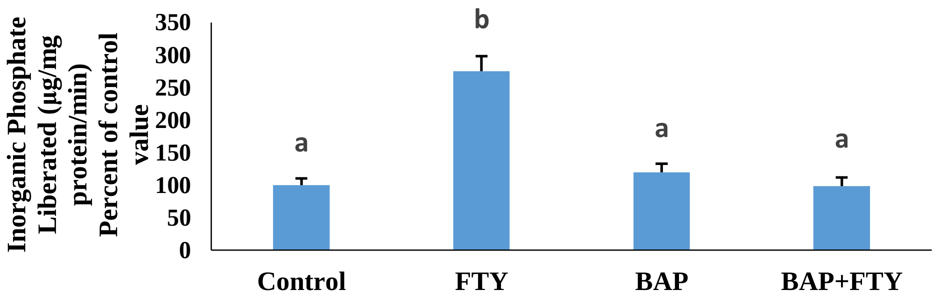


Figure 23**:** Effect of FTY720P (80 nM, 15 minutes) on the $Na^{+}/K^{+}$ ATPase activity in presence of BAPTA-AM (20 nM in DMSO), a $Ca^{2+}$ chelator. An equal volume of the vehicle was added to the control. Values are means ± SEM of 3 observations. Bars not sharing the same letter are considered significantly different from each other at P<0.001, as indicated by ANOVA followed by Tukey Kramer test. (FTY=FTY720P, BAP=BAPTA-AM)

**L. Position of calcium relative to NO**

Glyco-SNAP-1, a NO donor, mimicked the effect of FTY720P on the $Na^{+}/K^{+}$ ATPase. However, in presence of BAPTA-AM, a $Ca^{2+}$ chelator, its effect disappeared completely (figure 24).

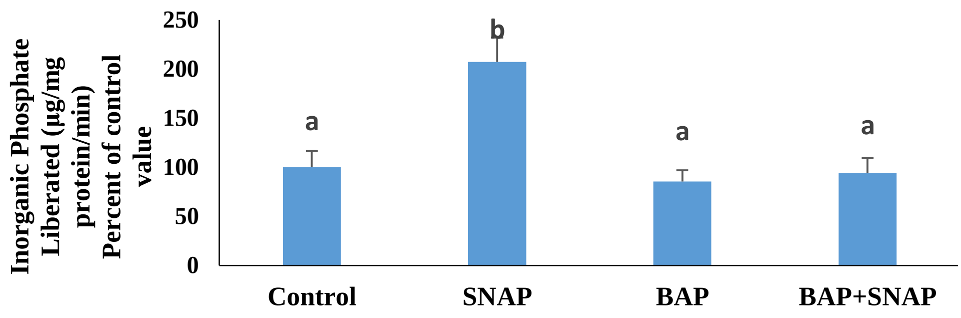


Figure 24: Effect of Glyco-SNAP-1 (4 µM, 15 mins) on the $Na^{+}/K^{+}$ ATPase alone or in presence of BAPTA-AM (20 nM in DMSO), a $Ca^{2+}$ chelator. An equal volume of the vehicle(s) was added to the control. Values are means ± SEM of 3 observations. Bars not sharing the same letter are considered significantly different from each other at $P<0.001$, as indicated by ANOVA followed by Tukey Kramer test. (FTY=FTY720P, SNAP=Glyco-SNAP-1, BAP=BAPTA-AM)

**M. PKG is along the signaling pathway**

KT5823, a PKG inhibitor eliminated FTY720P's activation of the $Na^{+}/K^{+}$ ATPase (figure 25).

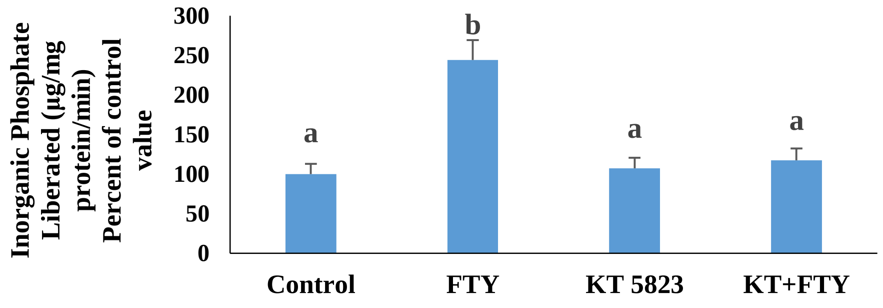


Figure 25: Effect of FTY720P (80 nM, 15 minutes) on the $Na^+/K^+$ ATPase activity in presence of KT-5823 (2.34 µM in DMSO), a PKG inhibitor. An equal volume of the vehicle was added to the control. Values are means ± SEM of 3 observations. Bars not sharing the same letter are considered significantly different from each other at P<0.001, as indicated by ANOVA followed by Tukey Kramer test. (FTY=FTY720P, KT=KT-5823)

## N. Position of PKG relative to calcium

8-bromo-cGMP, a cell permeable analog of cGMP capable of activating PKG, mimicked the effect of FTY720P on the $Na^+/K^+$ ATPase. This effect was not however maintained in presence of BAPTA-AM, a $Ca^{2+}$ chelator (figure 26).

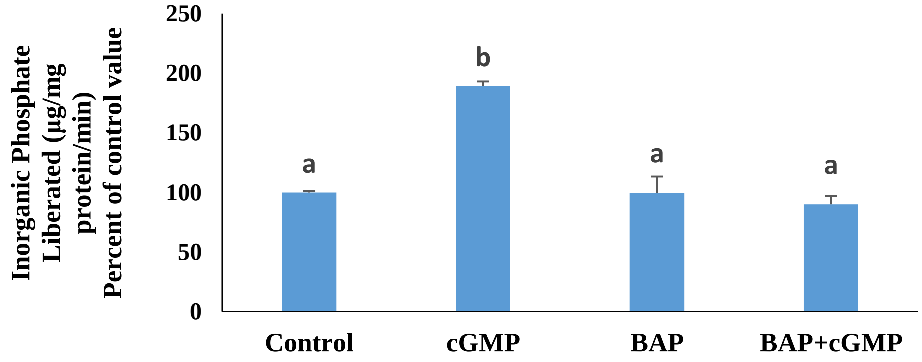


Figure 26: Effect of 8-bromo-cGMP (0.5 mM, 15 mins) on the $Na^+/K^+$ ATPase in presence or absence of BAPTA-AM (20 nM in DMSO), a $Ca^{2+}$ chelator. An equal volume of the vehicle(s) was added to the control. Values are means ± SEM of 3 observations. Bars not sharing the same letter are considered significantly different from each other at P<0.001, as indicated by ANOVA followed by Tukey Kramer test. (FTY=FTY720P, cGMP=8-bromo-cGMP, BAP=BAPTA-AM)

# CHAPTER V

# DISCUSSION

Proper functioning of the $Na^+/K^+$ ATPase in the kidneys is required to establish the sodium gradient needed to provide the energy for all secondary active transport processes such as transport of calcium, glucose, amino acids, etc (Jorgensen, 1986). Any alterations in the $Na^+/K^+$ ATPase's activity is expected to impair kidney functions (Clausen et al., 2017) and result in kidney failure. Renal IRI was reported to be accompanied with a decrease in the expression of the $Na^+/K^+$ ATPase (Kristensen et al., 2016) and activation of S1PRs could limit the damage induced. Therefore, this work investigated if the renal $Na^+/K^+$ ATPase is a target for S1P using LLC-PK1 cells as a model and FTY720P as an analogue for S1P.

Dose and time response studies conducted on LLC-PK1 cells revealed a stimulatory effect of FTY720P on the $Na^+/K^+$ ATPase with the highest effect observed at a dose of 80 nM applied for 15 minutes (figures 2 and 3). Higher concentrations of FTY720P restored the activity of the $Na^+/K^+$ ATPase to the control level. This might be due to the activation of one or more different S1PRs since these receptors have different binding affinities. In addition, incubating the cells with FTY720P for longer time periods showed similar results to the control. This can be explained by the internalization and desensitization of the receptors to prevent further stimulation. However, FTY720P had a dual effect on the ATPase in other cell lines. A dose of 7.5 nM applied for 15 minutes, inhibited the $Na^+/K^+$ ATPase in Caco-2 cells (Rida et al., 2018) and in HepG2 cells (Al Alam et al., 2016) and stimulated it when applied for 2 hours (Chakkour, 2018; Noureddine, 2018).

FTY720P exerts its effect via 5 types of receptors (S1PR1-5) (Albert et al., 2005; Brinkmann et al., 2002; Sobel et al., 2013). Western Blot analysis revealed that all S1PRs were expressed in LLC-PK1 cells (figure 4).

S1PR1-4 were shown to be expressed in mouse kidneys according to the following order of abundance: S1PR1 >S1PR3 >S1PR2 >S1PR4 (Awad et al., 2006). However, our results show a different order in LLC-PK1 cells where S1PR4 has the highest expression.

To determine the type of S1PR responsible for FTY720P's stimulatory effect, S1PR1 and S1PR3 were blocked using W146 and CAY-10444 respectively (figures 5 and 6). FTY720P still activated the $Na^+/K^+$ ATPase suggesting that these two receptors are not involved in FTY720P's signaling pathway. In addition, when an agonist of each of these two receptors was added individually to the cells (SEW 2871 and CYM 5541 respectively) (figures 7 and 8) no change in the activity of the ATPase was observed.

Next, S1PR2 's involvement in the signaling pathway was checked. Blocking S1PR2 using JTE-013 totally eliminated the activation of the pump induced by FTY720P (figure 9). Furthermore, CYM 5520, a S1PR2 agonist activated the $Na^+/K^+$ ATPase in a similar way to FTY720P (figure 10). It was concluded, based on these results, that S1PR2 is the only receptor involved in FTY720P's signaling pathway since its blockage eliminated completely the effect of FTY720P and brought the activity of the pump back to control values.

Previous studies also reported the involvement of S1PR2 in the inhibitory effect of FTY720P on the $Na^+/K^+$ ATPase in Caco-2 cells (Rida et al., 2018).

S1PR2 can act via Gi/o, Gq or G12/13. Signaling via Gi/o leads to the inhibition of PKA (Brinkmann, 2007). Inhibiting PKA using RpcAMP didn't simulate FTY720P's pump activation and adding FTY720P when PKA is inhibited did not eliminate the stimulatory effect (figure 11) indicating that FTY720P does not inhibit nor activate PKA. dbcAMP, a PKA activator did not have any effect on the pump's activity and didn't counteract the effect of FTY720P on the $Na^+/K^+$ ATPase (figure 12) when added simultaneously confirming the previous finding. Taken together, these results exclude Gi/o from FTY720P's signaling pathway.

Gq signals through activation of PKC (Brinkmann, 2007). Many PKC isoforms were reported to have an effect on the trafficking of the $Na^+/K^+$ ATPase and its activity (Abraham et al., 2008; Chibalin et al., 1999; Chibalin et al., 1998; Khundmiri et al., 2004; Ridge et al., 2002). Previous work in our lab also reported that leptin inhibits the $Na^+/K^+$ ATPase in Caco-2 cells via inhibition of PKC (El-Zein et al., 2015). Based on these previous studies, the involvement of PKC was next investigated. In presence of Calphostin C, a PKC inhibitor, FTY720P exerted its usual stimulatory effect on the $Na^+/K^+$ ATPase (figure 13) while PMA, a PKC activator didn't show any significant stimulation (figure 14) implying that PKC is not a mediator in FTY720P's signaling pathway thus ruling out the involvement of Gq.

Rho kinase can be a possible mediator in the signaling pathway if S1PR2 is acting via G12/13. In addition, activation of the RhoA pathway was previously shown to induce endocytosis of the $Na^+/K^+$ ATPase in response to hypoxia in human alveolar cells through a process that generates mitochondrial ROS (Dada et al., 2007). Therefore, the following mediator tested in the signaling cascade was Rho kinase. Y-27632, a Rho kinase inhibitor, abolished the effect of FTY720P and the activity of the pump went back to the control level (figure 15) indicating that FTY720P activates Rho kinase. The literature also reports a role for Rho kinase in the regulation of the intracellular sodium since it activates the $Na^+/K^+$ ATPase through a process dependent on the phosphorylated cofilin and triose-phosphate isomerase (Jung et al., 2002).

Previous studies in our lab demonstrated that PGE2 modulates the activity of the $Na^+/K^+$ ATPase in different cell types (Kreydiyyeh et al., 2007; Rida et al., 2018; Skayian et al., 2006). PGE2's involvement in the signaling pathway of FTY720P in LLC-PK1 cells was next determined by inhibiting with indomethacin, the enzyme COX-2 responsible for its production. Inhibiting PGE2 synthesis had no effect on the stimulatory effect of FTY720P (figure 16). Moreover, exogenous PGE2 exerted no effect on the ATPase (figure 17). The results suggest that PGE2 is not a mediator of FTY720P in LLC-PK1 cells.

Several studies revealed PI3K as a modulator for the activity and the trafficking of the $Na^+/K^+$ ATPase. In fact, Serhan et al. reported an inhibition of the pump and a decrease in its abundance following activation of PI3K by insulin in Caco-2 cells (Serhan et al., 2011). Whether PI3K is a mediator downstream of FTY720P has been addressed in the present study. LLC-PK1 cells treated with FTY720P following inhibition of PI3K by wortmannin showed no significant stimulation of the pump (figure 18) confirming the involvement of PI3K in the signaling cascade. In fact, the literature also reports an increase in the activity and the expression of the $Na^+/K^+$ ATPase upon activation of the PI3K pathway in rats treated with estradiol (Obradovic et al., 2015).

PI3K acts by activating Akt (Manning et al., 2007), thus increasing the expression of its phosphorylated form. The expression of p-Akt increased following treatment with FTY720P, but was not manifested when Rho kinase was inhibited with Y-27632 (figures 19 and 20) which implies that PI3K/Akt act downstream Rho kinase. Similar results were reported in endothelial cells where Rho kinase induced their motility via activation of PI3K/Akt (Basile et al., 2007).

Because NO was shown to be a modulator of the activity of the $Na^+/K^+$ ATPase in other tissues (Gupta et al., 1994), we wanted to determine if it is also  involved in the signaling pathway of FTY720P. LLC-PK1 cells were incubated with carboxy-PTIO, a NO scavenger before adding FTY720P (figure 21). The stimulatory effect of FTY720P disappeared indicating a role of NO downstream FTY720P. In contrast, previous work demonstrated an inhibitory effect of NO on the $Na^+/K^+$ ATPase in Caco-2 and HepG2 cells treated with FTY720P (Al Alam et al., 2017; Rida et al., 2018).

The NO donor, glyco-SNAP-1 stimulated the $Na^+/K^+$ ATPase in presence or absence of wortmannin, a PI3K inhibitor (figure 22) revealing that NO acts downstream PI3K. In fact, the literature reports many cases where PI3K activated NOS leading to NO production (Dimmeler et al., 1999; Fulton et al., 1999). Notch-PI3K/Akt activation leads to tumorigenesis via overexpression of NOS (Villegas et al., 2018). In palmitic acid-induced HUVECs, the endothelial dysfunction was reduced following activation of the PI3K/Akt pathway that subsequently led to a higher NO production (Li et al., 2018).

$Ca^{2+}$ was reported to have an inhibitory effect on the $Na^+/K^+$ ATPase dependent on the ATP and $MgCl_2$ concentration present (Beauge et al., 1983). Therefore, the participation of $Ca^{2+}$ in the effect of FTY720P on the pump was determined using a

$Ca^{2+}$ chelator, BAPTA-AM. FTY720P, in absence of free $Ca^{2+}$, did not increase the activity of the $Na^+/K^+$ ATPase (figure 23) revealing that $Ca^{2+}$ is a mediator in the signaling cascade. In rat jejunal crypt cells, $Ca^{2+}$ had an opposite effect; since epinephrine stimulated the activity of the $Na^+/K^+$ ATPase by reducing the intracellular levels of $Ca^{2+}$ (Kreydiyyeh, 2000).

Glyco-SNAP-1, a NO donor, had no effect on the pump in absence of free $Ca^{2+}$ (figure 24) suggesting that NO acts upstream $Ca^{2+}$. These results are in line with Huang et al. who reported that NO induced $Ca^{2+}$ influx and $Ca^{2+}$ release from intracellular stores thus increased cytosolic $Ca^{2+}$ in HeLa cells (Huang et al., 2014). In rat intestinal epithelial cells, non-agglutinable Vibrio cholerae heat-stable enterotoxin induced mobilization of stored intracellular $Ca^{2+}$ mediated by inositol triphosphate. Increase in $Ca^{2+}$ release activates NOS leading to NO synthesis, increase in cGMP and $Ca^{2+}$ influx (Hoque et al., 2004).

Since NO is known to signal through sGC/cGMP/PKG (Denninger et al., 1999), PKG seemed a possible mediator in the signaling pathway. In presence of KT 5823, a PKG inhibitor, the activity of the $Na^+/K^+$ ATPase was restored back to the control level (figure 25) implicating that PKG participates in FTY720P's effect on the pump in LLC-PK1 cells. An opposing effect for PKG is observed in tubular kidney epithelium where the atrial natriuretic peptide inhibits the $Na^+/K^+$ ATPase through activation of the cGMP/PKG pathway (Scavone et al., 1995).

To locate PKG and $Ca^{2+}$, cells were treated with 8-bromo-cGMP, a cell permeable analogue for cGMP, alone or in presence of BAPTA-AM, a $Ca^{2+}$ chelator. 8-bromo-cGMP could not activate the $Na^+/K^+$ ATPase when free $Ca^{2+}$ is absent (figure

26) indicating that PKG is upstream $Ca^{2+}$. In fact, several studies reported the regulatory effect of PKG on $Ca^{2+}$. Rat models suffering from heart failure were subject to infusions of exendin-4 that improved $Ca^{2+}$ homeostasis via activation of the eNOS/cGMP/PKG cascade (Chen et al., 2017). In human pancreatic enterochromaffin cells, PKG's activation decreased $Ca^{2+}$ levels in response to serotonin (Kalbe et al., 2016).

## Conclusion

In summary, LLC-PK1 cells treated with 80 nM FTY720P for 15 minutes exhibited an increase in the activity of the $Na^+/K^+$ ATPase. The downstream signaling pathway is summarized in the figure below (figure 27). FTY720P was shown to have a protective effect against IRI that was previously shown to be associated with a decrease in the expression of the $Na^+/K^+$ ATPase. FTY720P, through the pathway shown below, might be inducing the translocation of more $Na^+/K^+$ ATPase molecules from intracellular stores to the plasma membrane leading to an increased activity. The drug may also increase the activity of the $Na^+/K^+$ ATPase molecules in the plasma membrane by phosphorylation / dephosphorylation processes. Through increasing the activity of the $Na^+/K^+$ ATPase, FTY720P might be re-establishing the $Na^+$ gradient across the membrane along with the proper function of other secondary active transporters dependent on this gradient thus restoring proper kidney functions. Future work should investigate the effect of FTY720P on the translocation of the $Na^+/K^+$ ATPase and the link between the increased activity and its protective effect.

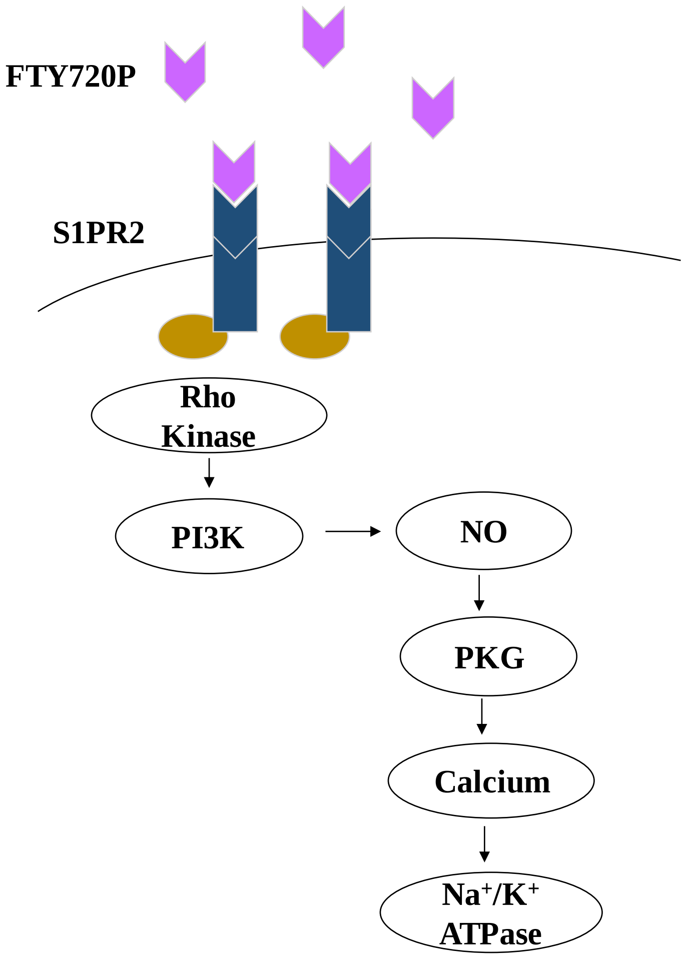


Figure 27: Summary of the signaling pathway downstream FTY720P in LLC-PK1 cells.

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