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THE SIGNALING PATHWAY MEDIATING THE STIMULATORY EFFECT OF FTY720-P ON HEPATIC Na^+/K^+ ATPase

by
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ABSTRACT OF THE THESIS OF

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Title: The Signaling Pathway Mediating the Stimulatory Effect of FTY720-P on Hepatic Na^+/K^+ ATPase.

Alterations in hepatocytes' volume contribute to the pathophysiology of several hepatic disorders including liver insufficiency, diabetic ketoacidosis, hyper-catabolism and infection. The Na^+/K^+ ATPase is a key regulator of ionic homeostasis in hepatocytes and consequently of cell volume. Emerging evidence ascribes a role for sphingosine 1-phosphate (S1P) in the development and progression of liver diseases. Previous studies in our lab showed that S1P modulates time-dependently the activity of the Na+/K+ ATPase in HepG2 cells, with an inhibitory effect appearing at 15min and a stimulatory one at 2hrs. This study focuses on the effect of S1P at 2 hours using the S1P analogue FTY720P.

HepG2 cells were incubated with FTY720P for 2 hrs and the activity of the pump was assayed by measuring the amount of inorganic-phosphate liberated in presence and absence of ouabain, a specific inhibitor of the Na^+/K^+ ATPase. FTY720P induced a 2.5 fold increase in the activity of the ATPase which was maintained in the presence of JTE-013, a specific blocker of S1PR2, but disappeared completely in presence of CAY 10444, a specific S1PR3 antagonist. The involvement of S1PR3 was confirmed by the stimulatory effect observed with Cym5541, a S1PR3 agonist. FTY720P increased the expression level of COX2, an enzyme involved in PGE2 synthesis, and its effect on the ATPase disappeared in presence of indomethacin, an inhibitor of COX enzymes, suggesting that FTY720P acts by promoting PGE2 production. The involvement of PGE2 was confirmed by the ATPase stimulation induced by exogenous PGE2. Inhibiting PKC and ERK with respectively calphostin and PD98059 abolished the effect of FTY720P on the Na+/K+ ATPase, but not that of exogenous PGE2 indicating that the two kinases are upstream of PGE2.The PKC activator PMA increased the activity of the Na+/K+ ATPase as well as the expression levels of phopho-ERK inferring that PKC is upstream of ERK. The effect of PGE2 disappeared in presence of PF-04418948 (blocker of EP2 receptor), RpcAMP (PKA inhibitor) and carboxy-PTIO (NO scavenger), indicating that PKA and NO are downstream mediators of PGE2. dbcAMP (PKA activator), SNAP (NO donor) and 8-bromo cGMP (cGMP analogue) mimicked the stimulatory effect of PGE2 on the pump. Western blot analysis and treating the cells with FTY720P in presence of an inhibitor of NF-κB revealed an involvement of the transcription factor in PGE2 and NO synthesis.

It was concluded that in HepG2 cells, FTY720P, when applied for 2 hours, binds to S1PR3 and activates sequentially PKC, ERK, NF-κB leading to a higher expression level of COX-2 enzyme and PGE2 release. The latter binds to EP2 receptor and activates PKA/NF-κB/NOS/cGMP pathway resulting in a stimulation of on the Na^+/K^+ ATPase.

ABREVIATIONS

15-PGDH	15-hydroxyprostaglandin Dehydrogenase
AA	Arachidonic Acid
AC	Adenylyl Cyclase
ADP	Adenosine Diphosphate
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
CaM	Ca^{2+} / Calmodulin
cAMP	Cyclic Adenosine Mono-phosphate
cGMP	Cyclic Guanosine Monophosphate
CICR	Calcium Induced Calcium Release
CNS	Central Nervous System
COX	Cyclooxygenase
DAG	Diacyl Glycerol
DAGK	Diacylglycerol Kinase
DCA	Dichloroacetic Acid
DMEM	Dulbecco Modified Eagle's medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
eNOS	Endothelial NO Synthase
ER	Endoplasmic Reticulum

ERK	Extracellular Signal Regulated Kinase
FAD	Flavin Adenine Dinucleotide
FBS	Fetal Bovine Serum
FTY720	Fingolimod
FTY720-P	Fingolimod Phosphate
Gi	Inhibitory G protein
GPCR	G-Protein Coupled Receptor
GSLs	Glycosphingolipids
HDACs	Histone Deacetylases
HepG2	Human hepatocellular liver carcinoma cell line
HGF	Hepatocyte Growth Factor
I/R	Ischemia-Reperfusion
Igf-1	Insulin Growth Factor 1
IL-1β	Interleukin 1 Beta
iNOS	Inducible Nitric Oxide Synthase
IP3	Inositol Triphosphate
IκB	Inhibitor of NF- κB
JNK	c-Jun N-terminal Kinase
LPPs	Lipid Phosphate Phosphohydrolases
LPS	Lipopolysaccharide
LSEC	Liver Sinusoidal Endothelial Cells
MAPK	Mitogen-Activated Protein Kinase

mRNA	Messenger RNA
MS	Multiple Sclerosis
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NF-κB	Nuclear Factor-kappa B
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
PARP	Poly(ADP-ribose) Polymerase
PE1	Phosphorylated E1
PE2	Phosphorylated E2
PEPCK	Phospho*Enol*Pyruvate Carboxykinase
PGE2	Prostaglandin E2
PGs	Prostaglandins
PGT	Prostaglandin Transporter
PI3-K	Phosphoinositide 3-Kinase
PKA	Protein Kinase A
PKC	Protein Kinase C
PKG	Protein Kinase G
PLC	Phospholipase C
PLD	Phospholipase D
ROS	Reactive Oxygen Species
RVD	Regulatory Cell Volume Decrease
RVI	Regulatory Cell Volume Increase

S1P	Sphingosine 1-Phosphate
S1PRs	Sphingosine 1-Phospahte Receptors
SAPK	Stress Activated Protein Kinases
SDK1	Sphingosine-Dependent Protein Kinase
sGC	Soluble Guanylate Cyclase
SM	Sphingomyelin
Sphk	Sphingosine Kinase
SPl	Sphingosine 1-Phosphate lyase
Spns2	Spinster 2
SPPs	S1P Phosphatases
TCA	Trichloroacetic Acid
TM	Transmembrane
TNF-α	Tumor Necrosis Factor Alpha

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CHAPTER I

INTRODUCTION

The liver, a heterogeneous tissue composed mostly of parenchymal cells (Nowatari et.al, 2015), plays an important role in protein synthesis, bile production, carbohydrates metabolism, and in the processing of hormones and foreign substances such as drugs and alcohol which, if not cleared, can build up in the body and cause significant damage (American Cancer Society, 2016).

Intracellular ion homeostasis in hepatocytes is accomplished by the synchronized activity of various carriers and transporters (Graf et.al, 1996). Alterations in ion transport rates may affect cell volume, leading to cell swelling or shrinkage with several disruptive effects on cell metabolism. In fact, cell shrinkage is considered a hallmark of apoptosis (Graf et.al, 1996). In hepatocytes, changes in cell volume are associated with altered hepato-cellular functions including changes in bile flow, bile acid transport, cytoplasmic and endosomal pH, metabolism of carbohydrates, proteins, lipids, and gene expression, as well as changes in the cytoskeletal components (Graf et.al, 1996) resulting in many disorders such as liver failure, diabetic ketoacidosis, hyper-catabolism, fibrosis, and infection (Lang et.al, 2007).

A major regulator of ionic homeostasis in the liver is the Na^+/K^+ pump or Na^+/K^+ ATPase, which is ubiquitously expressed in all mammalian cells but in differing amounts (Mobasheri et.al, 2000). The Na^+/K^+ ATPase is a member of the P-type family of ATPase, a family of evolutionary conserved proteins in prokaryotic and eukaryotic cells (Mobasheri et.al, 2000). The pump uses the energy derived from the hydrolysis of one ATP to drive the cellular uptake of $2/K^+$ ions in exchange with $3/Na^+$ ions creating a trans-membrane

ionic gradient involved in growth, survival, and cell differentiation (Mobasheri et.al, 2000). The gradient is used also to generate action potentials, regulate cell volume, pH, and drive secondary active transport processes (Mobasheri et.al, 2000). Because the pump is involved in such a wide array of activities, its role in the proper functioning of the liver is significant.

Emerging evidence provides a role for sphingolipids in hepatocellular death and liver diseases (Wang et.al, 2015). Several studies have demonstrated that sphingosine 1-phosphate (S1P) is involved in inflammatory diseases such as LPS induced liver injury and hydrophobic bile acid apoptosis (Tian et.al, 2016; Webster, et.al, 2016). S1P is a lipid mediator and a by-product of the sphingomyelin metabolism that gets phosphorylated by sphingosine kinases (Mandelson et.al, 2013). S1P acts via five identified G protein-coupled receptors which are expressed in a wide variety of tissues (Mandelson et.al, 2013). The widespread expression of S1P receptors in mammals reflects the broad regulatory role of S1P in many cellular activities (Kihara et.al, 2008), such as cell migration, differentiation, survival (Mandelson et.al, 2014), increased DNA synthesis, G1-S transition, mitogenesis, cytoskeletal rearrangement, vascular maturation, embryonic development of the heart, immunity, and lymphocyte trafficking, as well as growth arrest and apoptosis (Mandelson et.al, 2013).

Recently, a novel sphingosine analog FTY720, was developed and shown to be an effective immune-modulator approved by the US Food and Drug Administration as an oral treatment for relapsing forms of multiple sclerosis (Kihara et.al, 2008; Mandelson et.al, 2013; Singer et.al, 2011). FTY720 is rapidly phosphorylated in vivo by sphingosine kinase to FTY720-P which acts as an agonist at 4 S1P receptors namely $S1P_1$, $S1P_3$, $S1P_4$, and $S1P_5$. FTY720-P binds to S1P receptor 1 on lymphocytes inhibiting their egress from lymphoid organs into the CNS and sequesters them reversibly in the lymph nodes (Kihara

et.al, 2008; Mandelson et.al, 2013; Singer et.al, 2011). As an agonist of $S1P_1$, FTY720-P activates downstream signaling with similar to slightly higher affinity as S1P (Singer et.al, 2011).

FTY720 was reported to be effective in the treatment of hepatic ischemia – reperfusion injury, concanavalin A induced liver injury, and liver fibrosis (Kong et.al, 2014). Some adverse effects are however associated with this compound such as headache, influenza, diarrhea, back pain, elevations in the level of liver enzymes and bradycardia (Singer et.al, 2011); such events could be due to interference with S1P signaling in other organ systems (Mandelson et.al, 2013).

We have shown previously in our lab that treatment of HepG2 cells, a human hepatocellular carcinoma cell line, with S1P for 2 hrs, results in a significant increase in the activity of the Na^+/K^+ ATPase (Kreydiyyeh et.al, 2014). Whether its analogue, FTY720-P, exerts a similar stimulatory effect is a question that will be addressed in this work. An attempt will be made also to delineate the signaling pathway involved. Elucidating the mode of action of FTY720-P will help in reducing any undesirable or adverse effect of the drug on the proper functioning of the liver resulting because of its effect on Na^+/K^+ ATPse activity, specifically by inhibiting the mediators involved.

The present work aims to:

1. Studying the effect of FTY720-P on the activity of the Na^+/K^+ pump.
2. Determining the type of S1P receptors involved in affecting the activity of the Na^+/K^+ pump.
3. Determining key mediators involved in the signaling pathway underlying the effect

CHAPTER II

LITERATURE RIVIEW

A. The Liver

1. Overview

The liver is the largest internal organ, and is highly involved in the regulation of metabolism. Blood rich in nutrients, toxins and other substances absorbed from the small intestine enters the organ *via* the portal vein, and exits to the heart *via* the hepatic vein. The liver has special regeneration ability: one quarter of the organ is enough to regenerate the liver back to its original size within several weeks (Nowatari et.al, 2015, Mazoff et.al, 2015).

2. Liver Functions

The liver plays an important role in sugar and fat metabolism, digestion, immunity, and in the processing of hormones and foreign substances such as drugs and alcohol which, if not cleared, can build up in the body and cause considerable damage. It is also involved in the synthesis of proteins, carbohydrates and lipids, and the storage of some nutrients such as glucose (Mazoff et.al, 2015).

Liver hepatocytes produce a greenish-yellow fluid called bile that aids in the emulsification of lipids and consequently facilitates the absorption of lipid-soluble nutrients. Bile is delivered to the small intestine via the bile duct, and extra bile is stored in the gall bladder. Products left from the clearance of drugs, alcohol and toxic substances are excreted through bile to the outside of the body. When red blood cells are broken down,

heme, a component of hemoglobin, is changed to bilirubin which is conjugated in the liver with glucuronic acid to a water soluble form that becomes a component of bile and then released in the small intestine and egested with fecal matter. Impaired liver functions may lead to improper nutrients' absorption and abnormal bilirubin elimination (Mazoff et.al, 2015).

3. Hepatic cell volume

a) Overview:

While undergoing their normal functions, cells may accumulate a number of osmotically active substances which cause them to swell. Cellular life is always a struggle against cell swelling and if cells lose the battle they die. Changes in cell volume lead to changes in bile flow, bile acid transport, cytoplasmic and endosomal pH, metabolism of carbohydrates, proteins and lipids, gene expression as well as alteration in the cytoskeletal components (Graf et.al, 1996). Shrinkage and swelling exert opposite patterns of metabolic changes (Theodoropoulos, et.al, 1992; Tohyama, et.al, 1991). Cell shrinkage is considered less hazardous than cell swelling, but still adversely affects cellular activities. Consequently, in order to defend the constancy of their volume and normal functioning, cells have developed volume regulatory mechanisms mostly involving the activity of Na^+/K^+ ATPase.

b) Regulators and modulators of liver cell volume:

Hormones such as insulin, epinephrine and leptin, in addition to oxidative stress induce the movement of various molecules across hepatocyte membranes using various carriers and transporters. Most of these transport processes depend on the sodium gradient created by the Na^+/K^+ pump.

i. Mechanisms of regulatory cell volume decrease (RVD):

When hepatocytes are exposed to hyposmotic media they swell for minutes before they go back to their original cell volume, a behavior called regulatory cell volume decrease (RVD). It occurs as a result of the extrusion of osmotically active substances via various transport systems, including: parallel activation of K^+ and Cl^- channels, activation of electrogenic bicarbonate exit, activation of K^+ - Cl^- cotransport, coupled transporters like the K^+/H^+ and Cl^-/HCO_3^- exchangers, Na^+/Ca^{2+} exchanger, Ca^{2+}- ATPase, and loss of cellular amino acids (Häussinger. et.al, 1991-a; Häussinger. et.al, 1994). Most of these transport systems are dependent on the Na^+ gradient established and maintained by the Na^+/K^+ pump.

ii. Mechanisms of regulatory cell volume increase (RVI):

When hepatocytes are suddenly exposed to hyperosmotic media the cells shrink, but within minutes they undergo a regulatory cell volume increase which brings them back to their original cell volume. RVI is accomplished in part by the uptake of ions across the cell membrane using Na^+ gradient dependent transporters such as Na-K-2Cl-symporter, the coupled activity of the Na^+/H^+ and Cl^-/HCO_3^- exchangers, and the inhibition of K^+ and Cl^-channels (Häussinger. et.al, 1991-a; Häussinger. et.al, 1994).

iii. Cumulative substrate transport:

One of the important modulators of cell volume in the liver is the cumulative uptake of osmotically active molecules such as amino acids (Bakker-Grunwald, 1983) and the uptake may be Na^+ dependent. The Na^+ dependent transport can result in a 20-fold increase intracellular to extracellular concentration of amino acids. Sodium that is co-transported with the amino acid, exits the hepatocytes in exchange with K^+ ions *via* the activity of the Na^+/K^+ ATPase leading to intracellular accumulation of K^+ and amino acids and causing cell swelling which triggers volume regulatory K^+ efflux to bring cell volume

back to normal (Häussinger et.al, 1992-a; Bakker-Grunwald, 1983; Häussinger et.al, 1990).

iv. Cell volume control by hormones:

Hormones are known to modulate the activity of membrane transporters. In hepatocytes, insulin stimulates Na^+/H^+ exchange, Na^+-K^+-$2Cl^-$ co-transport, and Na^+/K^+-ATPase activity (Jakubowski et. al, 1990; Fehlmann et.al, 1981), leading to cellular accumulation of K^+, Na^+, and Cl^- ions and cell swelling. Alternatively, glucagon, through the activation of quinidine-sensitive K^+ channels may cause a depletion of cellular Na^+, K^+ and probably Cl^- ions leading consequently to cell shrinkage (Hallbrucker et.al, 1991). Thus, hormones cause either cell swelling or shrinkage depending on the type of transport system activated.

c) Modulation of hepatic metabolism by cell volume:

Hepatic metabolism is known to be regulated by enzymes, substrates abundance, transporters and hormones. Hepatic cell volume is a newly recognized regulator added to the list. Changes in hepatic cell volume, which may occur physiologically within minutes, can profoundly modify hepatocellular functions like protein turnover, carbohydrates' metabolism, lipogenesis, acidification of intracellular compartments, gene expression and bile acid excretion (Häussinger et. al, 1994). Cell volume changes are escorted with a variety of alterations in hepatocellular functions that are maintained even after cells go back to their original volume. Thus, alterations in cellular volume in response to physiological stimuli are considered an important signal which helps cellular metabolism adapt to hormones and environmental changes (substrates, tonicity) (Häussinger, et.al, 1992-a).

i. Cell volume and protein turnover:

There is a close relationship between protein synthesis and the extent of cell volume changes regardless of how this change is induced (Stoll, B. et.al, 1992). The anti-proteolytic role of insulin is prevented in the presence of inhibitors of Na^+/K^+ ATPase and Na^+-K^+-$2Cl^-$ co-transporter (Häussinger et.al, 1990). Thus, the Na^+/K^+ ATPase through its role in cell volume regulation can affect protein turnover.

ii. Cell volume and bile acid excretion:

Conjugated bile acids are taken up by a Na^+ dependent secondary active transport process across the hepatocytes' canalicular membrane. This type of transport is energized by the Na^+ gradient created by the Na^+/K^+ ATPase (Erlinger, 1982). While the intracellular movement of bile acids is accomplished by protein carriers such as ligandin, which move them to the canalicular area of the hepatocyte (Strange, 1981), their excretion into the ducts is accomplished by an ATP - dependent transport system (Nathanson, et.al, 1991). The Na^+ gradient which is the major energy source driving bile acid transport, is maintained by the activity of the Na^+/K^+ ATPase. Thus it is reasonable to conclude that the Na^+/K^+ pump plays a key role in bile acid excretion (Erlinger, 1982).

In addition to that, hepatocellular shrinkage inhibits the excretion of taurocholate into bile, which is a rate limiting step in the excretion process, while hepatic cell swelling stimulates this process regardless of the way cell volume is modified. A 10% cell swelling is enough to double, within minutes, the maximal velocity of bile acid excretion into bile (Häussinger, et.al, 1992-b).

B. Na^+/K^+ ATPase

Living cells work optimally under specific conditions that need to be maintained. Among these can be listed the constancy of the cytoplasmic ionic composition including: low sodium, low calcium, high potassium and constant pH (Mulkidjanian et al, 2012). Changes in extracellular ionic concentrations will result in changes in intracellular ionic concentration that require perpetual ion pumping to maintain the constancy of the intracellular milieu leading to the formation of trans-membrane ionic gradients that store energy. The sodium gradient established by the Na^+/K^+ pump in animal cells drives several cellular processes including the transport of molecules (sugars, neurotransmitters, amino acids, metabolites, bile) and ions (H^+, Ca^{2+}, Cl^-) (Clausen MV. et al, 2017). The Na^+/K^+ ATPase, a membrane protein complex is ubiquitously expressed in animal cells. It breaks down ATP and utilizes the energy derived from one ATP hydrolysis to move $2K^+$ ions in and $3Na^+$ ions out, resulting in the creation of a sodium / potassium electro-chemical gradient (Mobasheri et.al, 2000).

1. P-type ATPase Family of Proteins:

The Na^+/K^+ ATPase belongs to the P-type family of ATPase (phospho-intermediate type), an evolutionary conserved family of proteins found in prokaryotes and eukaryotes (Mobasheri et al., 2000; Xie et al., 2003). Members of the P-type family transport different cations and share a similar structure and transport mechanism (Clausen MV et al., 2017). They possess a catalytic subunit which is membrane bound and which contains the binding site for ATP, the selected cation (s) to be transported, and the specific inhibitors (Mobasheri et al, 2000). They hydrolyze the terminal phosphate bond of ATP and use the

derived energy to transport cations such as Na^+, K^+, H^+, Ca^{2+}, Cu^{2+}, and Cd^{2+} (Fagan M. & Saier M., 1994) against their concentration gradient.

2. *Na^+/K^+ ATPase: Structure*

The Na^+/K^+ pump is composed of three polypeptide subunits (α, β, γ). The alpha subunit consists of ten transmembrane helices important for ion binding, occlusion, and movement, in addition to three cytosolic domains called the N-domain (nucleotide binding), P-domain (phosphorylation) and A-domain (actuator) involved in the dephosphorylation step (Cui et al., 2017). It has a total molecular mass of 110 kD, and the N- and C- terminals are located in the cytosol (Fambrough et al., 1994) (Fig-1). Being the catalytic subunit, the α-subunit holds the binding sites for ATP and Mg^{2+}, cardiac glycosides, as well as Na^+ and K^+ ions (Mobasheri et al, 2000). The β-subunit is glycosylated and considered a regulatory subunit because it is involved in the stabilization of the enzymatic complex in the plasma membrane (Benarroch, 2011). Three isoforms (β1, β2, and β3) of the β-subunit have been identified. The γ-subunit belongs to the FXYD family with 7 identified isoforms ($FXYD_{1-7}$). It is considered an auxiliary regulatory subunit whose function seems to be associated with the regulation of enzymatic affinity to various ligands, with a positive direct effect on the rate of ATP hydrolysis (Mishra et al., 2011).

In the human liver the α1 and β1 subunits prevail and are expressed on the basolateral and canalicular membranes of hepatocytes (Baker Bechmann et al, 2016; Brisse J. et al, 1995). The various alpha subunits are regulated differently and have differencing kinetic properties allowing the pump to function at various rates. The beta subunit isoforms are considered the main regulators for the pump's activity, expression, trafficking, and positioning sites in the membrane (Mobasheri et al, 2000). The different combinations of the different isoforms produce several pump isozymes which are

differently expressed in different tissues, thus allocating to each one a specific physiological function (signal transduction) in addition to their common transport activities (Mobasheri et al, 2000).

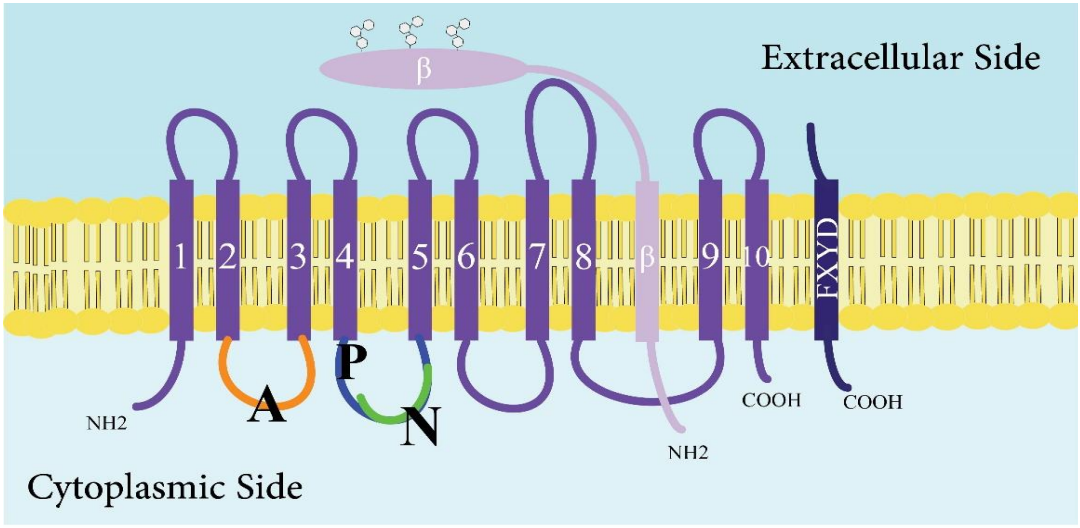

Figure 1: Schematic representation of the subunit domains of Na/K-ATPase. Modified from Horisberger, J. (2004)

3. Na^+/K^+ ATPase: Mechanism of ion transport

During one cycle of ATP hydrolysis, the pump alternates between two conformations: E1 and E2. In the E1 conformation the pump is phosphorylated and 3 Na^+ ions are bound while in the E2 conformation, the Na^+ ions are released extracellularly, 2 K^+ ions bind and the pump is dephosphorylated (Fig-2).

Binding sites for Na^+ and K^+ are present in the transmembrane part of the pump and are accessible via two gates: an inner gate facing the cytoplasm and an outer gate facing the extracellular milieu. When both are closed the transporter is said to be in an occluded state, and ions cannot move inward or outward. When the inner gate is open, Na^+ binds to three high affinity binding sites in the transmembrane helices. This binding induces the cleavage of ATP already attached to the N domain, and the phosphorylation of an aspartate residue in the P domain. The inner gate then closes and ADP is released, changing the pump to the occluded state which prevents the movement of ions. The outer gate then opens and the sodium ions are released to the outside. The release of Na^+ ions induces a conformational change that increases the affinity of the K^+ binding sites to K^+. The binding of two K^+ ions is accompanied by dephosphorylation of the aspartate residue and the closure of the outer gate, changing the pump to another occlusion state. Binding of another ATP to the P-domain causes the opening of the inner gate releasing the two potassium ions and the pump is then ready to undergo another cycle (Fig-2) (Poulsen et al., 2010; Clausen MV et al., 2017).

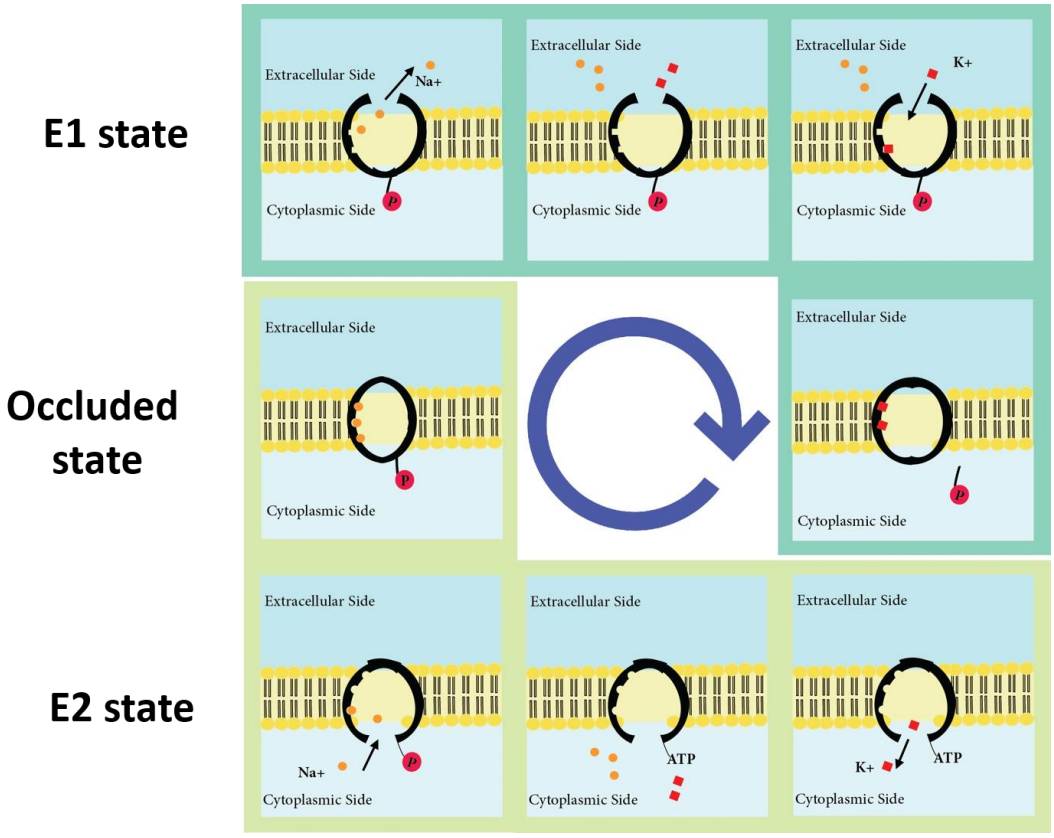

Figure 2: Schematic representation illustrating the alternating access model of the Na/K-ATPase.
Modified from Horisberger, 2004

4. Na^+/K^+ ATPase: Function

The Na^+/K^+ ATPase discovered 60 years ago by Skou, is ubiquitously expressed in animal cells but at different levels. It is highly expressed in excitable tissues such as muscles, brain, and epithelia and less abundant in other types of cells (Cui & Xie, 2017; Mobasheri et al, 2000). The Na^+/K^+ pump plays an important role in controlling the constancy of the ionic intracellular milieu which is required for the regulation of several biological functions and most critically cell volume (Erlinger, 1982). In addition the gradient is used to regulate pH, to fire action potentials and to control the activity of other transporters. In the kidneys it is utilized to reabsorb glucose and amino acids, regulate electrolytes' level and regulate blood pH; in sperm cells it is used for motility and acrosomal reaction; in neurons it is important for the generation of action potentials, and in astrocytes it is needed for neurotransmitters uptake (Clausen MV et al, 2017). In the liver, the Na^+ gradient established by the Na^+/K^+ ATPase is responsible for the transport and release of bile salts (Erlinger, 1982), and for controlling the activity of the Na^+/H^+ exchanger and the Na^+-K^+2Cl^- co-transporter which are involved in the regulation of hepatocellular pH and cell volume, respectively (Dallenbach et al., 1994).

Cell shrinkage is one of the common features of apoptosis: apoptotic cells lose K^+ and gain Na^+ in a process involving changes in the activity of the Na^+/K^+ ATPase (Panayiotidis et al., 2006) and the Na^+-K^+2Cl^- symporter (Leist, M., et al, 1995). On the other hand, hepatocellular shrinkage leads to the uptake of Na^+, K^+, and Cl^- ions by the action of Na^+/K^+ pump, Na^+-K^+ $2Cl^-$ symporter, ion channels, and Na^+/H^+ exchanger coupled to Cl^-/HCO_3 and Na^+/HCO_3^- exchangers causing an increase in cell electrolytes and water content (Friedrich et al, 2006). Thus, the Na^+/K^+ pump plays an important cell volume regulatory role.

5. Na^+/K^+ ATPase Activity Regulation:

The Na^+/K^+ ATPase activity is regulated by many factors including hormones, neurotransmitters (thyroid hormones, insulin, dopamine, norepinephrine…), phosphorylation state, changes in substrate concentration, and alterations in the membrane biophysical properties (Zhang et al., 2008). All these factors act via different signaling molecules such as mitogen-activated protein kinases (MAPKs), protein kinase A (PKA), protein kinase C (PKC), protein kinase G (PKG), Ca^{2+}/Calmodulin (CaM), phosphoinositide-3-kinase (PI3K), Src kinase, and reactive oxygen species (ROS) (Zhang et al, 2008; Sergio et al, 2009).

a) <u>Hormonal regulation:</u>

A number of circulating hormones control the activity of the Na^+/K^+ ATPase and exert either short-term or long-term regulatory effects. Environmental changes may lead to alterations in the activity of the pump and necessitate a rapid regulatory response using hormones like adrenaline, insulin and aldosterone (Ewart et al, 1995) to maintain the constancy of the internal milieu. Aldosterone, promotes in the kidneys K^+ release and Na^+ reabsorption via a stimulation of the Na^+/ K^+ pump (Ewart et al, 1995). A similar stimulatory effect was observed in rat cardiomyocytes, vascular smooth muscle cells, and rat brain hippocampus (Ikeda U., et al, 1991; Oguchiet al, 1993; Farman et al, 1994).

Continuous regulation of the pump's activity is required during periods of growth and repair, and such regulation is mediated by thyroid hormones and growth factors that mainly modulate the pump's expression. Thyroid hormones maintain normal and steady functioning of the Na^+/K^+ ATPase in the heart, skeletal muscle, fat, liver and kidney (Fehlmann et al, 1981). An increase in thyroid hormones' concentration is correlated with an increase in the pump's activity resulting from an increase in the number of the pump's molecules and not from a change in its catalytic activity (Lingrel et al, 1990). In fact, the

regulatory effect of thyroid hormones is Na^+/K^+ pump's isoform specific and tissue-dependent (Ewart et al, 1995).

In addition, catecholamines provide a fine tuning of the pump's activity (Ewart et al., 1995) and their effect varies with the signaling pathway activated by each hormone. For instance, dopamine reduces the activity of the pump in kidney tubules leading to a decrease in Na^+ reabsorption, an effect that is mediated via cAMP and its downstream effectors: DRAPP-32 or PKA / phospholipase A2 (Satoh et al., 1992; Satoh et al., 1993). Epinephrine induces a stimulatory effect on the pump in skeletal muscles through an increase in cAMP levels and PKA activity (Ewart et al., 1995). Similarly, another catecholamine such as norepinephrine, as well as vasopressin and angiotensin II, increase the pump's activity in the liver by increasing the levels of intracellular Ca^{2+} and activating calcineurin, a Ca^{2+}/Calmodulin – dependent protein phosphatase (Lynch et al., 1986; Aperia et al., 1992).

Insulin, also an important modulator of the Na^+/K^+ ATPase, its effect may be stimulatory or inhibitory depending on the tissue in which it acts. Insulin was shown to reduce the activity of the Na^+/K^+ pump in enterocytes through an activation of PKC, PI3K, and MAPKs (Serhan et al., 2011), but increased it in hepatocytes, adipocytes, and skeletal muscle cells by elevating the intracellular concentration of Na^+ ions. In myocytes insulin increased the activity of the ATPase by triggering its phosphorylation, while in kidney cells and adipocytes, it acted via increasing in the pump's affinity to Na^+ (Ewart et al, 1995).

b) Role of Protein Kinases:

Several protein kinases are involved in the signaling pathways mediating the regulatory effects of hormones, neurotransmitters and cytokines on the pump. For instance,

in sheep pulmonary artery, PKC was shown to be involved in the arachidonic acid inhibitory effect on the Na^+/K^+ ATPase activity (Singh et al., 2012). In Caco-2 cells leptin exerted an inhibitory effect on the pump mediated by an inhibition of PKC and activation of p38MAPK (El-Zein et al., 2015). Insulin was found to reduce the activity of the Na^+/K^+ ATPase in Caco-2 cells but through a different pathway involving activation of PKC, PI3K and MAPKs (Serhan et al., 2011). PKC and PI3K were also shown to be involved in the insulin-like growth factor I (IGF-I) increase in Na^+/K^+ pump activity in the vascular smooth muscle cells (VSMC) (Li et al., 1999). In proximal tubules, activation of PKC by the sequential activation of phospholipase D, and Ca^{2+} insensitive phospholipase A (2) was also behind the stimulatory effect of angiotensin II on the Na^+/K^+ ATPase (Souza et al., 2010), while the sequential activation of PI3K / protein kinase B and protein kinase C resulting in an inhibition of protein kinase A (Peruchetti et al., 2011), was behind the increase in the expression and activity of the pump by albumin.

Many studies have shown that nitric oxide (NO) and its downstream signaling pathways, mainly cGMP and protein kinase (PKG), regulate the activity of the Na^+/K^+ ATPase in a cell type dependent manner (Balon et al., 1994). Liang and Knox (1999) reported an inhibition of the Na^+/K^+ ATPase in a renal tubule cell line by NO, acting via cGMP. Similarly, the activity of the pump was modulated in the rat striatum by glutamate acting via cGMP and PKG (Munhoz et al., 2004; Ferreira et al., 1998). It has been shown that cGMP and PKG are responsible for the age-related stimulation of Na^+/K^+ ATPase activity in rat cerebellum (Scavone et al, 2005), and the inhibition of the ATPase in renal tubules by the atrial natriuretic peptide (ANP) (Scavone et al, 1995).

TNF-α was reported to stimulate the Na^+/K^+ ATPase in HepG2 cells (Kassardjian et al., 2010), and reduce it in LLC-PK1 using the same mediators JNK and NF-κB (Ramia et al., 2010).

c) Phosphorylation state:

Several studies have demonstrated that the Na^+/K^+ ATPase can be regulated via the reversible phosphorylation of its catalytic and/or regulatory subunits. The α subunit contains several phosphorylation sites that are targeted by many kinases including PKA, PKC, PKG, ERK1/2 and AMP activated protein kinase (Al-Khalili et al., 2004; Bertorello et al., 1991; Fotis et al., 1999; Benziane et al., 2012). Hormones' regulatory effect on the pump is a result of an alteration in the activity of various kinases leading to changes in the phosphorylated state of the ATPase. PKA and PKC can phosphorylate the α-subunit of the ATPase causing either an increase or a decrease in its activity in a tissue dependent manner (Ewart et al., 1995). PKG also was shown to increase the activity of the pump via directly phosphorylating its α-subunit in mammalian kidneys (Fotis et al., 1999; Young et al., 1998). Direct phosphorylation of Na^+/K^+ ATPase FXYD2 regulatory subunit by PKC alone or PKA alone or both kinases simultaneously was also shown to affect the pump's activity (Cortes et al., 2011).

6. Na^+/K^+ ATPase: The signal transducer

In human cells the Na^+/K^+ ATPase may be involved in dynamic interaction with other cytosolic and membrane proteins. At least three biological roles are associated with these interactions: (1) regulation of ionic concentrations including Na^+, K^+, and Ca^{2+} resulting from change in the Na^+/K^+ pump's activity; (2) signal transduction due to the pump's interaction with signaling molecules; (3) signal integration by organizing specific membrane micro-domains and bridging several affecters and effectors together by pump's scaffolding function (Cui & Xie, 2017). As such, caveolins bind to the pump and other signaling molecules concentrating them into one region in the cell membrane forming a large signaling complex called caveola (Xie et al., 2003; Zhang, et al., 2008).

For instance, binding of ouabain (Na^+/K^+ pump inhibitor) or changes in extracellular K^+ levels induce the attachment of Src to the pump and consequently its activation (Zand et al., 2002; Cui &Xie, 2017). This interaction allows the Na^+/K^+ ATPase to regulate several different signal transduction pathways, in a cell type dependent manner (Haas et al., 2002; Xie et al., 2003; Cui &Xie, 2017). These signaling cascades have a wide array of biological effects on the cells leading to the expression of some genes, or some transcription factors such as AP1 and NF-κB (Xie et al., 2003) and may affect even the Na^+/K^+ pump itself (Zand et al., 2002; Cui &Xie, 2017).

C. Sphingosine

1. Overview:

Sphingolipids exist in almost all eukaryotic cell membranes. Some sphingolipid metabolites such as ceramide, sphingosine, sphingosine 1-phosphate (S1P), and ceramide 1-phosphate have diverse functions and are involved in a variety of signaling pathways (Kihara, A. et al., 2008; Spiegel et al., 2003).

Sphingosine is considered a backbone of most sphingolipids (Kihara, A. et al., 2008; Spiegel et al., 2003). There exist two main routes for Sphingosine (Sph) production, it is either produced from the degradation of plasma membrane glycosphingolipids (GSLs) and sphingomyelin (SM) in the endocytic (Endosome) recycling pathway (Stunff et al., 2002), or synthesized from ceramide, in a pathway in which sphingomyelinase changes sphingomyelin into ceramide, which is then further processed into sphingosine by the action of the enzyme ceramidase as shown in Figure 3 (Mendelson et al, 2014).

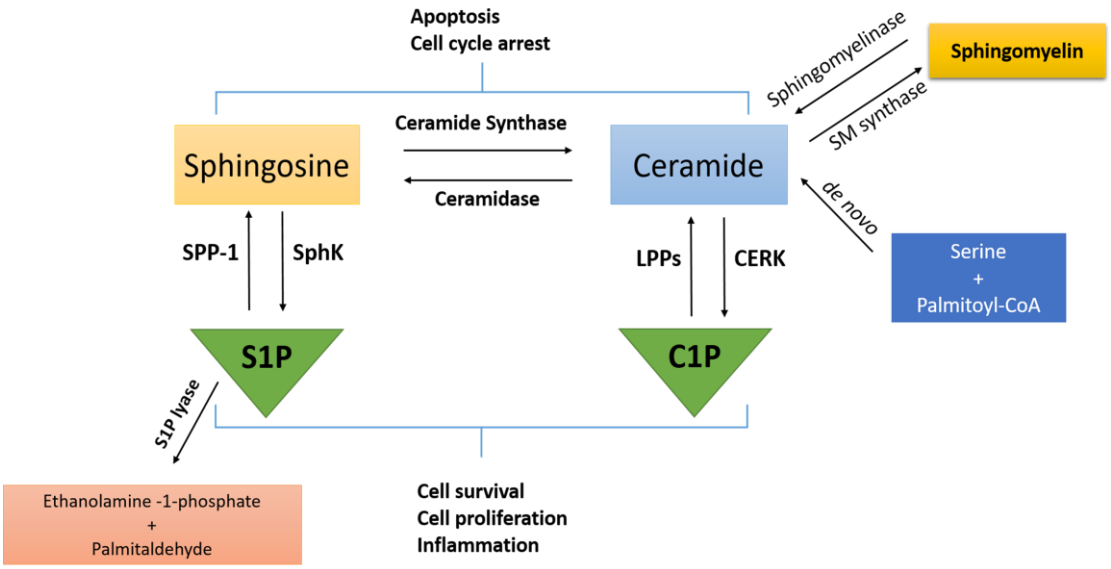

Figure 3: Bioactive sphingolipid metabolites

The scheme depicts the sphingolipid biosynthetic pathway: LPP, lipidphosphate phosphatase; SPP-1, S1P phosphatase-1; CERK, ceramide kinase; SphK, sphingosine kinase; SM, sphingomyelin. Differential/opposing signaling roles of sphingosine, S1P, ceramide and C1P are indicated. Because of the inter-convertibility of these molecules, the activity of a single enzyme in the depicted pathways may affect the fate of the cell.
Modified from Chalfant E.C. et al, 2005.

2. Sphingosine Effects

Sphingosine is considered a key signaling molecule due to its interaction with several kinases. It weakly activates JNK and exerts a strong inhibitory effect on ERK (Taha et al., 2006). It can also target and change the activity of many enzymes such as diacylglycerol kinase (DAGK) (Munoz, 2006), Ca^{2+}/Calmodulin-dependent protein kinase, protein kinase B (Akt) (Mao et al., 2008), v-Src or c-Src tyrosine kinase, phospholipase D (PLD) (Munoz, 2006), and PKCζ isoform (Taha et al., 2006).

3. Sphingosine and Cell Death

Through the classical mitochondrial pathway, sphingosine can mediate a caspase dependent cell death. The mechanism involves Bid cleavage, mitochondrial cytochrome c release, activation of downstream effectors caspases 3 or 7, and finally the cleavage of Poly-ADP-ribose polymerase (PARP) leading to apoptosis (Taha et al., 2006).

Sphingosine can also mediate cell death by inhibiting the full length PKCζ and stimulating its truncated version the sphingosine-dependent protein kinase (SDK1), allowing the release of the pro-apoptotic factors Bax and Bad (Taha et al., 2006). In addition, sphingosine is capable of downregulating the major mitochondrion gatekeepers Bcl-2 and Bcl-xl (Taha et al., 2006).

In some cell types and when added exogenously, sphingosine was reported to stimulate massive Golgi fragmentation and inhibit α1 integrin glycosylation and transport to the cell surface, before inducing apoptosis (Taha et al., 2006).

D. Sphingosine 1-Phosphate

The bioactive sphingolipid metabolite sphingosine 1-phosphate (S1P) is the phosphorylated form of sphingosine; it is considered a very important lipid mediator implicated in diverse biological processes. S1P has been detected in almost all living organisms including mammals, plants, yeast, worms, slime molds and flies (Spiegel et al., 2003).

In vertebrates, S1P is present in the extracellular milieu where it interacts with cell surface receptors to influence a wide array of cellular responses starting from cell migration and differentiation to survival, proliferation and apoptosis (Blaho V. A., & Hla T., 2011; Chun J. et al., 2002). It plays a critical role in morphogenetic mechanisms during development such as collective cell migration, tissue inductive events and biomechanical signaling (Mendelson et al., 2014).

1. Synthesis and Export

Sphingosine is phosphorylated by sphingosine kinase (Sphk) (Spiegel et al., 2002), yielding sphingosine 1-phosphate. There exist two sphingosine kinase isoforms: Sphk1 and Sphk2, each one has five conserved domains including the unique catalytic domain containing a consensus sequence for ATP binding (Spiegel et al., 2003).

Sphk1 is mainly located in the cytosol but can be recruited to the plasma membrane. Sphk2 is however, associated with the nucleus and internal membranes (Spiegel et al., 2003; Kihara A., et al., 2008). The translocation of sphingosine kinases to the membrane where their substrate, sphingosine, resides seems to be a part of the activation process (Spiegel et al., 2003; Contreas et al., 2006).

Despite their structural similarity, Sphk1 and Sphk2 different from each other in many aspects including tissue distribution, temporal expression patterns during development, and kinetic properties, indicating that they might be responsible for different cellular processes and might be regulated differently (Spiegel et al., 2003). While Sphk1 is known for its pro-survival role, Sphk2 possesses a BH3 motif allowing it to mediate growth inhibitory and pro-apoptotic functions (Taha et al., 2006).

Platelets and erythrocytes accumulate S1P and are considered in mammals to be the major source of plasma sphingosine 1-phosphate (Kihara, A. et al., 2008). Platelets have a high Sphk activity and no S1P lyase while erythrocytes have no lyases and no phosphatases (Kihara, A. et al., 2008). Extracellular biosynthesis of S1P also occurs because of the activity of secreted Sphk1 by some cells (Venkataraman et al., 2006), contributing to the enrichment of the vertebrates' plasma in S1P. Sphk1 was shown to be constitutively secreted from vascular endothelial cells (Ancellin, et al., 2002), and contributes to vascular development (Hla et al., 2008; Venkataraman et al., 2006).

S1P cannot cross the plasma membrane of mammalian cells and is transported to the extracellular environment by specific transporters called the ABC transporters. Different transporters are active in different cells: ABCC1 transporters export S1P from mast cells (Mitra et al., 2006), while ABCA1 transporters are the ones involved in astrocytes (Sato et al., 2007), and ABCA7 transporters in platelets (Kobayashi et al., 2006). Other types of transporters exist such as spinster 2 (Spns2), and still additional transporters are suspected to be involved in the export of S1P but have not been identified yet (Kawahara et al., 2009).

2. Sphingosine 1-phosphate Effects / Signaling

S1P has a multitude of biological effects that are exerted in a cell type dependent manner; it promotes proliferation, G1-S transition, increase in DNA synthesis, cell survival (Taha et al., 2006; Spiegel et al., 2003), inhibition of ceramide induced cell death (Chalfant et al., 2005), cell motility (Mao et al., 2008), in addition to the induction of growth arrest, differentiation and apoptosis (Mao et al., 2008). In fact S1P signaling plays an essential role in development, and was shown to be involved in angiogenesis (Gaengel et al., 2012), cardiogenesis (Kupperman et al., 2000), limb development (Chae et al., 2004) and neurogenesis (Mizugishi et al., 2005). S1P regulates major players in the cell survival and cell death pathways (Muñoz 2006) and can act as a first or second messenger.

a) <u>First Messenger:</u>

As a first messenger, secreted S1P binds to a family of G-protein coupled receptors (S1PRs). Five receptors ($S1PR_1/EDG_1$, $S1P_2/EDG_5$, $S1PR_3/EDG_3$, $S1PR_4/EDG_6$, and $S1PR_5/EDG_8$) have been identified to date, expressed in a wide variety of tissues (Chalfant et al., 2005; Spiegel et al., 2003; Kihara et al., 2008). These G-protein coupled receptors allow S1P to influence a diversity of cellular activities (Spiegel et al., 2002). Upon binding to any of its receptors, S1P induces the dissociation of the α-subunit from the βγ subunits of the specific G-protein to which it is coupled. S1PR1 couples to $G_i\alpha$; S1PR2 to $G_{12/13}\alpha$, $G_q\alpha$, and $G_i\alpha$; S1PR3 to $G_q\alpha$, $G_i\alpha$, and $G_{12/13}\alpha$; S1PR4 to $G_i\alpha$; S1PR5 to $G_i\alpha$ and $G_{12}\alpha$ (Fig-4) (Kihara et al., 2008).

Downstream effectors of the dissociated α subunit, depending on the type of G protein activated, include adenylate cyclase, phospholipase C, extracellular signal regulated kinase (ERK), stress activated protein kinases (JNK), phosphatidylinositol 3-kinase (PI3K), Akt and nitric oxide (Chalfant et al., 2005; Spiegel et al., 2003, Kihara A. et al., 2008; Kwon, et al., 2001). The Gβγ subunit, especially Giβγ, is also involved in

signaling. It drives the activation of phospholipase C, as well as the activation of Ras and ERK and the activation of the PI3K/Akt pathway (Igarashi et al., 2001; Taha et al., 2004). As illustrated in Figure 4, G_i inhibits adenylate cyclase through its α subunit and activates ERK and PI3K through the $\beta\gamma$ subunit, while Gq activates PLC which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) generating IP3 and DAG which in turn activates PKC (Jeong-Hun Kang, 2014).$G_{12/13}$ activates Rho GTPase (Nagahashi M. et al, 2014) as illustrated in Figure 4.

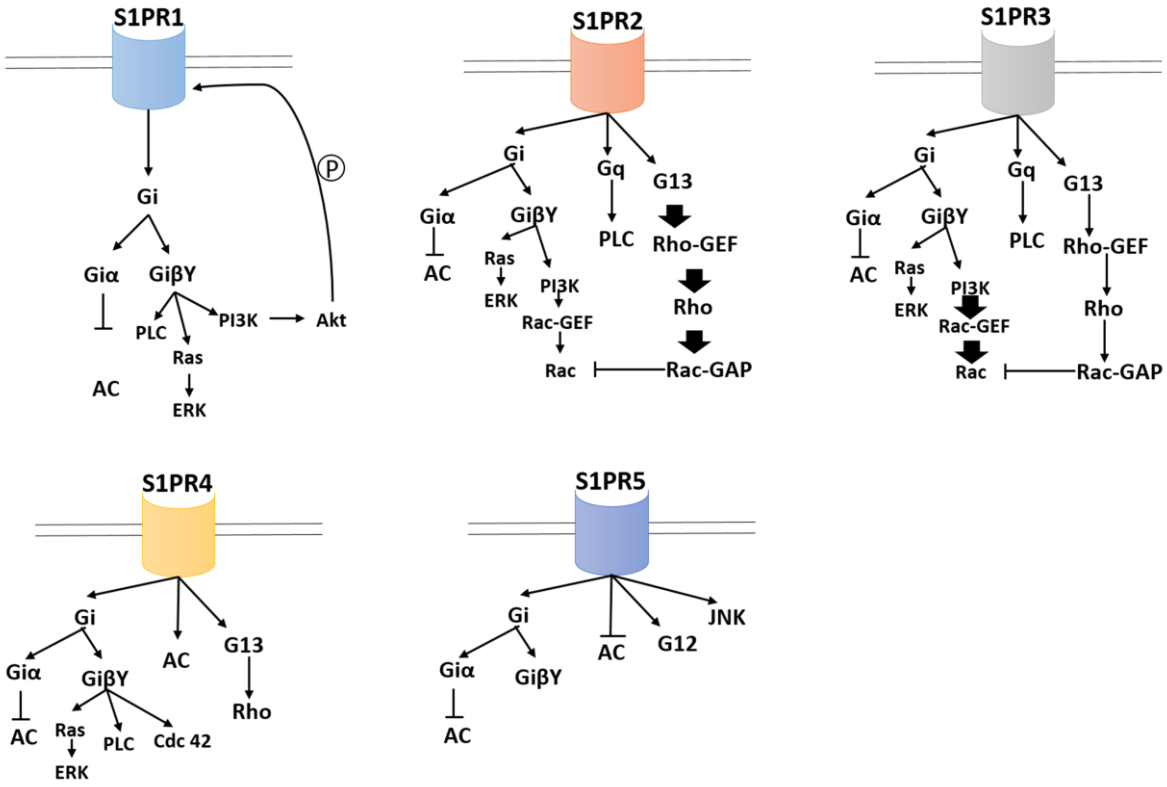

Figure 4: The coupling pathways of the different S1P receptors.

(A) S1P1 couples only to Gi. The arrow from Akt to the receptor indicates phosphorylation (p) of the receptor by Akt. (B and C) S1P3 and S1P2 couple to Gi, Gq, and G13. The thick arrows show the predominant pathway regulating Rac. While S1P3 activates Rac, S1P2 inhibits it. (D) S1P4 couples to Gi and G13. It also activates adenylate cyclase (AC) through an unknown mechanism. (E) S1P5 couples to G12 and Gi. It inhibits ERK and activates JNK
Modified from Taha et al., 2004.

It is worth mentioning that ERK activation starts by the activation of a MAP-kinase kinase (Ser/Thr), mainly Raf, which in turn activates another MAP (Tyr/Thr) kinase that will lead to the activation of ERK (Shaul et al., 2007). Another MAPK module activated by stress involves MEK kinase which activate SEK (Tyr/Thr kinase) leading to JNK activation (Himes et al., 2006). Adenylate cyclase, which is inhibited by G_i, is an enzyme involved in the production of cAMP, a cyclic nucleotide responsible for the activation of PKA (Trewhella, 2006). In addition to mediating several biological functions PKA is known to activate endothelial nitric oxide synthase (eNOS) via phosphorylation (Dixit, et al, 2005), leading to NO synthesis (Moncada et al., 1991). NO activates soluble guanylate cyclase (sGC), which in turn induces the production of cGMP that activates PKG which mediates the downstream biological effects of NO (Francis et al., 2010).

$S1PR_1$, $S1PR_2$ and $S1PR_3$ are ubiquitously expressed in mammals; while $S1PR_4$ and $S1PR_5$ expression is restricted to certain tissues. $S1PR_4$ is mainly present in lungs and lymphoid tissue, while $S1PR_5$ is expressed in brain and skin (Gräler et al., 1998; Ishii et al., 2001).

b) Secondary Messenger:

Under normal conditions, intracellular levels of S1P are low and spatiotemporally regulated due to the balance between synthesis and degradation (Spiegel et al., 2003). However, cell proliferation and differentiation inducers, such as growth factors, GPCR agonists, cytokines, phorbol-esters, vitamin D3, and antigens, activate Sphks causing an increase in S1P intracellular levels enabling it to act as an intracellular secondary messenger (Chalfant et al., 2005; Taha et al., 2006; Spiegel et al., 2003).

As an intracellular second messenger, S1P inhibits the mitochondrial death pathway and the stress activated protein kinases such as JNK, and activates NF-κB, thus protecting

the cell from apoptosis (Spiegel et al., 2003). S1P induces NF-κB activation specifically in response to various cytokines such as TNF-α, making S1P a key mediator of inflammatory responses (Cowrat et al., 2008). In addition, the downstream targets of S1P include IP3-independent calcium mobilization and DNA synthesis. Thus S1P as a second messenger regulates calcium homeostasis, tumorigenesis, cell growth, and apoptosis (Chalfant et al., 2005; Kee et al., 2005; Spiegel et al., 2003).

3. Sphingosine 1-Phosphate Regulation

S1P is regulated mainly by two major enzymes; S1P lyase (SPl) and S1P phosphatases (SPPs). S1P lyase is localized on the cytosolic side of the ER and is responsible for cleaving S1P at the C2-C3 bond (Pyne, et al, 2000) producing two non-sphingolipid products, hexadecanal and ethanolamine phosphate (Fig-3). S1P lyase forms the major route of irreversible S1P degradation and constitutes the only known exit from the sphingolipid pathway since lysosomal degradation of sphingolipids yields sphingolipid metabolites that can be recycled back into the sphingolipid pathway (Spiegel et al., 2003). Thus the only way to remove ceramide and sphingosine that accumulate in excessive amounts and relieve the cell from their toxic effect would be by converting them into S1P, which can be cleaved by S1P lyase and consequently exit the lipid pool. The Sphks play an important role in this exit because the lyase acts only on phosphorylated sphingosine. (Fig-3) (Taha et al., 2006).

S1P phosphatases that belong to the family of magnesium-dependent, N-ethylmaleimide-insensitive type 2 lipid phosphate phosphohydrolases (LPPs), are also localized to the ER and mediate the reversible dephosphorylation of S1P to sphingosine

which can either be converted to ceramide by ceramide synthase or converted back to S1P by the action of Sphk as illustrated in Figure 3 (Levade et al., 2002, Spiegel et al., 2003).

Because of the key roles of S1P in tissue development and cellular functions, alterations in S1P metabolism are linked to the pathophysiology of many diseases, like Sjögren-Larsson syndrome, a rare autosomal recessive neurocutaneous disorder with infancy onset (Proia et al., 2015), sickle cell disease (Platt, 2000; Zhang et al., 2014), inflammatory bowel disease which is characterized by chronic destructive inflammation of the gastrointestinal tract (Liang et al., 2013), and cancer in which S1P is responsible for several key hallmarks of cancer development (Chae et al., 2004; Lee et al., 2010; Pyne et al., 2010).

S1P was shown, on the other side, to decrease the severity of certain diseases such as multiple sclerosis (MS) (Garris et al., 2013) through its effect on the immune, vascular, and nervous systems (Brinkmann et al., 2010), severe influenza infection and other acute respiratory diseases (Teijaro et al., 2011;Teijaro et al., 2014), acute lung injury (McVerry et al., 2004; Peng et al., 2004), and vascular and cardiac diseases (Keul, et al. 2007; Poti, et al., 2013; Skoura et al. 2011).

Emerging evidence provides a role for sphingolipids in hepatic cell death, which contributes to the development of several liver diseases including ischaemia-reperfusion liver injury, steatohepatitis and hepato-carcinogenesis (Wang et al., 2015). Several studies have demonstrated that sphingosine 1-phosphate is involved in inflammatory diseases such as LPS induced liver injury and hydrophobic bile acid apoptosis (Tian et al., 2016; Webster et al., 2016). S1P and through the stimulation of SIPR2 impairs insulin signaling in hepatocytes specifically during palmitate-induced insulin resistance (Fayyaz et al., 2014). In addition, S1P is involved in acute liver failure,

metabolic syndrome, control of blood lipid and glucose homeostasis, nonalcoholic fatty liver disease, and liver fibrosis (Rohrbach et al., 2017). González-Fernández, et al. (2017) demonstrated that inhibition of SphK1/S1P pathway in human hepatic stellate cells protected from liver fibrogenesis. S1P also has proliferative and anti-apoptotic effects in human liver sinusoidal endothelial cells (LSEC) by promoting the production of IL-6 and VEGF (Nowatari et al, 2015).

E. FTY720 (Fingolimod)

1. Overview

Fingolimod or FTY720 is derived synthetically from myriocin (ISP-1), a fungal metabolite produced by ascomycete, Isariasinclarii (Kiuchi et al., 2000). It is a structural analogue of sphingosine that can be phosphorylated by sphingosine kinases in the cell yielding its active form FTY720-P that shares striking structural homology with S1P and acts as an agonist at S1P receptors (Mandala et al., 2002; Brinkmann et al., 2010). Fingolimod is a potent immune modulator that has been shown experimentally to be effective against autoimmune diseases and is under preclinical and clinical studies considering its use in transplantation rejection treatment (Chiba et al., 2006; Budde et al., 2006). In fact, FTY720 has been recently approved by FDA as an oral treatment for relapsing forms of multiple sclerosis (Brinkmann et al., 2010).

2. Mode of action:

Once inside the cell, FTY720 is phosphorylated to its biologically active form fingolimod phosphate (FTY720-P) by the action of sphingosine kinases. However, only

SphK2 efficiently phosphorylates FTY720 while SphK1 exhibits weak activity on the drug (Billich et al., 2003). Upon phosphorylation, FTY720-P was reported to bind to any of the four S1P receptors (S1PR1, S1PR3, S1PR4 and S1PR5), with S1PR1 exhibiting the highest affinity for the drug (Mandala et al., 2002; Brinkmann et al., 2002). However, several recent studies have demonstrated that FTY720-P may also act through the receptor subtype S1PR2 (Alam et al., 2016; Sobel et al., 2013; Sobel et al., 2015). Thus, FTY720-P can make use of the many different signaling pathways activated by downstream S1P G-protein coupled receptors, to interfere and alter cellular functions. The effect of the drug is however dependent on the type of receptor expressed in the cell, and thus may exert different effects in different cells (Brinkmann et al., 2002). FTY720-P can promote the endocytosis and degradation of S1P receptors leading to ultimately an antagonistic effect (Brinkmann et al., 2002; Oo et al., 2007) to that of S1P.

Platelets and erythrocytes are able to efficiently import exogenous FTY720, but only platelets are able to phosphorylate it into FTY720-P and release it in plasma (Anada, et al, 2007), suggesting that platelets are the major source of plasma FTY720-P. The inability of erythrocytes to produce FTY720-P is because of the lack of SphK2 (Anada et al., 2007). FTY720-P release from the platelets may be stimulation-dependent and independent (Anada et al., 2007). Stimuli can induce the export of FTY720P, as in the case of S1P via ABC transporters (Anada et al., 2007).

3. FTY720: The immune-modulator

FTY720 was originally synthesized to minimize the toxicity of a highly potent and structurally related immunosuppressive agent, myriocin (ISP-1) (Fujita et al., 1994). Mechanistically, unlike other immune-suppressants, FTY720 does not impair T- and B-cell activation, proliferation or function but exerts its beneficial effects by preventing the

entry of auto-immune lymphocytes into the central nervous system (CNS) and their sequestration in the thymus and secondary lymphoid organs (Brinkmann et al., 2010; Chiba et al., 2006). Following their maturation in the thymus, T-lymphocytes circulate in the blood stream until they "home" to a secondary lymphoid organ such as lymph nodes where they react with antigens to become active, and proliferate and egress again into the circulation in response to S1P gradient. S1P and S1PR1 are highly expressed in both T-lymphocytes and endothelial cells, and are responsible for the release of lymphocytes from both the thymus and lymphoid organs (Sanchez et al., 2003; Matloubian et al., 2004; Allende et al, 2004).

After phosphorylation in vivo, FTY720-P prevents the egress of T-Lymphocytes out of the secondary lymphoid organs via two proposed models. The antagonistic model in which FTY720-P (Brinkmann et al., 2002) binds to S1PR1s on lymphocytes, causing their internalization and degradation thus, blocking the effect of S1P gradient. The agonistic model ascribes the effect of FTY720-P to its binding to S1PR1 on endothelial cells stimulating the assembly of cell-to-cell adherent junctions, strengthening barrier integrity and decreasing the permeability of endothelial cells to lymphocytes, preventing their exit from the lymphoid tissue (Sanchez et al, 2003).

Multiple sclerosis (MS) is an auto-immune disease in which the immune system attacks and destroys myelin sheath delaying the propagation of electric nerve impulses and resulting in communication problems between the nervous system (NIoNDaS, 2017) and the effectors. FTY720 sequesters T-lymphocytes in the lymphoid organs preventing them from attacking neurons as a result it is considered an effective treatment for relapsing forms of multiple sclerosis.

4. Medical Significance of FTY720

In addition to being an immune-modulator, FTY720 was shown to possess a neuro-protective effect in various murine disease models (Brunkhorst et al., 2014) including neuro-inflammation associated with axonal injury (Lee et al, 2009), ischemia (Hasegawa et al., 2010), prion (Moon et al., 2013), excitotoxicity and Rett syndrome (Deogracias et al., 2012). FTY720 was also found to modulate de – and/or re-myelination in MS mouse models, through a still unknown mechanism (Blanc et al., 2014). Moreover, Spiegel and colleagues have demonstrated recently that FTY720 in neurons inhibits specific histone deacetylases (HDACs) (Hait et al., 2014), but detailed molecular and cellular insights into its mode of action are not currently available. In fact, FTY720 was reported to affect gene expression in astrocytes (Cui et al., 2014) and Schwann cells (Heinen et al., 2015), axonal growth, as well as axonal regeneration in vivo (Anastasiadou et al., 2016). Thus, therapeutic use of FTY720 would affect both immune cells and neuronal functions and was found to be beneficial in various animal models of cardiac and renal fibrosis by attenuating the inflammatory response or inhibiting endothelial dysfunction in the microvasculature (Liu, et al., 2013; Ni, et al., 2013).

In the liver, FTY720 was reported to be effective in hepatic ischemia-reperfusion (I/R) and concanavalin A (conA)-induced liver injury (Man et al., 2005; Zeng et al., 2012), by improving acute phase inflammatory response and up-regulating several protective genes such as heat shock proteins and anti-apoptotic genes (Zhao et al., 2004; Man et al., 2005). Kong et al, have demonstrated that FTY720 is an effective therapy for liver fibrosis through suppressing the migration of bone marrow-derived mesenchymal stem cells (Kong Y. et al., 2014). FTY720 was also reported to suppress liver tumor metastasis by reducing the number of circulating endothelial progenitor cells (Li, C. X., et al., 2012) and exert a

strong antitumor effect on breast, liver, bladder and prostate cancer (Lee et al., 2005; Azuma et al., 2002; Azuma et al., 2003; Zhou et al., 2006).

5. Side effects of the drug

Some adverse effects are associated with FTY720 such as headache, diarrhea, back pain, liver enzyme elevations and bradycardia (Kappos et al., 2010), which suggests that the drug may interfere in the proper functioning of various organs (Mendelson et al., 2014).

F. Prostaglandin E_2 (PGE2)

Prostaglandins are active lipid metabolites that have multiple functions participating in normal tissue homeostasis, inflammation and cancer progression (Rundhaug et al., 2011; Li & Zhu, 2015). PGE2 particularly is involved in normal physiological functions such as regulation of renal blood flow and maintenance of the gastric mucosa (Rundhaug et al., 2011). The precursor of PGE2 is arachidonic acid (AA) which is released from diacylglycerol (DAG) by the action of phospholipase-A_2 and converted to prostaglandin H_2 through COX enzymes then further converted into PGE_2 by prostaglandin E synthase (Prescott & Fitzpatrick, 2000; Park et al, 2006). COX enzymes exist in two isoforms: COX-1 (Okuyama et al., 2002) and COX-2 (Lee et al, 2012). COX-1 is expressed in almost all tissues and is responsible for maintaining basal PGE2 levels required for carrying basic physiological functions. It maintains basal levels of COX products, mainly prostaglandins (PGs). COX-2 is not constitutively active but induced in response to various stimuli like cytokines, growth factors, and tumor promoters (Jones et al., 1993; Zhu et al., 2003), and plays a role in inflammation and carcinogenesis. Exceptionally, some cells exhibit a constitutive expression of COX-2 without stimulation like the interstitial cells of Cajal and

within muscle layers and neurons in the stomach (Porcher et al, 2002). PGE2 intracellular levels can be affected by its degradation via the action of 15-hydroxyprostaglandin dehydrogenase (15-PGDH) and its export via the prostaglandin transporter (PGT) (Nomura T, et al., 2004).

Many cytokines and growth factors such as IL-1β, TNF-α, hepatocyte growth factor (HGF), dichloroacetate (DCA) and lipopolysaccharide (LPS) enhance COX-2 expression. For example, IL-1β induces time dependently the expression of COX-2 mRNA by activating p38MAP kinase, kinase ½, extracellular signal regulated c-Jun kinase, or nuclear factor-kappa B (NF-κB) (Liu et al., 2003).

PGE2 binds and activates four G-protein coupled receptors called EP1, EP2, EP3, and EP4 (Sugimoto et al., 2007). The EP receptors have seven transmembrane domains and each receptor is coupled to different hetero-trimeric G protein. EP2 and EP4 are coupled to Gs, EP1 is linked to Gq, and EP3 to Gi. The binding of PGE2 to one of the different EP receptors leads to the activation of different downstream signaling pathways depending on the type of the coupled G protein (Fig-5) (Sugimoto et al., 2007).

EP1 receptor leads to the activation of phospholipase C (PLC) which in turn activates PKC and induces a rise in cytosolic free calcium via IP3 (Tang, et al, 2005). Both EP2 and EP4 receptors are known to activate adenylate cyclase resulting in higher cAMP levels and consequently higher PKA activity .Interestingly, EP4 but not EP2 is also coupled to phosphatidylinositol 3-kinase (PI3K) pathway known also as the PI3K/AKT pathway (Sugimoto, et al, 2007). Finally the EP3 receptor is coupled to Gi, which inhibits adenylate cyclase and its downstream pathway (Fig-5) (Narumiya, 2009). Different receptors have different binding affinities to PGE2 and thus the type of receptors and the signaling pathways activated depend on the level of PGE2 present. (Negishi, et al, 1993).

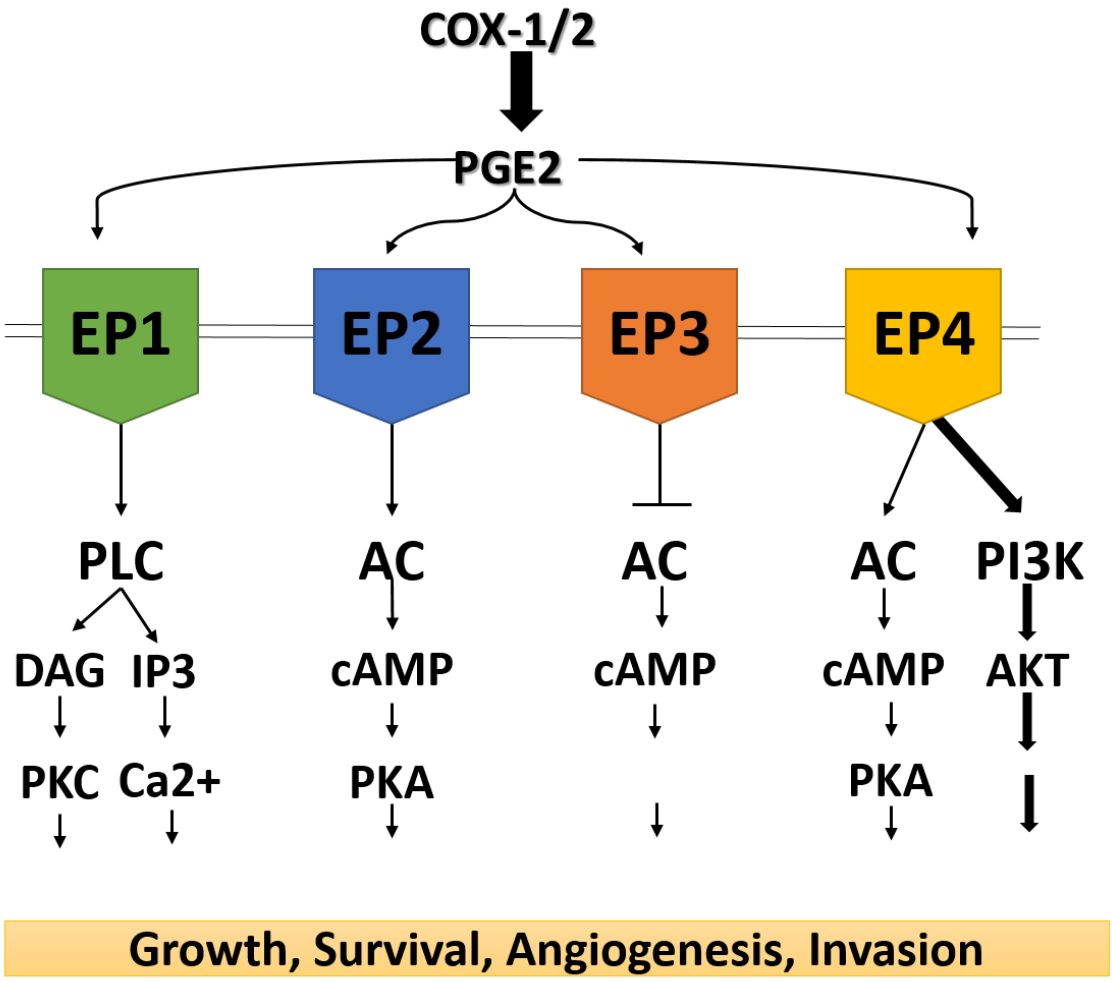

Figure 5: Canonical signaling pathways activated by the EP receptors of PGE2. Modified from Rundhaug J.E. et al, 2011.

Several studies have demonstrated a role for PGE_2 in the regulation of Na^+/K^+ ATPase activity. In fact, its effect in the kidneys has been extensively studied: PGE2 was shown to reduce the activity of the Na^+/K^+ pump in Madin-Darby canine kidney cells, a renal cell line with collecting duct properties (Cohen et al., 1993) as well as in the medullary thick ascending limb of Henle's loop (Wald et al., 1990). Also a modulatory role of the prostaglandin was shown in the proximal tubule cells (Libano-Soares et al., 2011). Moreover, Zeidel, et al (1991), have demonstrated a role for PGE2 in the interleukin-1-induced inhibition of the Na^+/K^+ ATPase in inner medullary collecting duct cells.

PGE2 regulates also the activity of the ATPase in other organs. In the intestine, the stimulatory effect of pentagastrin on intestinal adenylate cyclase and its inhibitory effect on the Na^+/K^+ ATPase were shown to be mediated via PGE2 (Sharon et al., 1981). In the rat colon (Markossian et al., 2005), liver (Kreydiyyeh et al., 2007) and cardiac myocytes (Skayain & Kreydiyyeh, 2006) it mediates the TNF-α effect on the Na^+/K^+ ATPase. FTY720P, an analogue of S1P was also found to inhibit the Na^+/K^+ ATPase by inducing PGE2 release in HepG2 cells (Alam et al., 2016).

CHAPTER III

MATERIALS AND METHODS

A. Materials:

FTY720P (FTY720P), Glyco-SNAP1and Carboxy-PTIO were purchased from Santa Cruz Biotechnology, CA, USA. PF-04418948 was obtained from Cayman Chemical Company, Michigan, USA.

Anti-ERK 1/2 rabbit polyclonal antibody was from Promega, WI, USA, while anti-p-ERK 1/2 rabbit polyclonal antibody was from Cell Signaling, MA, USA. Anti-COX2 rabbit polyclonal antibody was from Santa Cruz Biotechnology, TX, USA.

Phorbol-12-myrsitate-13-acetate (PMA), Adenosine-3',5'-cyclic Monophosphorothioate Rp-Isomer Triehtylammonium salt (RpcAMP), Calphostin C, and PD98059 were obtained from Calbiochem, San Diego, USA.

Prostaglandin (PGE2), Ouabain, Indomethacin, Dulbecco's Minimal Essential Medium (DMEM) with 4500mg/L Glucose and pyridoxine HCL, Trypsin-EDTA, Penicillin/Streptomycin, Fetal Bovine Serum (FBS), 10x Phosphate Buffered Saline (PBS) without magnesium and calcium, Adenosine 5'-triphosphate disodium salt (ATP), and 2'-O-Dibutryryladenosine 3',5'-cyclic monophosphate sodium salt (dbcAMP) were procured from Sigma, Chemical Co, St Louis Missouri, USA.

Protease inhibitor cocktail tablets were purchased from Boehringer Mannheim, Germany.

Biorad protein assay reagent, nitrocellulose membranes and western blotting luminol and peroxidase (Clarity TM Western ECL Substrate) reagent were obtained from Biorad, California, USA.

The Human hepatocellular carcinoma cell line, HepG2, was purchased from ATCC. All other chemicals were purchased from Sigma, Chemical Co, St Louis Missouri, USA.

B. Methods

1. Culture of HepG2 cells

HepG2 cells at passages 28-35 were grown in DMEM supplemented with 1% penicillin (100µg/ml) and streptomycin (100µg/ml) and 10% FBS at a density of 120,000 cells/ml on 100 mm culture plates. The cells were kept in humidified incubator (95% O2, 5% CO2) at 37°C and treated at 85-90% confluence after an overnight starvation.

2. Treatment of HepG2 cells:

a) Effect of FTY720P on the Na^+/K^+ ATPase activity

Following starvation, HepG2 cells were treated for 2 hours with 7.5 nM FTY720P. This concentration is the one used in our previous study showing an inhibitory effect of FTY720P on the activity of the Na^+/K^+ ATPase in HepG2 cells treated for 15 min with the drug. An equal volume of the carrier DMSO was added to the control. The cells were then washed with PBS buffer (pH=7.4), scraped in lysis buffer to which protease inhibitors were added (9.9 ml of 150mM histidine buffer pH=7.4, 400 µl protease inhibitor (1 tablet/2ml H2O), 100 µl Triton-X (1mg/mlH2O)), homogenized for 30 seconds using PRO

Homogenizer at maximum speed (around 30,000 rpm), and centrifuged for 30 min at 20000g and 4°C. Proteins in the cell lysates were quantified colorimetrically at a wavelength of 595 nm using the Bradford method and then the homogenates were used to assay for the ATPase activity.

b) Determination of the S1P receptors mediating FTY720P effect

In order to determine the type of S1P receptors involved in the FTY720P effect, S1P2 and S1P3 receptors were blocked respectively with JTE-013 (1 µM in DMSO) and CAY10444 (17.4 µM in dimethylformamide ,DMF). The blockers were added to the cells 15 min before FTY720P. The carriers were added in the same amounts to the controls.

The effect of specific agonist of the two receptors namely CYM5520 (2.5 µM in DMSO, S1PR2 agonist) and CYM5541 (2 µM in DMSO, S1PR3 agonist) was tested by adding them individually to the cells which were then incubated at 37°C for 2 hours. The carrier was added in the same amount to the controls.

c) Involvement of PGE2 and determination of the EP receptor involved

The involvement of PGE2 was determined by treating the cells, 30 min prior to FTY720P, with indomethacin, a COX inhibitor (100 µM, DMSO) or by direct treatment with exogenous PGE2 (100 nM in water, 2 hours).

To determine the type of EP receptor mediating PGE2 effect, EP2 receptors were blocked with PF-04418948 (1 µM in DMSO, 30 min before PGE2) or activated with the specific EP2 agonist Butaprost (4 µM in DMSO, 2 hours). Whenever DMSO was the carrier, it was added in the same amount to the controls.

d) Involvement of NO

Involvement of NO was tested by inhibiting its production by carboxy-PTIO (30µM, in DDW) or by using the NO donor Glyco-SNAP-1 (2 µM, in DDW).The cells were incubated with carboxy-PTIO for 20 minutes before addition of FTY720P.The incubation with SNAP was for 2 hours.

e) Involvement of PKA

Since receptors for S1P and PGE2 may be coupled to Gi or Gs, cAMP may be a potential mediator in the effect of FTY720-P on the ATPase. The involvement of cAMP/PKA was tested by treating the cells with an inhibitor of PKA namely RpcAMP added 20 min prior to FTY720P (30 µM in water) or by treating the cells with dbcAMP (10 µM in water), a cell permeable cAMP analogue for 2 hours.

f) Testing the involvement of PKC

Both S1PR and PGE2 receptors may be coupled to Gq proteins that activate PLC and eventually PKC. The involvement of PKC in the signaling pathway was tested by addition of a PKC inhibitor, calphostin (50 nM in DMSO) 20 min before FTY720P or by treating the cells with a PKC activator, PMA (100nM in DMSO) for 2 hours. Here again the carrier was added in the same amount to the control.

g) Involvement of ERK

Activation of the S1PR linked to Gi/o may lead to ERK activation (Rundhaug et al, 2011). The involvement of the kinase in the effect of FTY720P on the Na+/K+ ATPase was studied by inhibiting ERK with PD98059 (50 µM in DMSO), added 30 min before FTY720P. Activation of ERK was tested also by western blot analysis by investigating changes in the protein expression of phosphorylated ERK.

h) Determining NF-κB involvement

Since NF-KB is known to induce COX-2 expression, its involvement in the signaling pathway was investigated by incubating the cells with its specific inhibitor (15 μM, in DMSO) for 30 min before FTY720P and by studying the protein expression of I-κB, an inhibitory protein of the transcription factor, using western blot analysis.

i) Involvement of cGMP

The involvement of cGMP was investigated by incubating the cells with 8-bromo cGMP (0.5 mM in DMSO), a cell permeable cGMP analogue, for2 hours.

j) Locating the involved mediators with respect to each other in the signaling pathway

To know if PKC is upstream or downstream of PGE2, HepG2 cells were treated with PMA, a PKC activator in presence of indomethacin, an inhibitor of PGE2 synthesis, and with PGE2 in presence of calphostin, a PKC inhibitor.

To determine the position of PKA and NO relative to PGE2, cells were treated with PGE2 in presence of an inhibitor of PKA (RpcAMP) or a nitric oxide scavenger (PTIO). The inhibitors were added 20 minutes to the cell prior to PGE2. The cells were then incubated with PGE2 in presence or absence of the inhibitors for an additional 2 hour period. In addition, cells were treated with PKA activator (dbcAMP) or NO donor (SNAP) for 2 hours in the presence of indomethecin (COX inhibitor) which was added 20 minutes prior to the inhibitors addition.

To determine the position of phospho-ERK relative to PGE2 and COX-2, cells were treated with PGE2 in presence of an inhibitor of phosphor-ERK (PD98059). Western blot analysis was used also to study the protein expression of COX-2 enzyme in the presence of PD98059, using a specific anti-COX-2 primary antibody.

To determine the position of ERK relative to PKC, cells were treated with PMA (PKC activator) in the presence of phospho-ERK inhibitor (PD98059), and the activity of the ATPase was assayed. Western blot analysis was used also to investigate changes in the protein expression of phospho-ERK in cells treated with FTY720P in presence and absence of Calphostin, a specific PKC inhibitor.

To determine the position of NF-κB relative to phospho-ERK and COX-2, the protein expression of NF-κB was studied using western blot analysis in the presence of phopho-ERK inhibitor (PD98059) and COX-2 inhibitor (Indomethacin).

To determine the position of NO relative to PKA, cells were treated with SNAP (NO donor) in the presence of Rp-cAMP (a specific PKA inhibitor) added 20 minutes prior to the 2 hours incubation with SNAP. In addition, cells were treated with a PKA activator (dbcAMP) for 2 hours in the presence of an inhibitor of the NO scavenger, PTIO, which was added 20 minutes before dbcAMP.

3. The Na+/K+ ATPase Activity Assay:

At the end of the treatment, cells were scrapped after addition of lysis buffer, homogenized in the presence of histidine buffer (pH 7.4, 150mM) and the proteins were quantified. Cell homogenates were then diluted to a concentration 0.5 µg/µl with histidine buffer (pH 7.4, 150mM) and 65 µl of the homogenate were incubated with 17 µl of 1% saponin for 15 min at room temperature, followed by a 15 min incubation with 13 µl of phosphatase inhibitor cocktail [100µl of pyrophosphate (200mM), 100µlglycerophosphate (200mM), and 800µl of water]. Then 12 µl were taken from each sample and incubated with a mixture of NaCl (1240mM, 10µl), KCl (200mM, 10µl), $MgCl_2$ (40 mM, 10 µl), ATP (30mM, 10 µl), and histidine buffer (20 µl) for 30 min at 37°C in presence or absence

of 30 µl ouabain (15 mM, 30 µl). When ouabain was absent, it was replaced by 30 µl water. The reaction was stopped by adding 10µl of 50% Trichloroacetic acid.

The samples were then spun at a speed of 14000 rpm using Sigma 1-14 sartorius cintrifugator for 5 min and 90 µl were taken from the supernatant and added to 80 µl of Ferrous sulfate-molybdate reagent (0.5 mg Ferrous sulfate, 1 ml of ammonium molybdate (0.1g/L ammonium molybdate in 10N H_2SO_4), and 9 ml of water. This mixture gave a blue color reflecting the concentration of inorganic phosphate liberated that was quantified in a microplate reader at a wavelength of 750nm.

4. Western Blot Analysis

Forty micrograms of proteins were resolved on 10% SDS polyacrylamide gels (To 133 ml of 30% polyacrylamide were added 100 ml of Tris-HCl (1.5 M, pH= 8.8), 4 ml 10% SDS and 163 ml water) and then transferred to a nitrocellulose membrane. The membranes were then blocked for 30 min with blocking buffer (1L of 1x PBS, 1ml of Tween20, 3 g skimmed milk, and 10 ml 0.1% sodium azide), and then incubated overnight at 4°C with primary antibodies. The primary antibodies used were: rabbit polyclonal anti-ERK1/2, rabbit polyclonal anti-p-ERK1/2, rabbit polyclonal anti-IκB, rabbit polyclonal anti-COX-2, and mouse polyclonal anti-GAPDH. The membranes were then incubated with goat anti-rabbit or goat anti-mouse HRP conjugated IgG secondary antibodies for 1 hour at room temperature. The signal was detected by chemiluminescence by adding 1 ml luminol Clarity ECL Substrate. The intensity of the signal was detected using ChemiDoc^{TM}MP.

5. Statistical Analysis

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

CHAPTER IV

RESULTS

1. *Effect of FTY720-P on hepatic Na^+/K^+ ATPase activity:*

HepG2 cells treated with FTY720-P (7.5 nM) for two hours showed a significant increase in Na^+/K^+ ATPase activity ($p<0.0001$) (Fig-6).

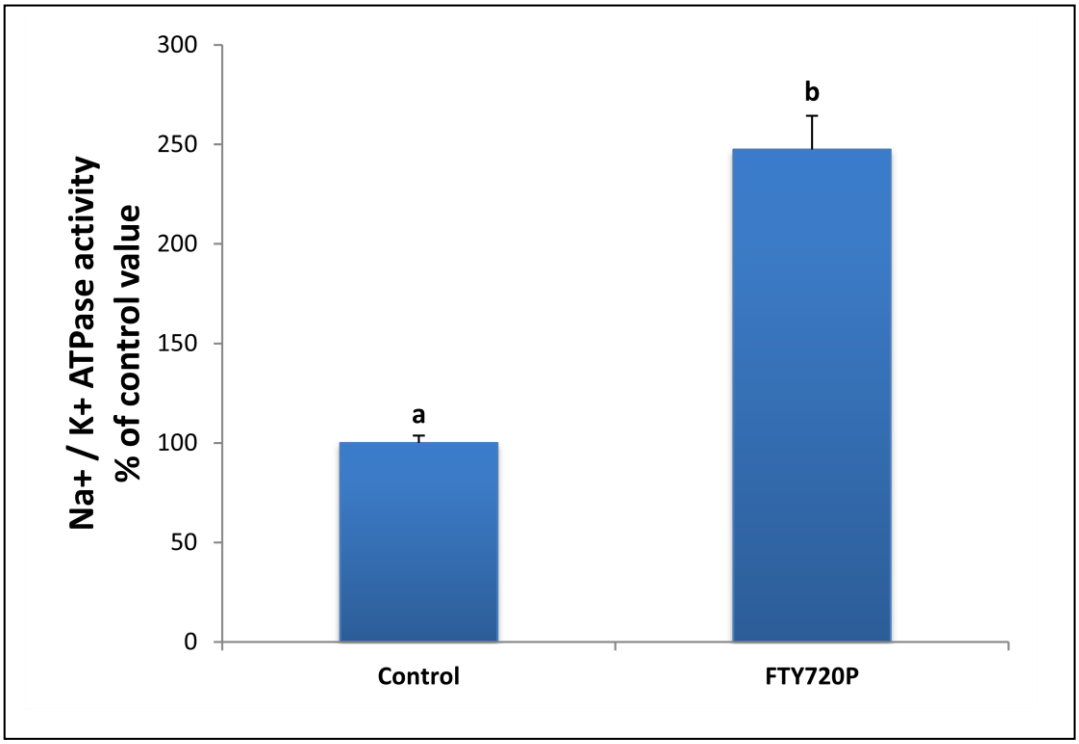

Figure 6: FTY720-P causes a 2.5 fold increase in the hepatic Na+/ K+ ATPase activity at 2 hours. Values are means ± SEM. N=14. Bars not sharing a common letter are statistically significant from each other as indicated by the one-tailed t-test. P< 0.0001.

2. *FTY720-P exerts its stimulatory effect via S1PR3*

To determine the type of receptors involved in FTY-720P's action, HepG2 cells were treated with FTY720-P in presence of blockers of the various receptors. The stimulatory effect of FTY720-P persisted in the presence of JTE-013 (blocker of S1PR2) ($q = 6.051$; $P<0.05$) (Fig-7) but disappeared totally in the presence of CAY1104 (17.4 µM, specific blocker of S1PR3s) (Fig-8). The S1PR3 agonist, Cym5541 (2 µM), exerted a similar stimulatory effect on the ATPase ($q= 13.309$; $P<0.001$) (Fig-8), while Cym5520 (2.5 µM), a S1PR2 agonist had no effect (Fig-8), confirming thus the involvement of S1PR3 in the stimulatory effect of FTY720P on the ATPase.

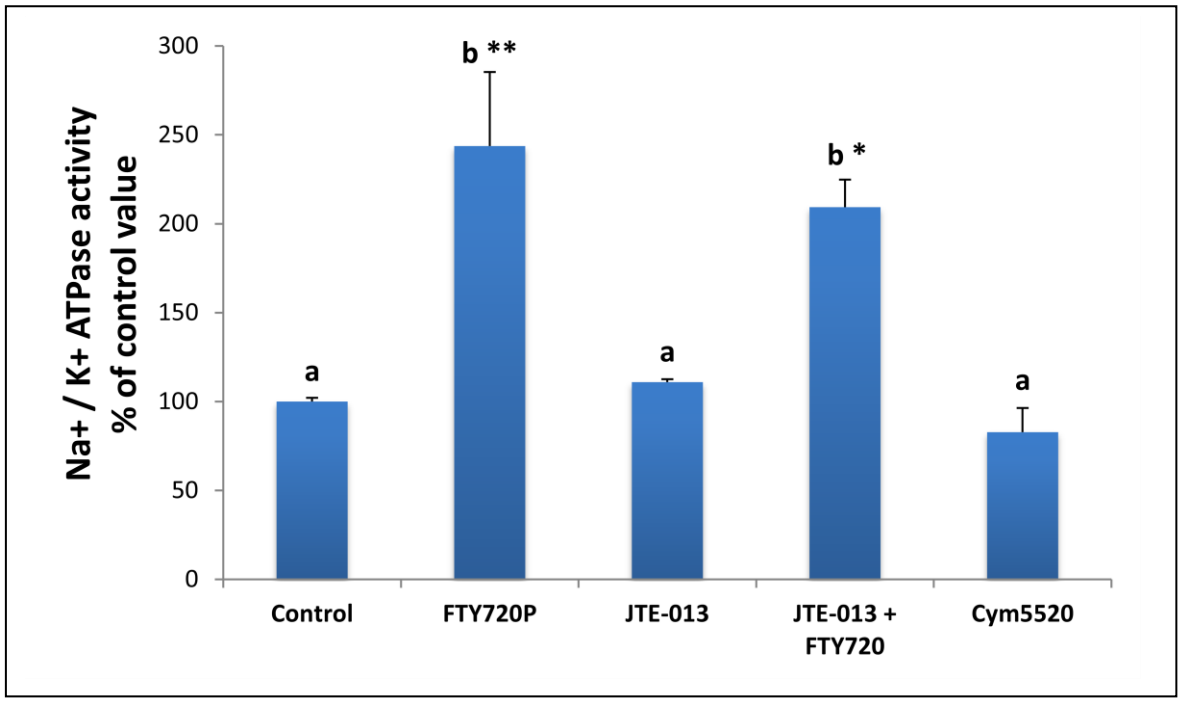

Figure 7: FTY720-P doesn't act *via* SIPR2. The stimulatory effect of FTY720-P (7.5 nM) persisted in the presence of S1PR2 antagonist (JTE-013: 1 µM) and the S1PR2 agonist (Cym5520: 2.5 µM) had no effect on the activity of the Na+/ K+ pump at 2 hours. Values are means ± SEM. N=3. Bars not sharing a common letter are statistically significant from each other.
*significantly different at $p<0.05$
**significantly different at $p< 0.01$

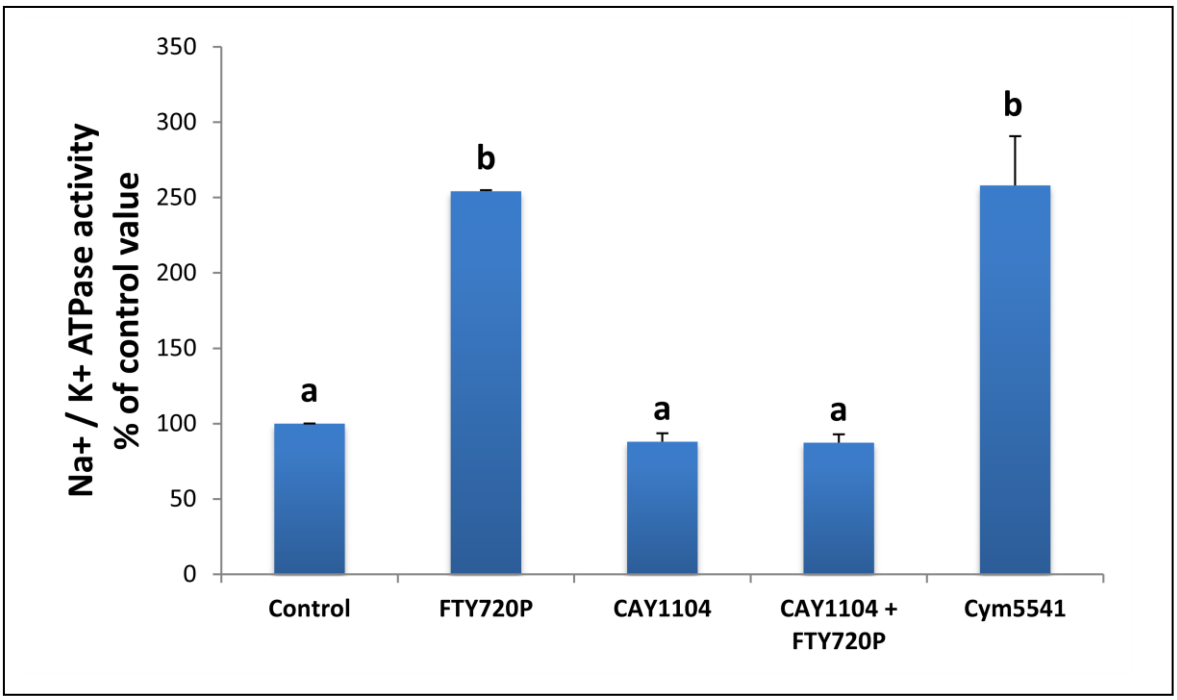

Figure 8: FTY720-P exerts its stimulatory effect *via* S1PR3. The stimulatory effect of FTY720-P (7.5 nM) disappeared in the presence of S1PR3 antagonist (CAY1104: 17.4 µM) while the S1PR3 agonist (Cym5541: 2 µM) exerted similar stimulatory effect on the Na^+/K^+ ATPase at 2 hours. Values are means ± SEM. N=3. Bars not sharing a common letter are statistically significant from each other.
*significantly different at $p<0.05$
**significantly different at $p< 0.01$

3. FTY720-P activates PKC

S1PR3 is coupled to Gq protein which is known to activate PKC (Jeong-Hun Kang, 2014). Treating the cells with PMA (100nM), a PKC activator resulted in a significant increase in the activity of the ATPase ($q = 7.2985$; $p<0.05$). Calphostin (50 nM) a PKC inhibitor, canceled the effect of FTY70-P (Fig-9).

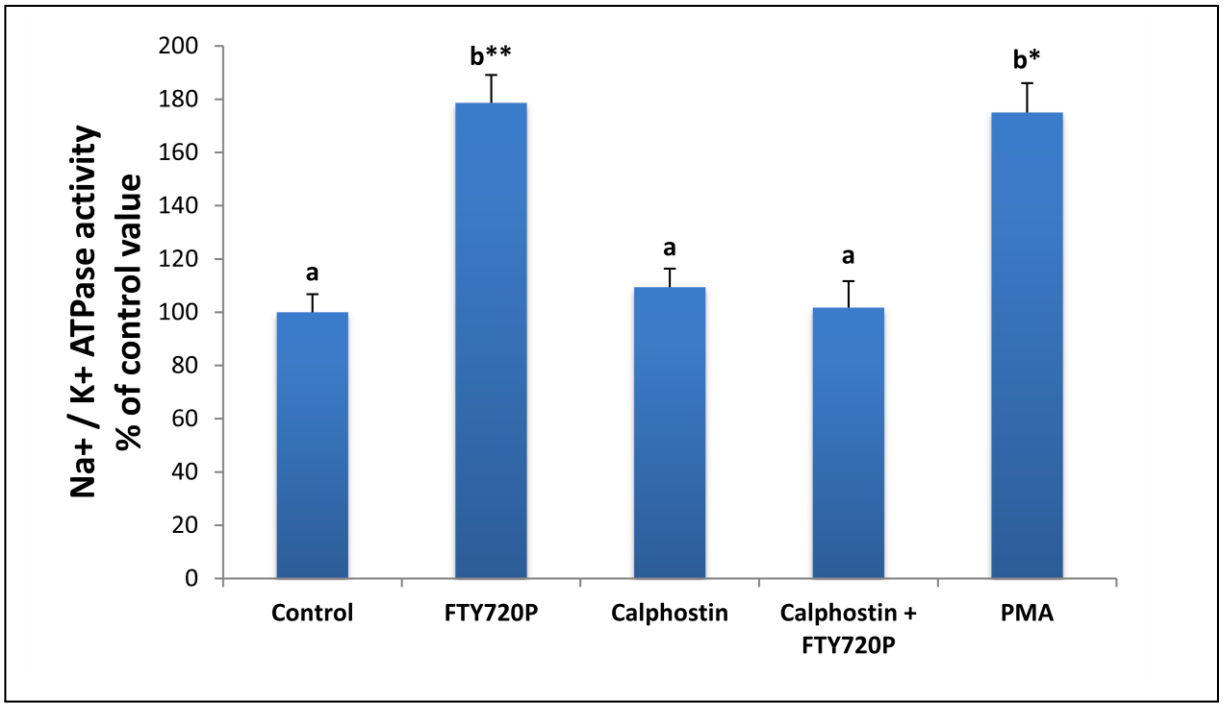

Figure 9: FTY720P activates the hepatic Na+/K+ ATPase by activating PKC. The PKC inhibitor (Calphostin: 50 nM) abolished the effect of FTY720P on the ATPase while the activator of PKC (PMA: 100nM) caused a similar stimulatory effect as FTY720P at 2 hours. The inhibitor was added 20 min before FTY720-P. Values are means ± SEM. N=3. Bars not sharing a common letter are statistically significant from each other.
*significantly different at $p<0.05$
**significantly different at $p< 0.01$

4. FTY720-P acts via PGE2

The effect of FTY720-P on the Na/+/K+ ATPase in HepG2 cells at 15 min was mediated by PGE2 (Alam et al, 2016). To check whether PGE2 is also a mediator of FTY720-P action at 2 hours, cells where treated with FTY720-P in presence of Indomecathin (100 µM), an inhibitor of COX enzymes. In this case the stimulatory effect of FTY720-P ($q = 22.7016$; $p<0.01$) did not appear (Fig-10). Indomecathin alone had no effect. Exogenous PGE2 (100 nM) exerted, similarly to FTY720-P, a stimulatory effect on the ATPase ($q = 11.8022$; $p<0.01$) (Fug-10). Western blot analysis showed a higher expression of COX-2 enzyme which is needed for PGE2 synthesis ($q = 8.074$, $P<0.01$) (Fig-11-a and 11-b).

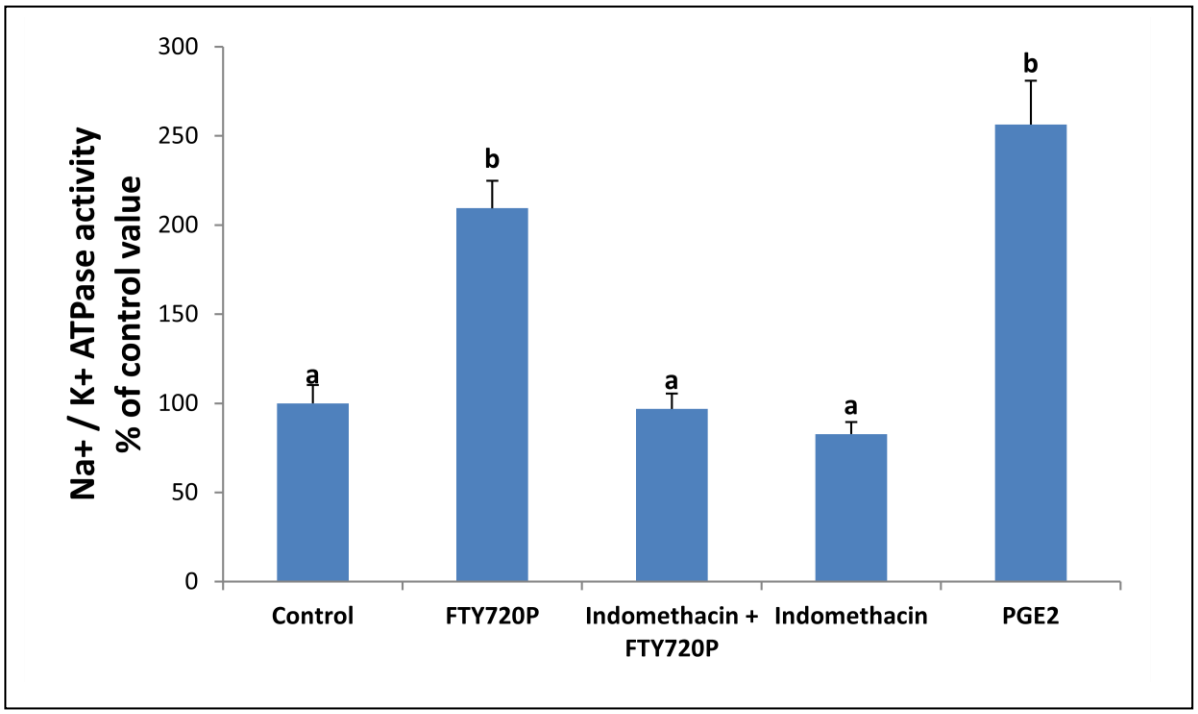

Figure 10: FTY720-P stimulatory effect on the hepatic Na^+/K^+ ATPase is mediated by PGE2. PGE2 (100 nM) increased the activity of the Na^+/K^+ ATPase, while the stimulatory effect of FTY720-P disappeared in the presence of COX enzymes inhibitor (Indomecathin: 100 µM) at 2 hours. Values are means ± SEM. N=3. Bars not sharing a common letter are statistically significant from each other at $p< 0.01$

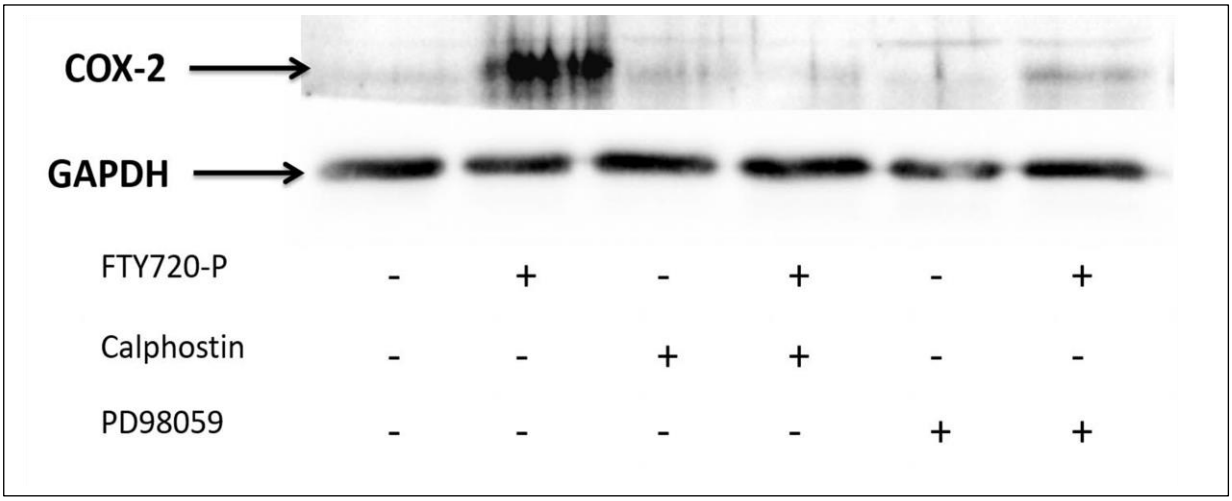

Figure 11-a: Effect of FTY720-P on COX-2 expression at 2 hours in presence of Calphostin and PD98059. The blot is representative of an experiment repeated 3 times. The blot is representative of an experiment repeated 3 times.

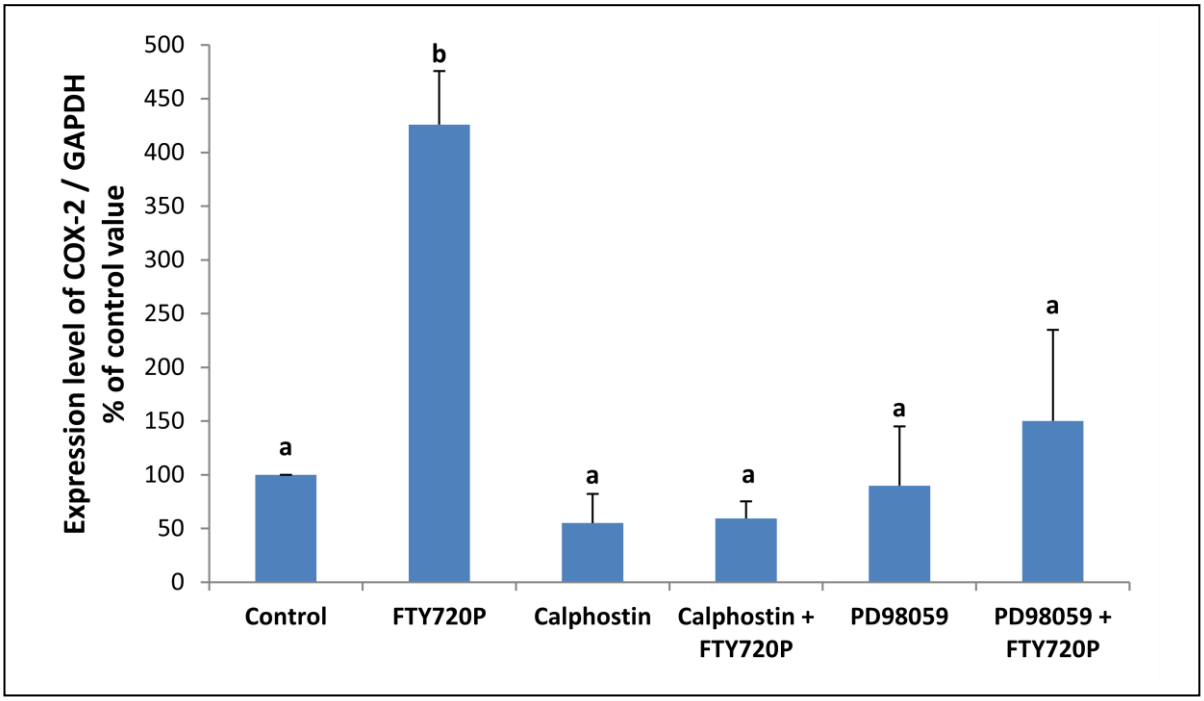

Figure 11-b: FTY720-P increases COX-2 expression relative to the control at 2 hours. This increase was diminished in the presence of PKC inhibitor (Calphostin: 50 nM) and ERK inhibitor (PD98059: 50 µM). Values were normalized to GAPDH using the image lab application, and reported as arbitrary densitometry units. N=3. Bars not sharing any common letter are statistically significant from each other at $P< 0.01$.

5. PGE2 acts via EP2 receptor

The stimulatory effect of PGE2 on the ATPase (q = 13.378; P<0.001) was mimicked by butaprost (4 µM), a specific EP2 receptor agonist, and abolished in presence of PF-04418948 (1 µM) (q = 6.309; P<0.01), an inhibitor of EP2 receptors. PF-04418948 alone had no effect on the pump (Fig-12).

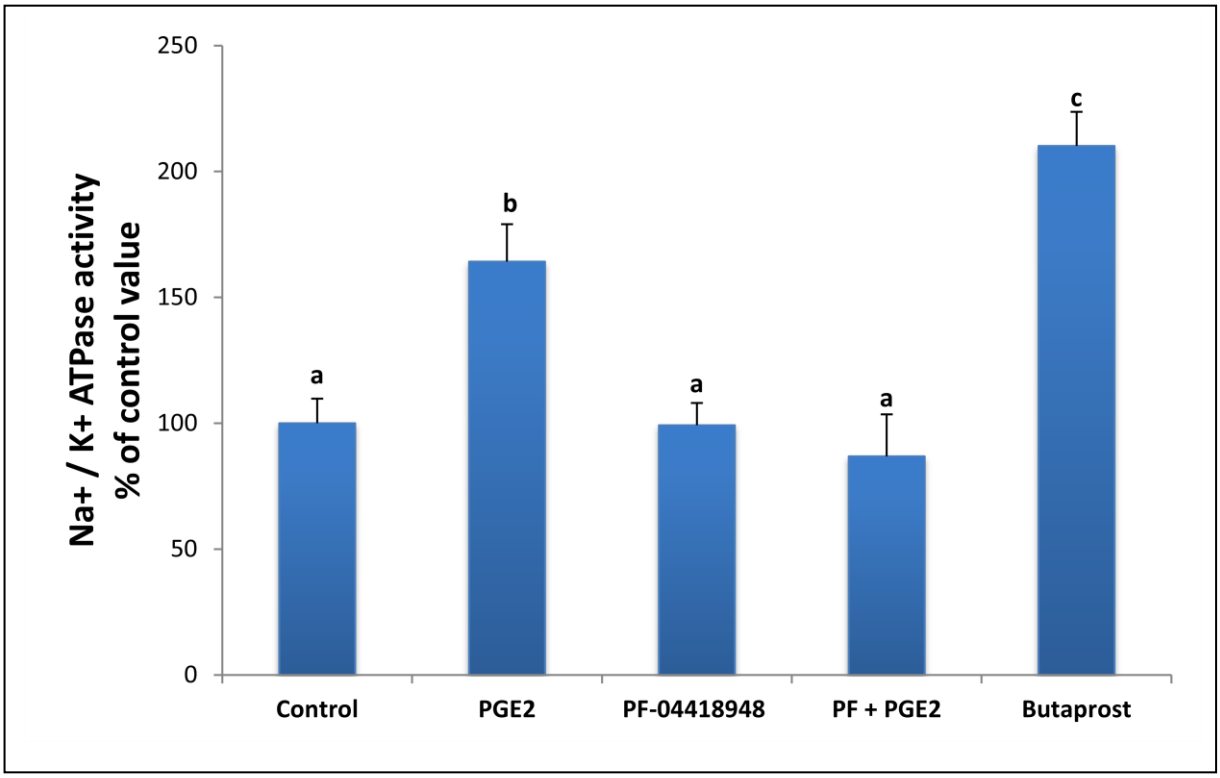

Figure 12: PGE2 acts *via* EP2 receptor. The stimulatory effect of PGE2 (100 nM) on Na^+/K^+ ATPase disappeared in the presence of EP2 receptor antagonist (PF-04418948: 1 µM) and EP2 agonist (Butaprost: 4 µM) mimicked the stimulatory effect of PGE2 at 2 hours. The inhibitor was added 20 min before PGE2. Values are means ± SEM. N=3. Bars not sharing a common letter are significantly different from each other at P< 0.01.

6. PKC is upstream of PGE2

The stimulatory effect of PGE2 (q =11.545; P<0.001) persisted in presence of Calphostin, a PKC inhibitor (Fig-13), while the PMA stimulatory effect on the pump (q =8.447; P<0.01) disappeared in presence of Indomecathin (COX enzymes inhibitor) (Fig-14). Calphostin and Indomecathin alone had no effect on the ATPase. Calphostin reduced the FTY720-P induced increase in the expression of COX-2 and brought it back to control levels (Fig-11-a, 11-b), indicating that PKC is upstream of PGE2.

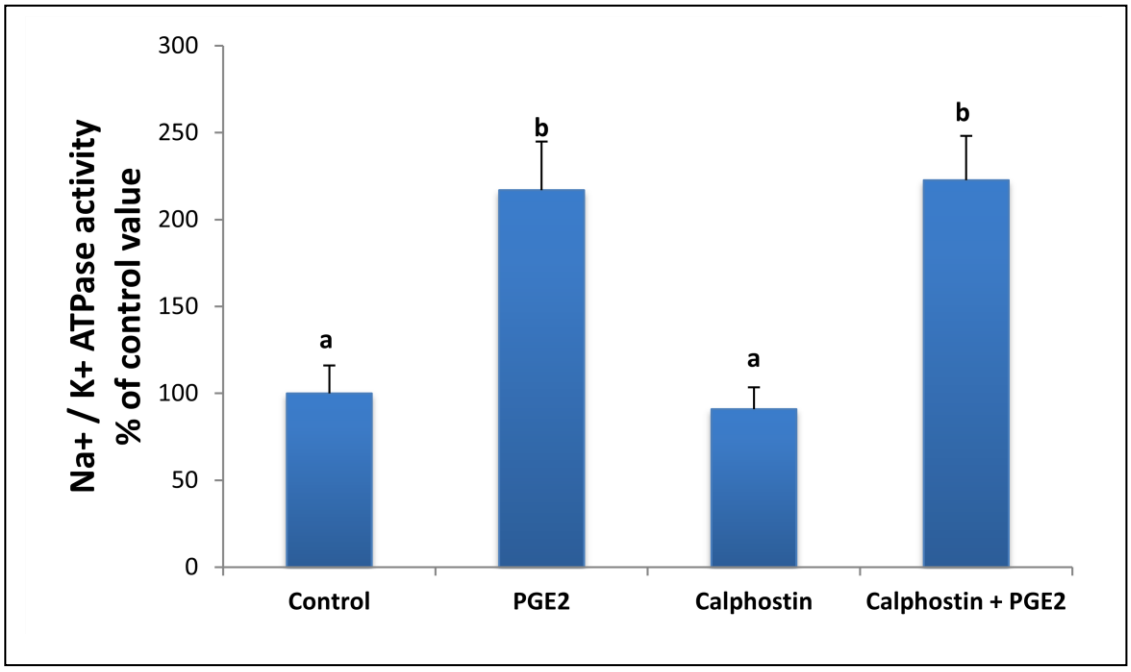

Figure 13: PKC acts upstream of PGE2. The stimulatory effect of PGE2 (100nM) on Na^+/K^+ ATPase at 2 hours disappeared in the presence of PKC inhibitor (Calphostin: 50 nM). The inhibitor was added 20 min before FTY720-P. Values are means ± SEM. N=3. Bars not sharing a common letter are statistically significant from each other at p< 0.001.

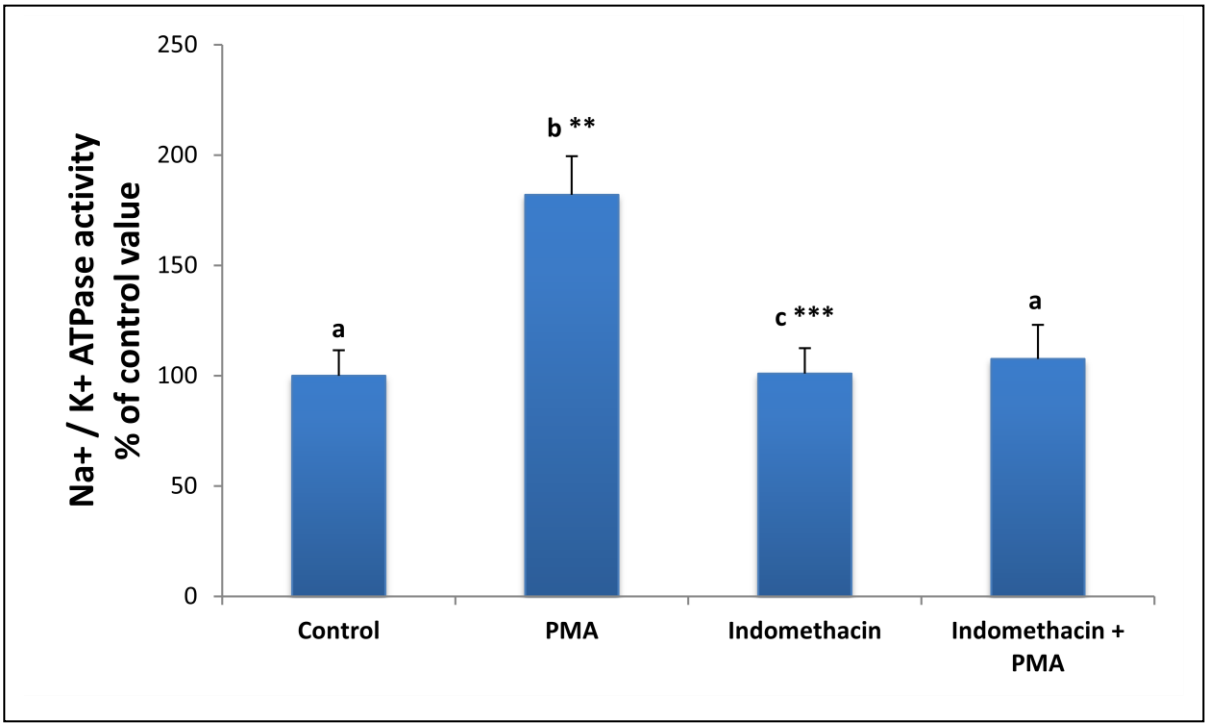

Figure 14: PKC acts upstream of COX enzymes. The stimulatory effect of the PKC activator (PMA: 100nM) on the Na^+/K^+ ATPase at 2 hours was lost in the presence of COX enzymes inhibitor (Indomecathin: 100 µM). Values are means ± SEM. N=3. Bars not sharing a common letter are statistically significant from each other at P< 0.01.

7. PGE2 acts via PKA

EP2 receptors are coupled to Gs proteins, which increase cAMP levels and activate PKA. Treating the cells with dbcAMP (10 µM), a PKA activator, increased significantly the activity of the ATPase. The stimulatory effect of FTY720-P (q =10.2083; P<0.01) disappeared in presence of RpcAMP (30 µM), an inhibitor of PKA. RpcAMP alone had no significant effect on the pump (Fig-15).

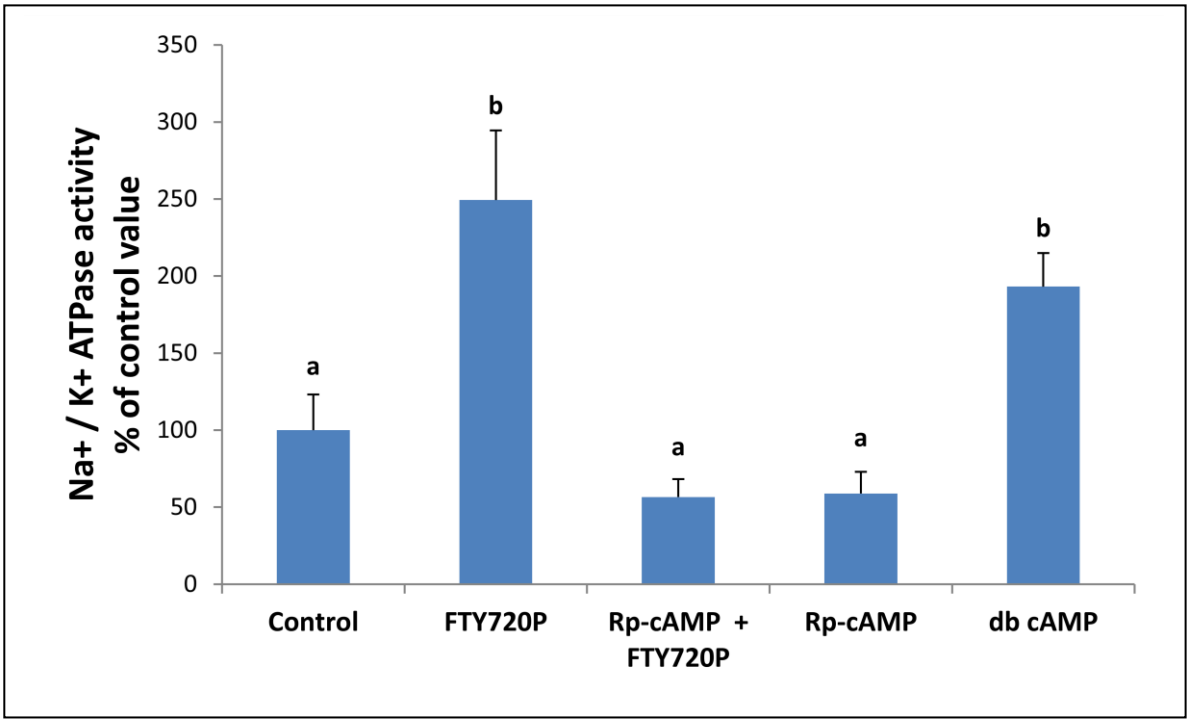

Figure 15: PGE2 acts *via* PKA. The stimulatory effect of FTY720-P (7.5 nM) on the Na^+/K^+ ATPase disappeared in the presence of the PKA inhibitor (Rp-cAMP: 30 µM) while the PKA activator (dbcAMP: 10 µM) increased the Na+/ K+ ATPase activity at 2 hours. The inhibitor was added 20 min before FTY720-P. Values are means ± SEM. N=3. Bars not sharing a common letter are significantly different from each other at p< 0.01.

8. PKA acts downstream of PKC and PGE2

The stimulatory effect of PMA (PKC activator) ($q = 15.198$; $P<0.001$) disappeared in presence of Rp-cAMP, a PKA inhibitor. Rp-cAMP alone had no effect on the pump (Fig-16). However, the stimulatory effect of db-cAMP (PKA activator) ($q = 7.554$; $P<0.01$) persisted in the presence of Calphostin, a PKC inhibitor, indicating that PKA acts downstream of PKC. Calphostin alone had no effect on the pump (Fig-17).

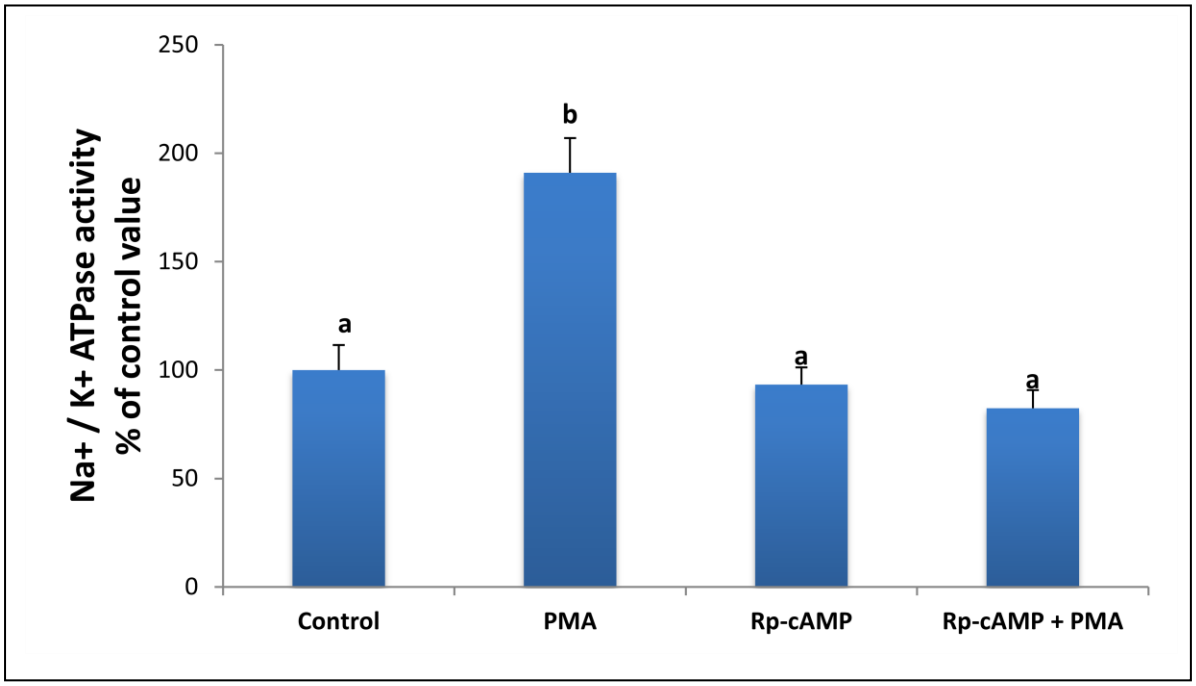

Figure 16: PKC acts upstream of PKA. The stimulatory effect of the PKC activator (PMA: 100nM) on the Na^+/K^+ ATPase at 2 hours disappeared in presence of the PKA inhibitor (Rp-cAMP: 30 µM). The inhibitor was added 20 min before PMA. Values are means ± SEM. N=3. Bars not sharing any common letter are significantly different from each other at $p< 0.001$.

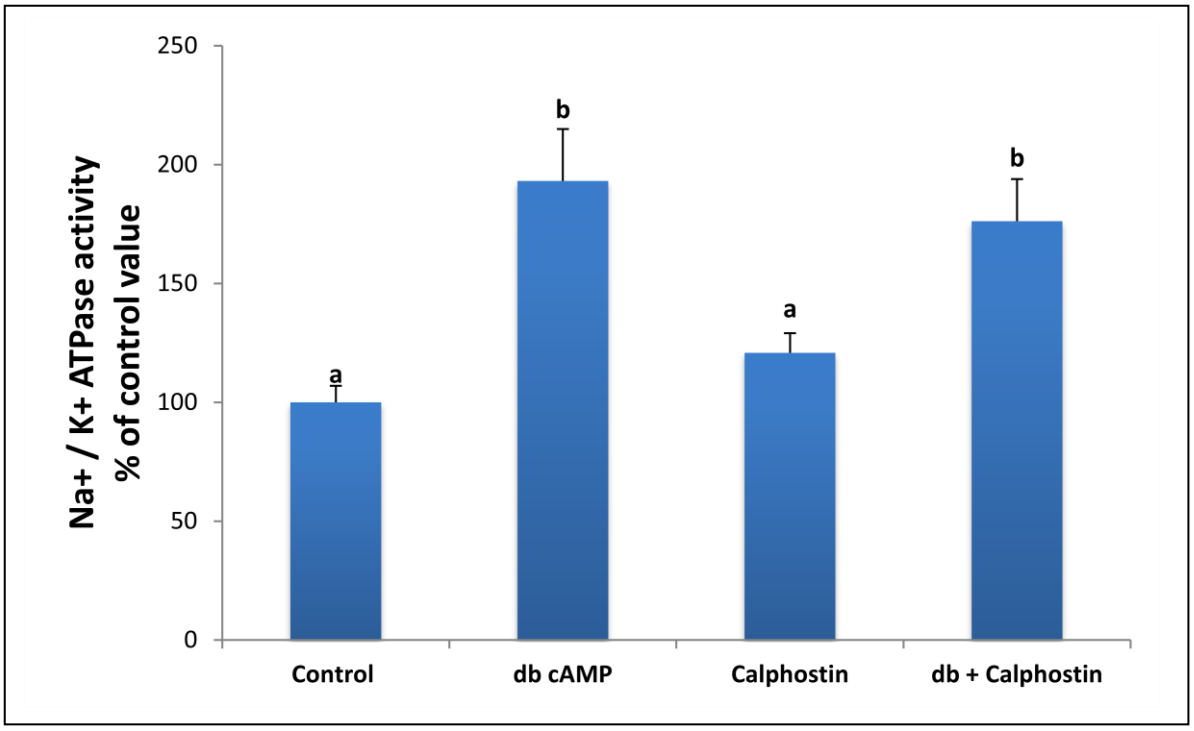

Figure 17: PKA acts downstream of PKC. The stimulatory effect of the PKA activator (db-cAMP: 10 µM) on Na^+/K^+ ATPase at 2 hours disappeared in the presence of the PKC inhibitor (Calphostin: 50 nM). The inhibitor was added 20 min before dbcAMP. Values are means ± SEM. N=3. Bars not sharing a common letter are significantly different from each other at $P< 0.01$.

The stimulatory effect of db-cAMP on the pump (q =5.170; P<0.05) was still observed in presence of Indomecathin (COX enzymes inhibitor) (Fig-18), while the stimulatory effect of PGE2 disappeared when PKA was inhibited with Rp-cAMP (q =13.281; P<0.001) (Fig19).

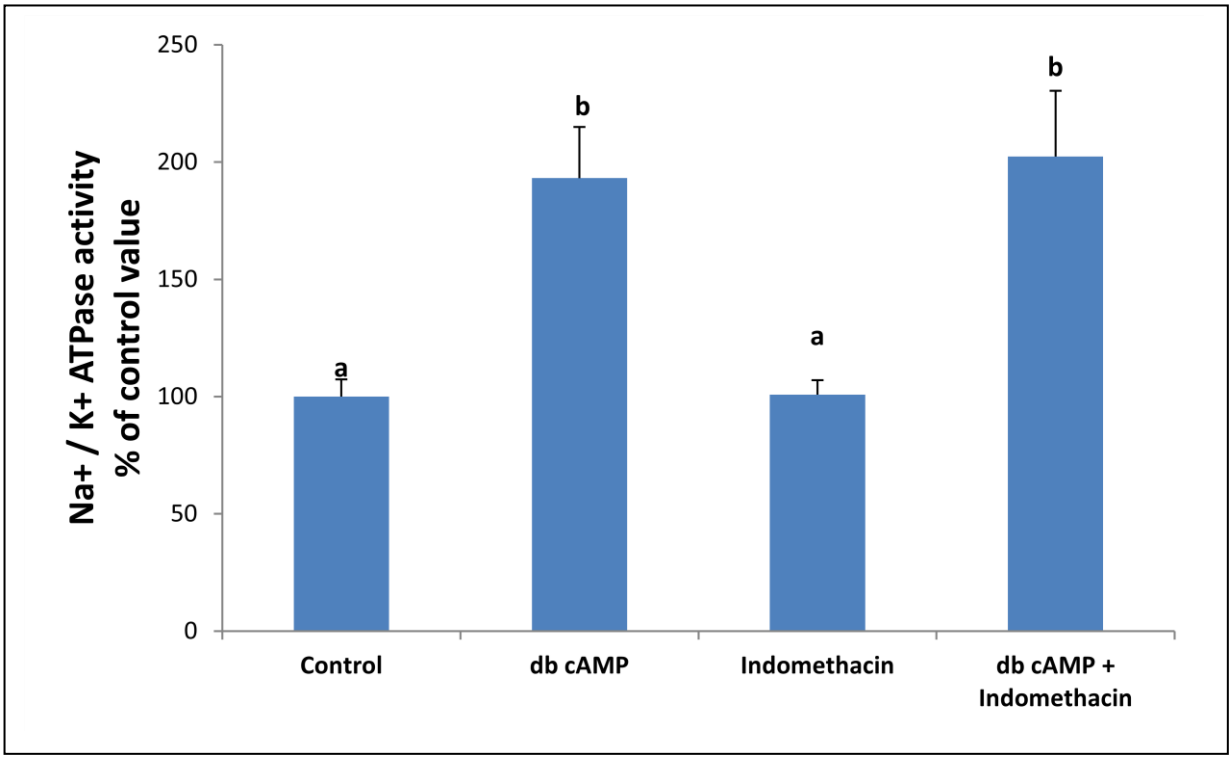

Figure 18: PKA acts downstream of COX enzymes. The stimulatory effect of the PKA activator (db-cAMP: 10 µM) on Na^+/K^+ ATPase at 2 hours disappeared in the presence COX enzymes inhibitor (Indomecathin: 100 µM). The inhibitor was added 20 min before. Values are means ± SEM. N=3. Bars not sharing a common letter are statistically significant from each other at p< 0.01.

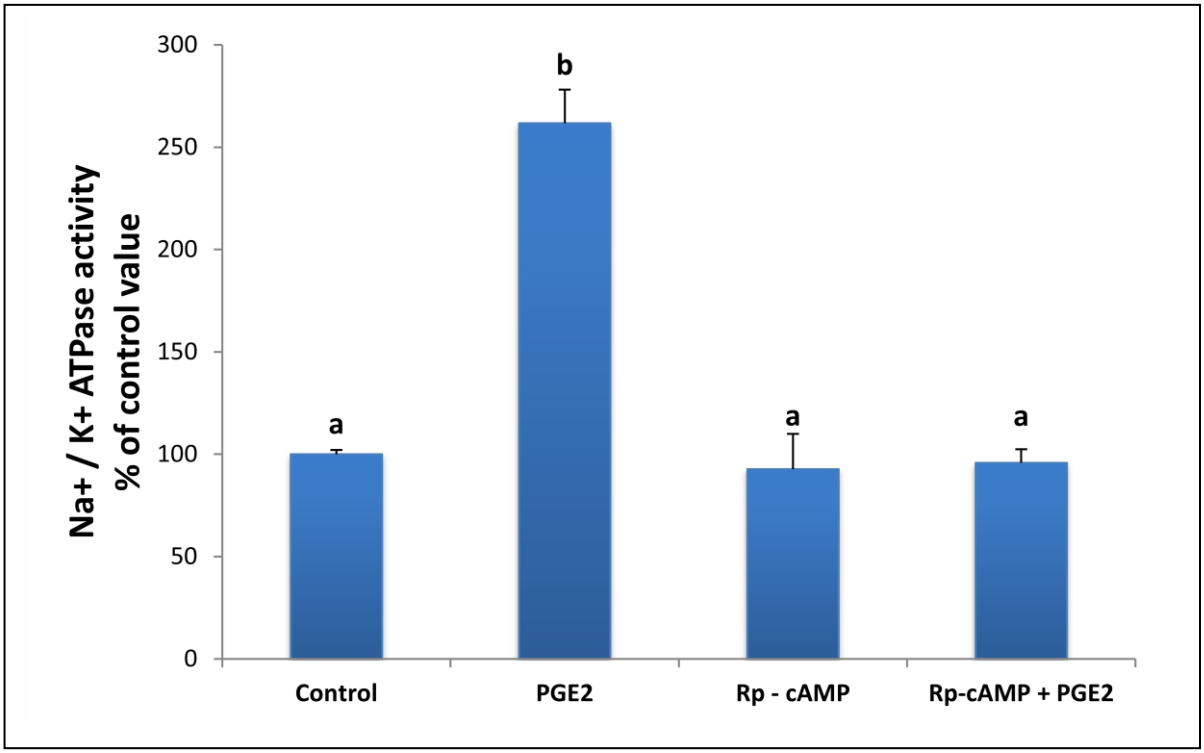

Figure 19: PGE2 acts upstream of PKA. The stimulatory effect of PGE2 (100 nM) on Na^+/K^+ ATPase at 2 hours disappeared in the presence of the PKA inhibitor (Rp-cAMP: 30 µM). The inhibitor was added 20 min before PGE2. Values are means ± SEM. N=3. Bars not sharing a common letter are statistically significant from each other at $p<0.001$.

Taken together, the results indicate that PKA is downstream of PGE2.

9. ERK is a mediator of FTY720-P and acts downstream of PKC

Activation of the $S1PR_3$ is linked to Gi/o in addition to Gq which may lead to ERK activation (Rundhaug et al, 2011). To study the involvement of ERK in the effect of FTY720-P on the ATPase, HepG2 cells were treated with FTY720-P in presence of PD98059 (50 µM), an inhibitor of ERK. FTY720P could not stimulate the ATPase when ERK was inhibited ($q = 10.654$; $P<0.01$) (Fig-20). Similarly, the stimulatory effect of PMA did not appear in presence of PD98059 ($q = 8.705$; $P<0.01$) (Fig-21). PD98059 alone had no effect on the pump. FTY720-P increased the expression of phospho-ERK ($q=7.099$, $P<0.01$). But the increase was not observed in the presence of Calphostin, PKC inhibitor (Fig 22-a, and 23-b).

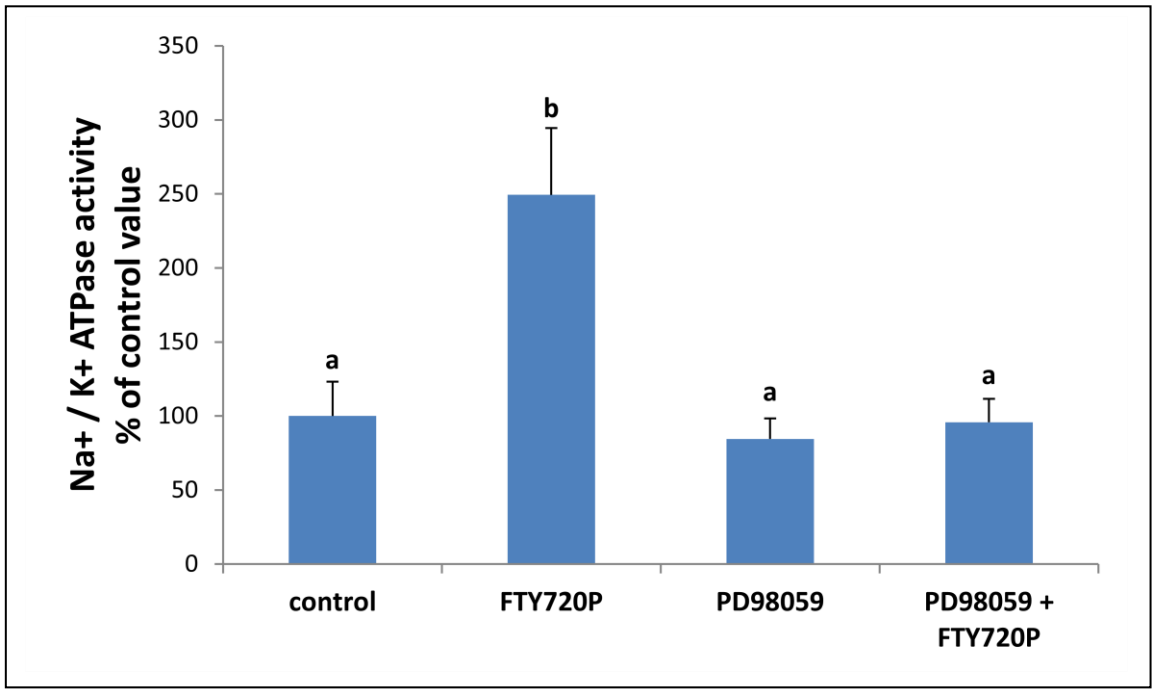

Figure 20: FTY720-P increases the activity of the hepatic Na^+/K^+ ATPase by activating ERK. The stimulatory effect of FTY720-P (7.5 nM) on Na^+/K^+ ATPase at 2 hours disappeared in the presence of the ERK inhibitor (PD98059: 50 µM). The inhibitor was added 20 min before FTY720-P. Values are means ± SEM .N=3. Bars not sharing a common letter are statistically significant from each other at $P< 0.01$.

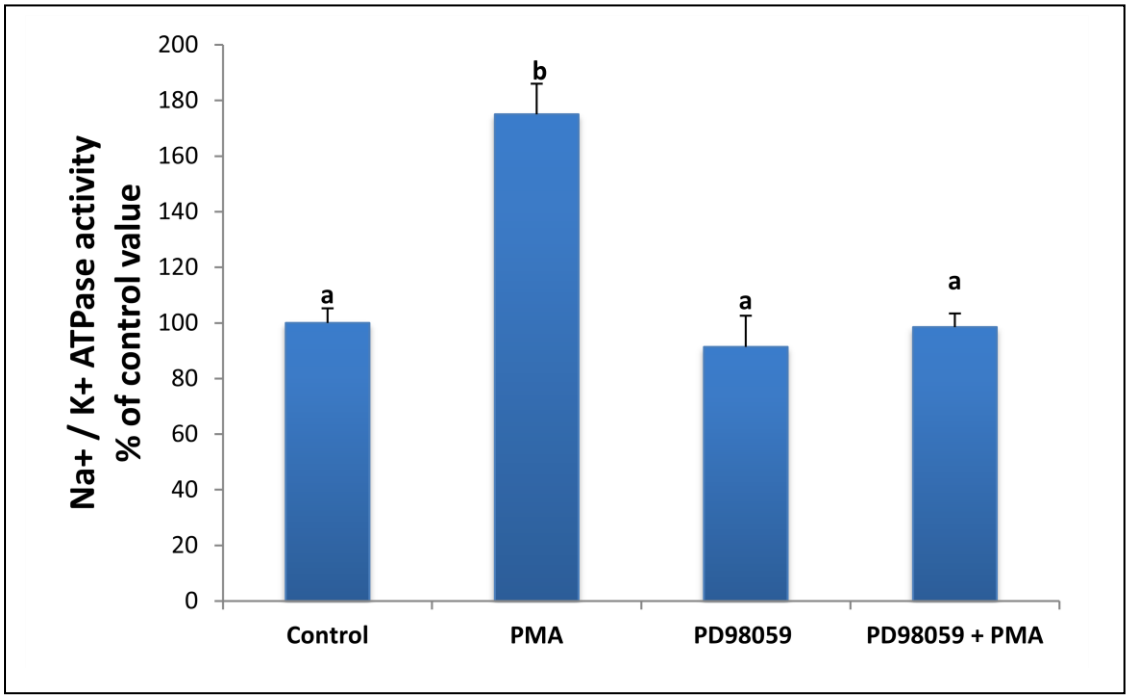

Figure 21: ERK acts downstream of PKC. The stimulatory effect of the PKC activator (PMA: 100nM) on the Na^+/K^+ ATPase activity at 2 hours was cancelled in the presence of ERK inhibitor (PD98059: 50 µM). The inhibitor was added 20 min before PMA. Values are means ± SEM. N=3. Bars not sharing a common letter are statistically significant from each other at $P<0.01$.

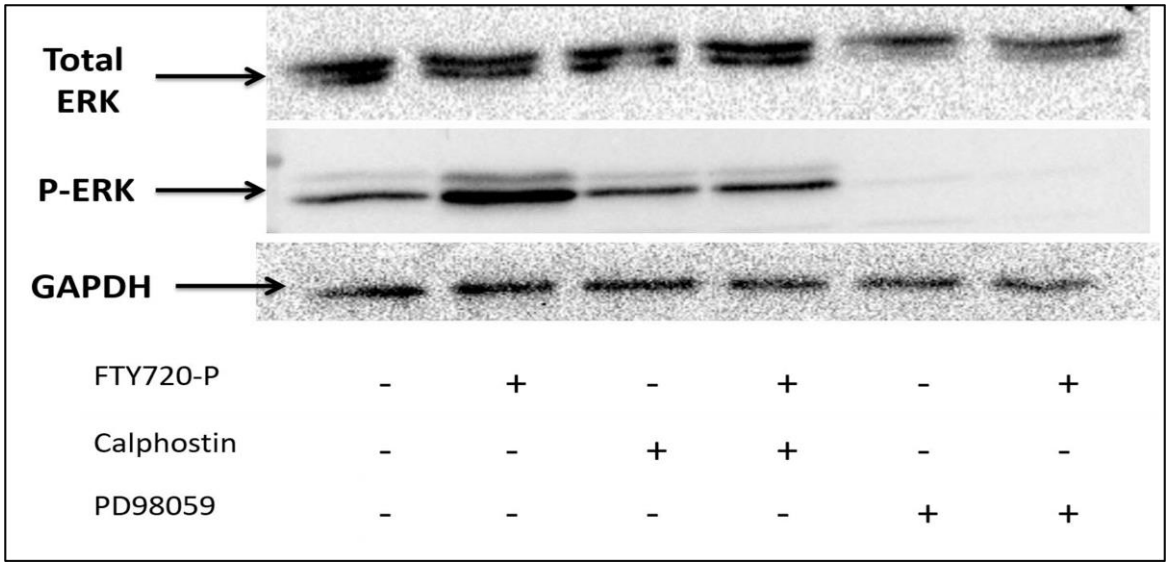

Figure 22-a: Effect of FTY720-P on phosphorylated ERK expression at 2 hours in the presence of PKC (Calphostin 50 nM) and ERK inhibitor (PD98059: 50 µM). The blot is representative of an experiment repeated 3 times.

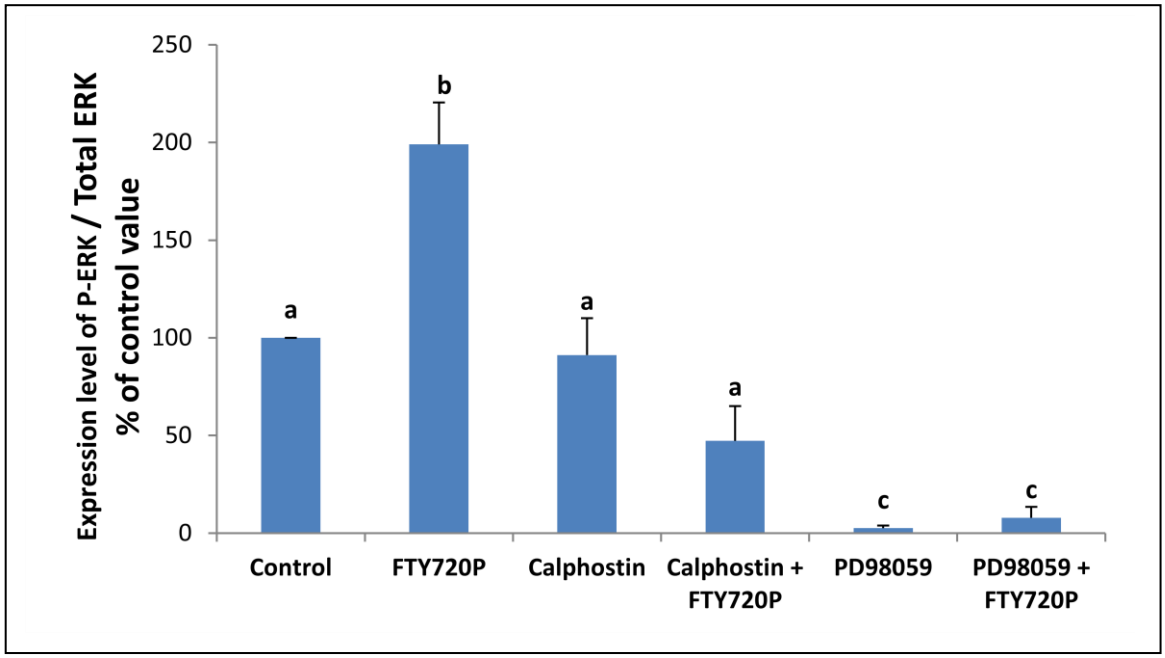

Figure 22-b: Effect of FTY720-P (7.5 nM) on phosphorylated ERK expression relative to the control at 2 hours in the presence of PKC inhibitor (Calphostin: 50 nM) and ERK inhibitor (PD98059: 50 µM). Values were expressed as arbitrary densitometry units and normalized to total ERK. N=3. Bars not sharing a common letter are statistically significant from each other at $P<0.01$.

10. P-ERK acts before PGE2

Inhibiting ERK with PD98059 did not affect the stimulatory effect of PGE2 on the ATPase ($q = 5.187$; $P<0.05$) (Fig-23), but abolished the FTY720P-induced increase in the expression of COX-2 (Fig11-a and 11-b). The results suggest that ERK is located before PGE2.

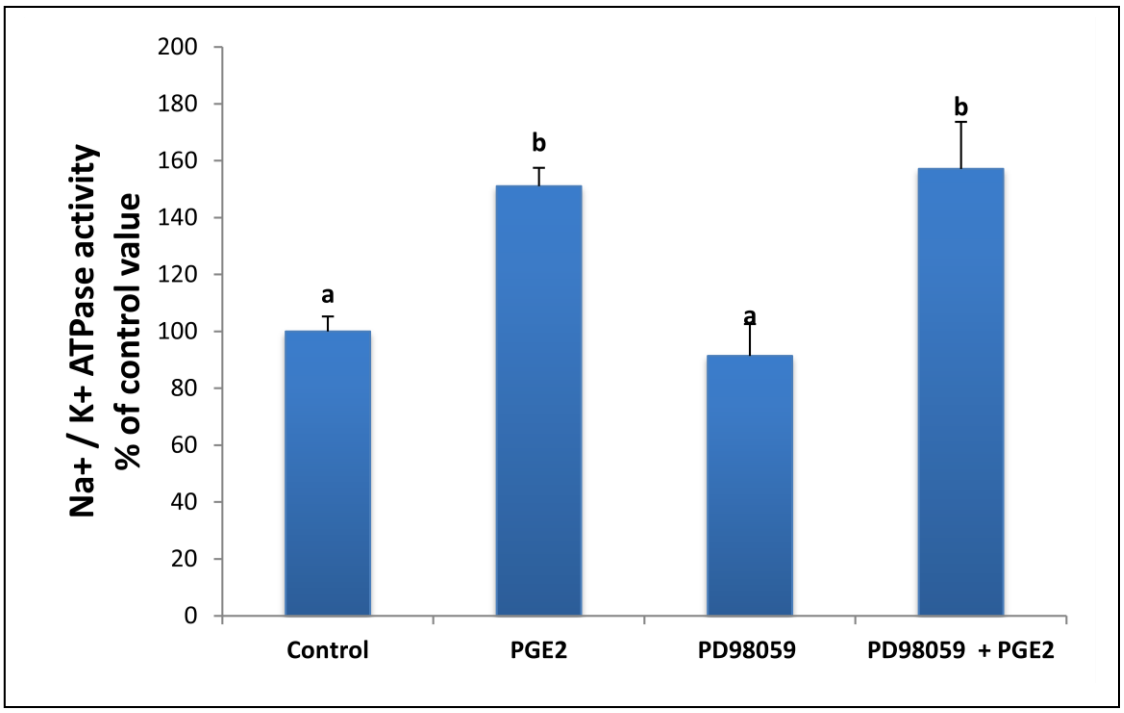

Figure 23: P-ERK acts upstream of PGE2. The stimulatory effect of PGE2 (100 nM) on the Na^+/K^+ ATPase at 2 hours disappeared in the presence of ERK inhibitor (PD98059: 50µM). Values are means ± SEM. N=3. Bars not sharing a common letter are significantly different from each other at $P< 0.05$.

11. NO is a downstream mediator of PGE2

Previous work in our lab identified NO as a downstream mediator of PGE2. Cells treated with the NO donor Glyco-SNAP-1 (2 µM) for 2 hours, showed a significant increase in their Na^+/K^+ ATPase activity (q =5.226; P<0.05) even when PGE2 synthesis was blocked with indomethacin, while the stimulatory effect of PGE2 at 2hrs (q =10.975; P<0.001) disappeared in presence of carboxy-PTIO (30µM), an NO scavenger. Carboxy-PTIO alone had no effect on the pump (Fig-24). Thus, NO acts downstream of PGE2.

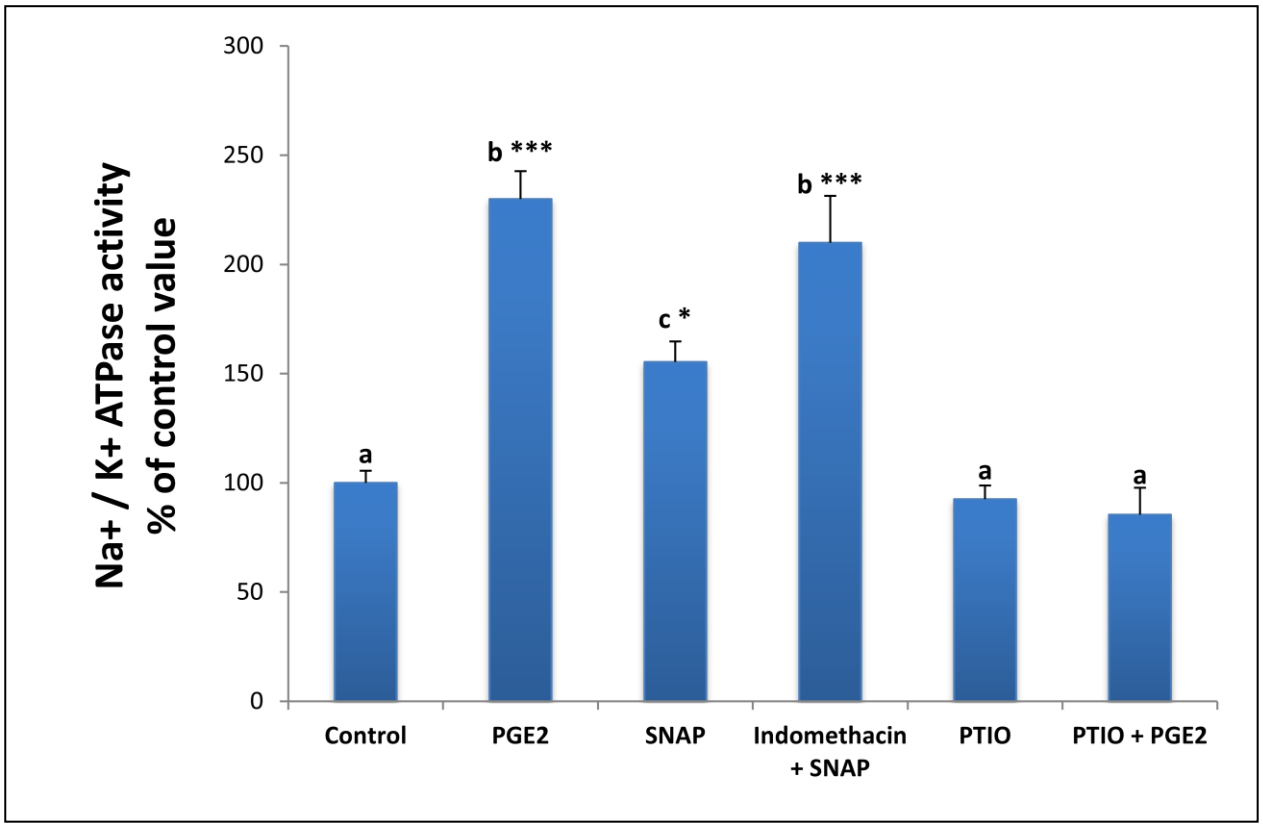

Figure 24: PGE2 exerts its stimulatory effect on the Na^+/K^+ ATPase *via* NO. The stimulatory effect of PGE2 (100nM) was cancelled in the presence of NO inhibitor (carboxy-PTIO: 30µM) and the NO donor (Glyco-SNAP-1: 2 µM) increased the Na^+/K^+ ATPase activity at 2 hours. Values are means ± SEM. N=3. Bars not sharing a common letter are significantly different from each other.
*significantly different at P< 0.05
***significantly different at P< 0.001

12. NO acts downstream of PKA

PKA inhibition with Rp-cAMP, did not affect the stimulatory effect of Glyco-SNAP-1 ($q = 5.226$, $P<0.05$) on the Na^+/K^+ ATPase (Fig-25). Moreover, the stimulatory effect of db-cAMP was not manifested in presence of carboxy-PTIO. Carboxy-PTIO alone and Rp-cAMP alone had no effect on the pump (Fig-26) indicating that NO acts downstream of PKA.

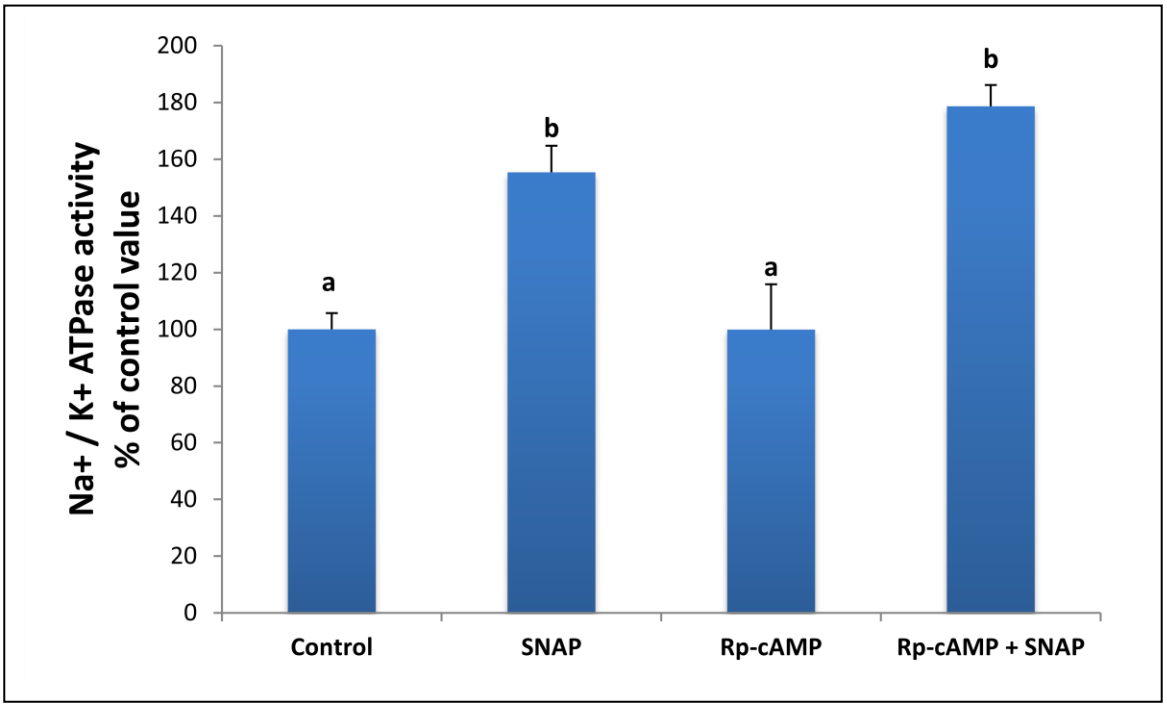

Figure 25: NO acts downstream of PKA. The stimulatory effect of the NO donor (SNAP: 2 µM) disappeared in the presence of PKA inhibitor (Rp-cAMP: 30 µM). Values are means ± SEM. N=3. Bars not sharing a common letter are significantly different from each other at $P<0.05$.

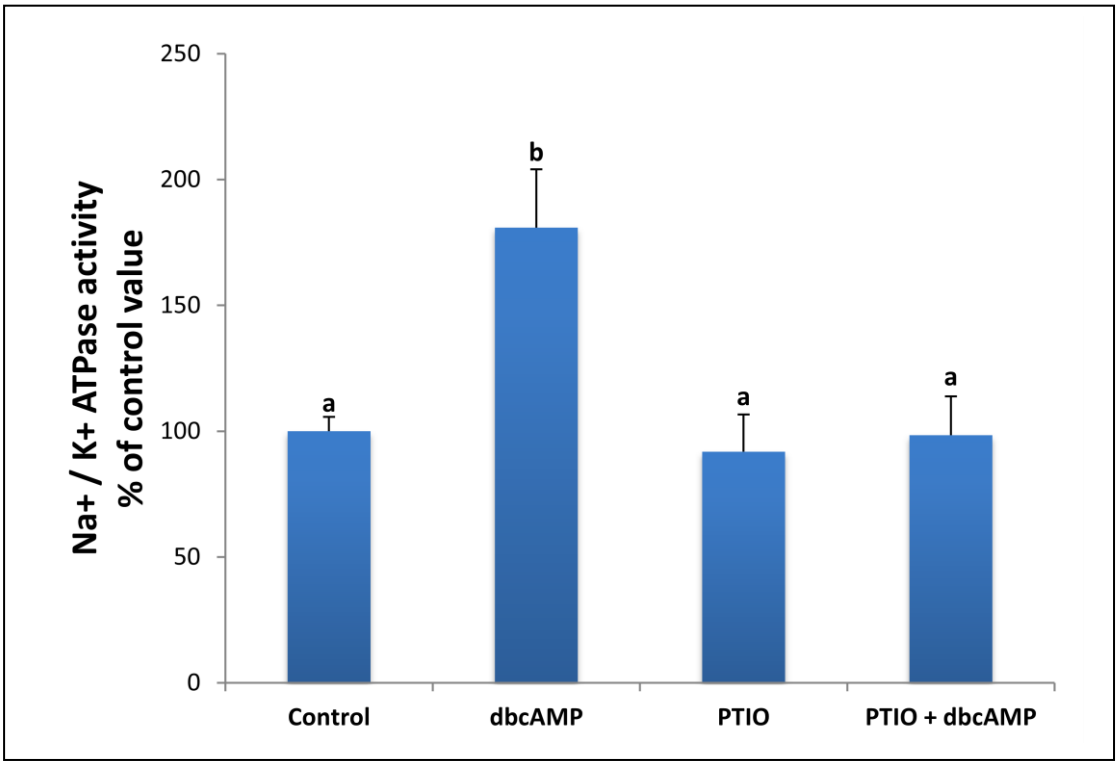

Figure 26: PKA acts upstream of NO. The stimulatory effect of the PKA activator (db-cAMP: 10 µM) on the Na^+/K^+ ATPase at 2 hours disappeared in the presence of NO inhibitor (PTIO: 30µM). Values are means ± SEM. N=3. Bars not sharing a common letter are significantly different from each other at $P<0.05$.

13. FTY72O-P acts via NF-κB

The stimulatory effect of FTY720-P at 2hrs (q =5.062; P<0.05) disappeared in presence of NF-κB inhibitor. The NF-κB inhibitor alone had no effect on the pump (Fig-27). The involvement of NF-κB in the stimulatory effect of FTY720P was further confirmed by the reduced expression of IκB. (q=11.013, P<0.001) (Fig 28-a and 29-b)

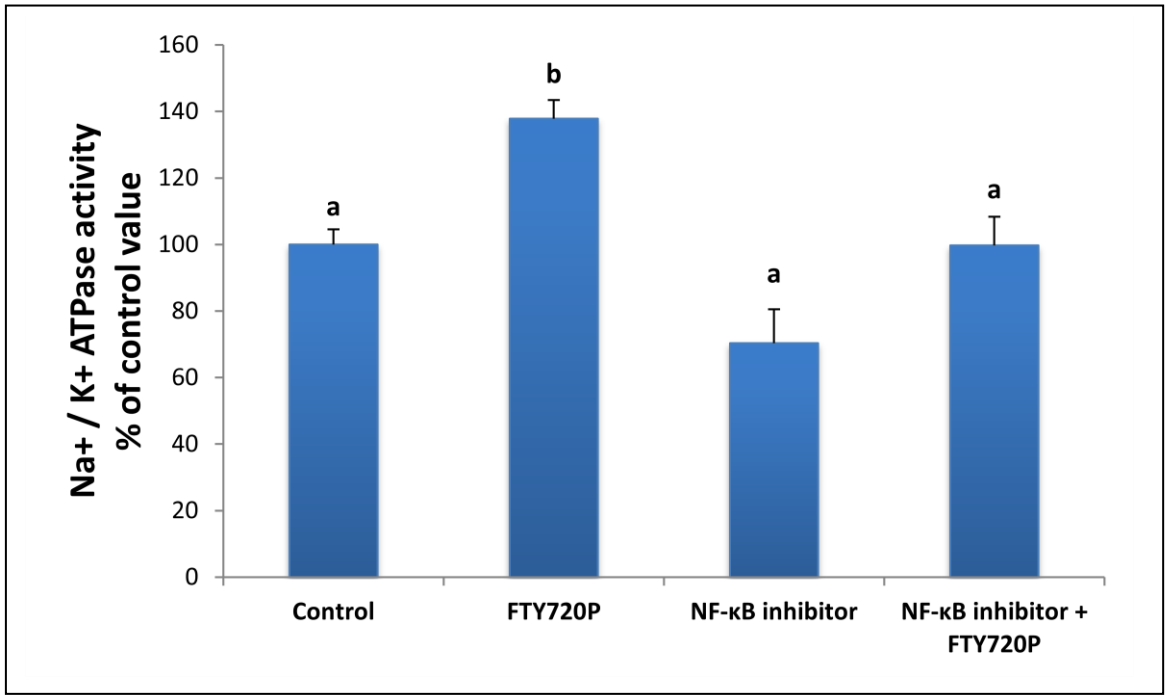

Figure 27: FTY720-P increases the activity of the hepatic Na^+/K^+ ATPase *via* NF-κB. The stimulatory effect of FTY720-P (7.5 nM) was annulled in the presence of NF-κB inhibitor (15 µM). Values are means ± SEM. N=3. Bars not sharing any common letter are significantly different from each other at P< 0.05.

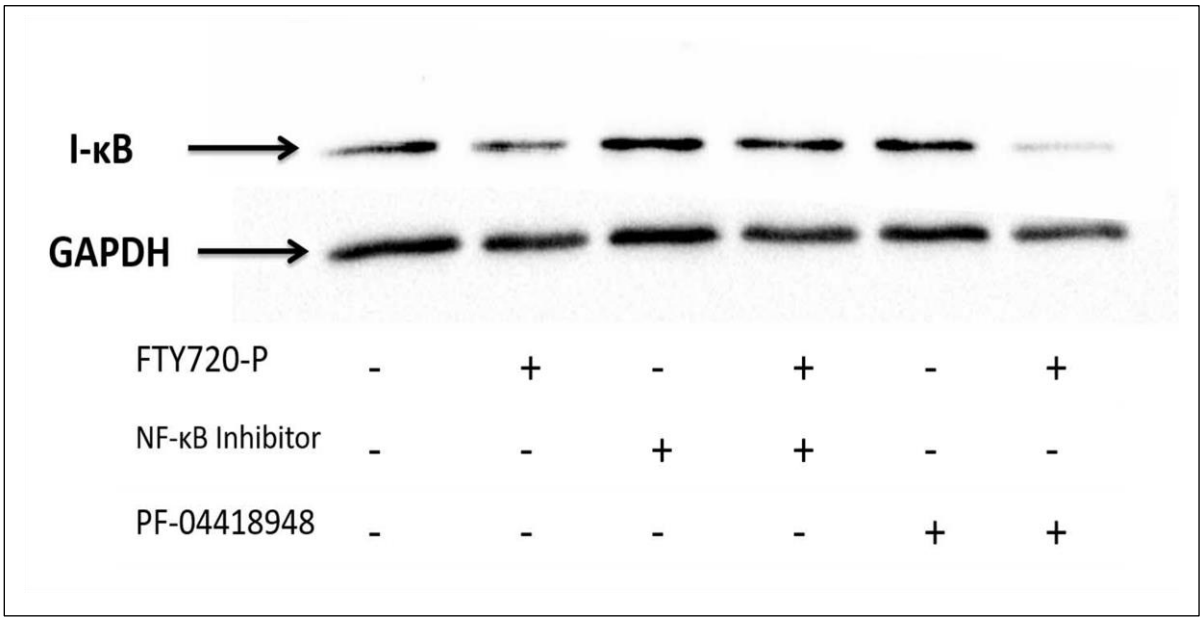

Figure 28-a: Effect of FTY720-P on I-κB expression at 2 hours in the presence of NF-κB inhibitor (15 µM) and EP2 receptor specific antagonist (PF-04418948 :1 µM). The blot is representative of an experiment repeated 3 times.

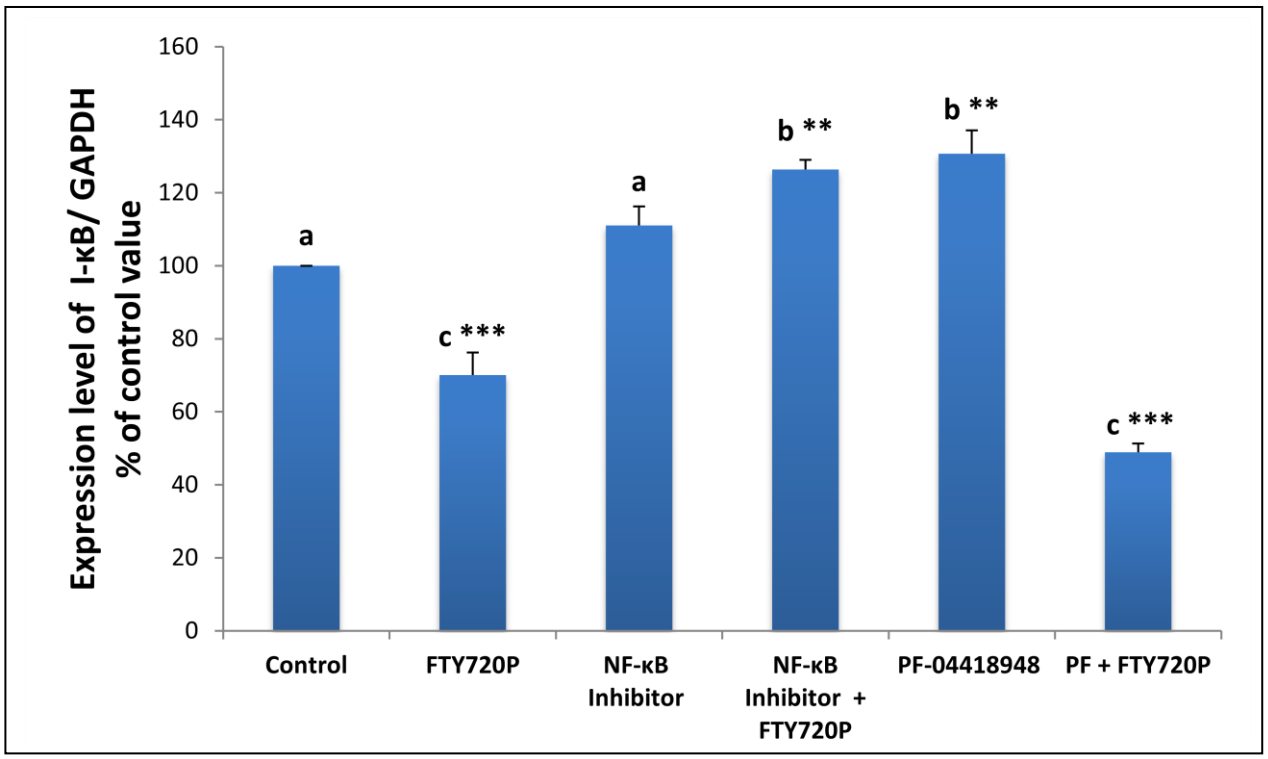

Figure 28-b: Effect of FTY720-P (7.5 nM) on I-κB expression relative to the control at 2 hours in the presence of NF-κB inhibitor (15 µM) and EP2 receptor specific antagonist (PF04418948:1µM).Values are reported as arbitrary densitometry units and normalized to GAPDH. Values are means ± SEM. N=3. Bars not sharing a common letter are statistically significant from each other at P< 0.001.

14. NF-κB acts before and after PGE2

Inhibiting NF-KB abolished not only the effect of FTY720P but also that of PGE2 (q = 9.591, P<0.01) indicating that NF-κB acts also downstream PGE2 (Fig-29). However, FTY720P reduced the expression of Iκ-B even when EP2 receptors were blocked with PF-04418948 indicating that NF-κB also acts above PGE2 (q=15.665, P<0.001) (Fig 28-a and 29-b).

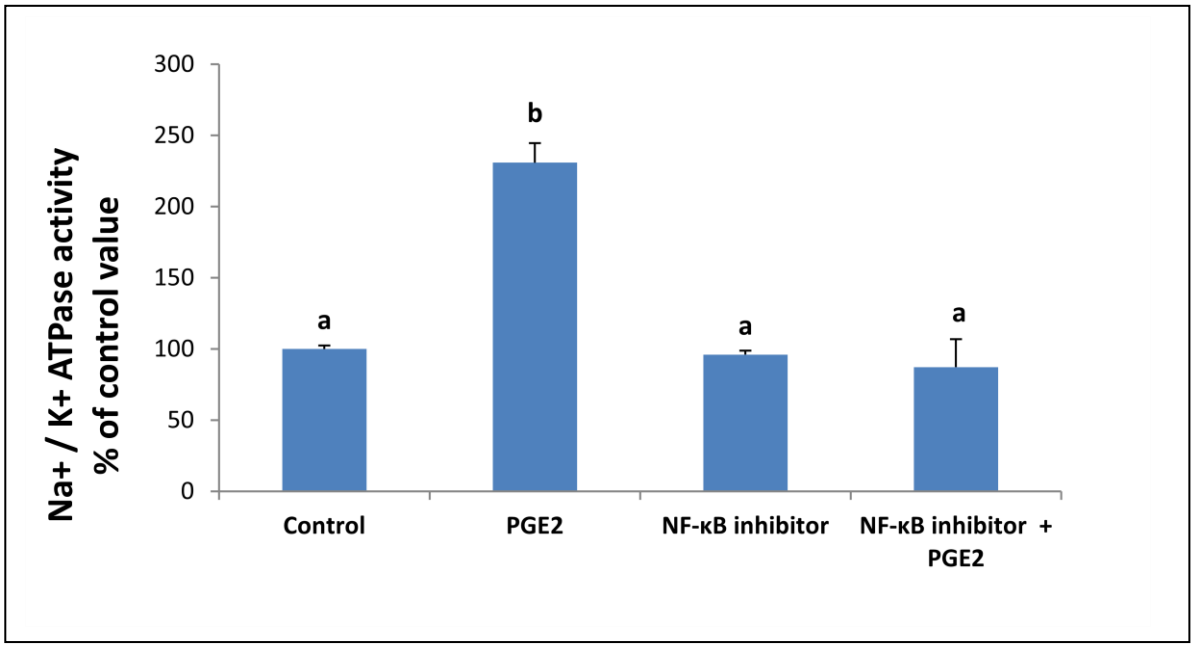

Figure 29: NF-κB acts downstream of PGE2. The stimulatory effect of PGE2 (100 µM) on Na^+/K^+ ATPase at 2 hours was cancelled in the presence of NF-κB inhibitor. Values are means ± SEM. N=3. Bars not sharing a common letter are significantly different from each other at p< 0.01.

a) NF-κB acts downstream of PKC and ERK and upstream of COX-2

FTY720P did not reduce the expression of IKB when PKC and ERK were inhibited respectively with Calphostin and PD98059 (Fig 30-a and 30-b) thus, NF-κB acts downstream of PKC and ERK. Moreover, the FTY720P-induced increase in COX-2 expression ($q = 7.266$, $P<0.01$) was not observed any more when NF-κB was inhibited (Figure 31-a, Figure 31-b) indicating that NF-κB acts upstream of COX-2.

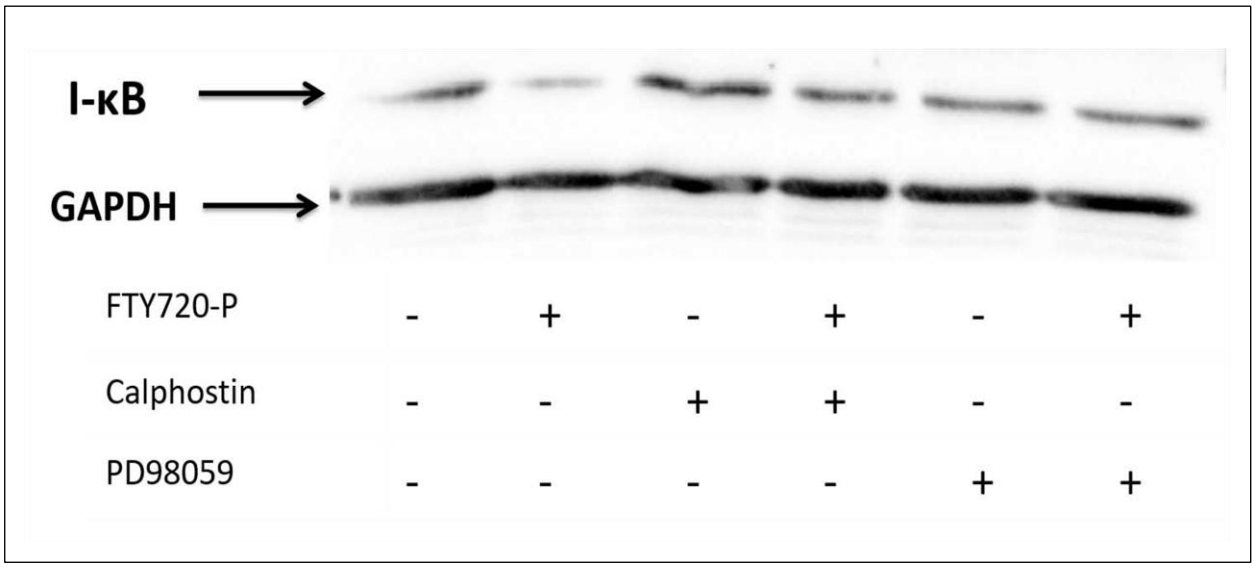

Figure 30-a: Effect of FTY720-P on I-κB expression at 2 hours in the presence of PKC inhibitor (Calphostin 50 nM) and ERK inhibitor (PD98059: 50 µM). The blot is representative of an experiment repeated 3 times.

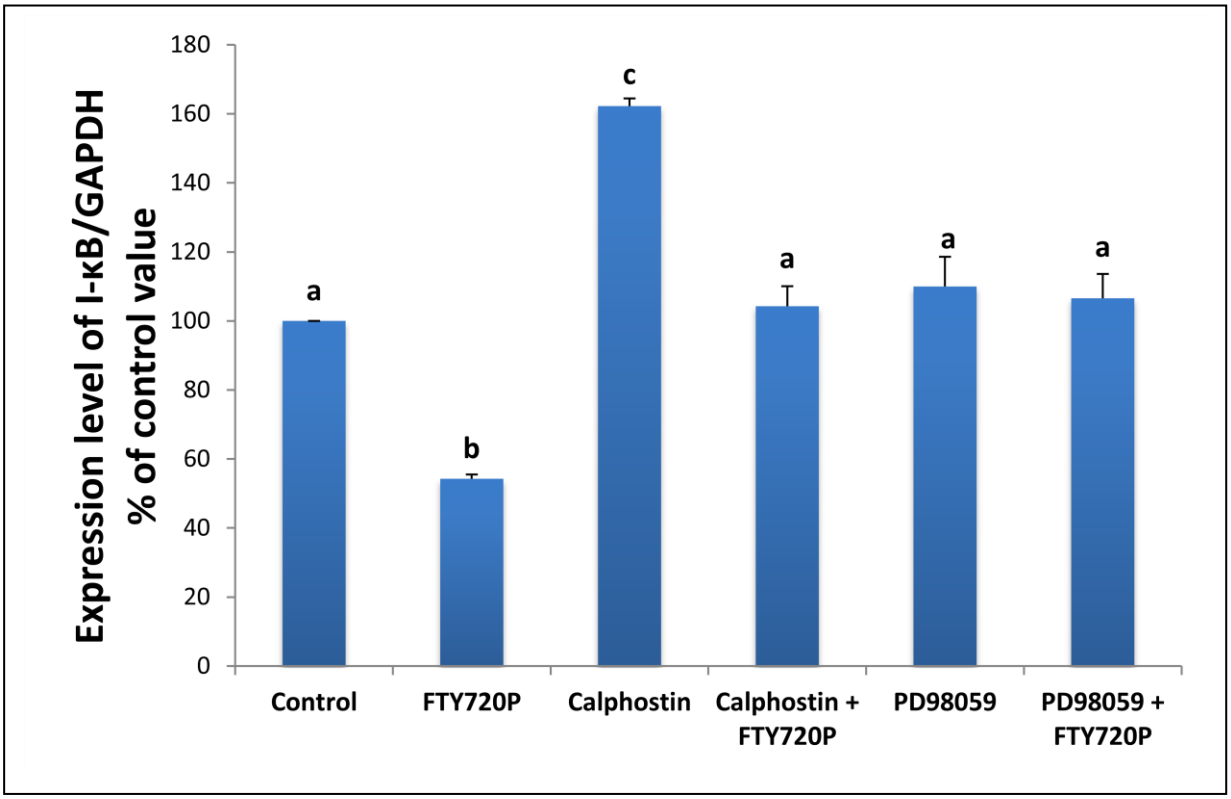

Figure 30-b: Effect of FTY720-P (7.5 nM) on I-κB expression relative to the control at 2 hours in the presence PKC inhibitor (Calphostin 50 nM) and ERK inhibitor (PD98059: 50 µM). Values are reported as arbitrary densitometry units and normalized to GAPDH. Values are means ± SEM. N=3. Bars not sharing any common letter are significantly different from each other at P< 0.01.

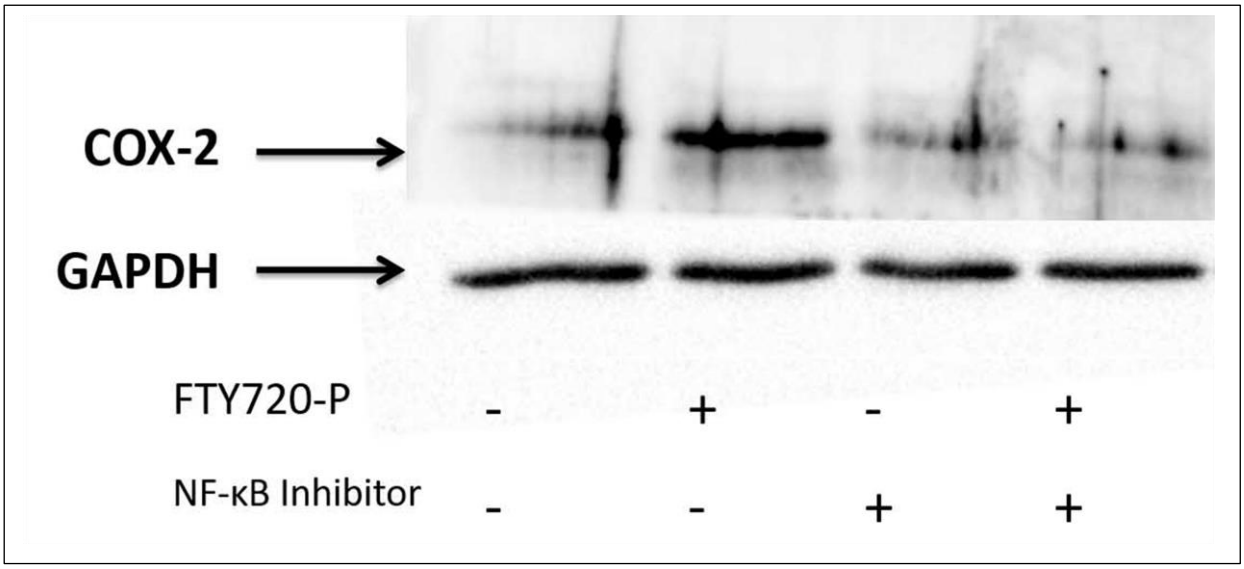

Figure 31-a: Effect of FTY720-P on COX-2 expression at 2 hours in the presence of NF-κB inhibitor (15 µM) and EP2 receptor specific antagonist (PF-04418948 :1 µM). The blot is representative of an experiment repeated 3 times.

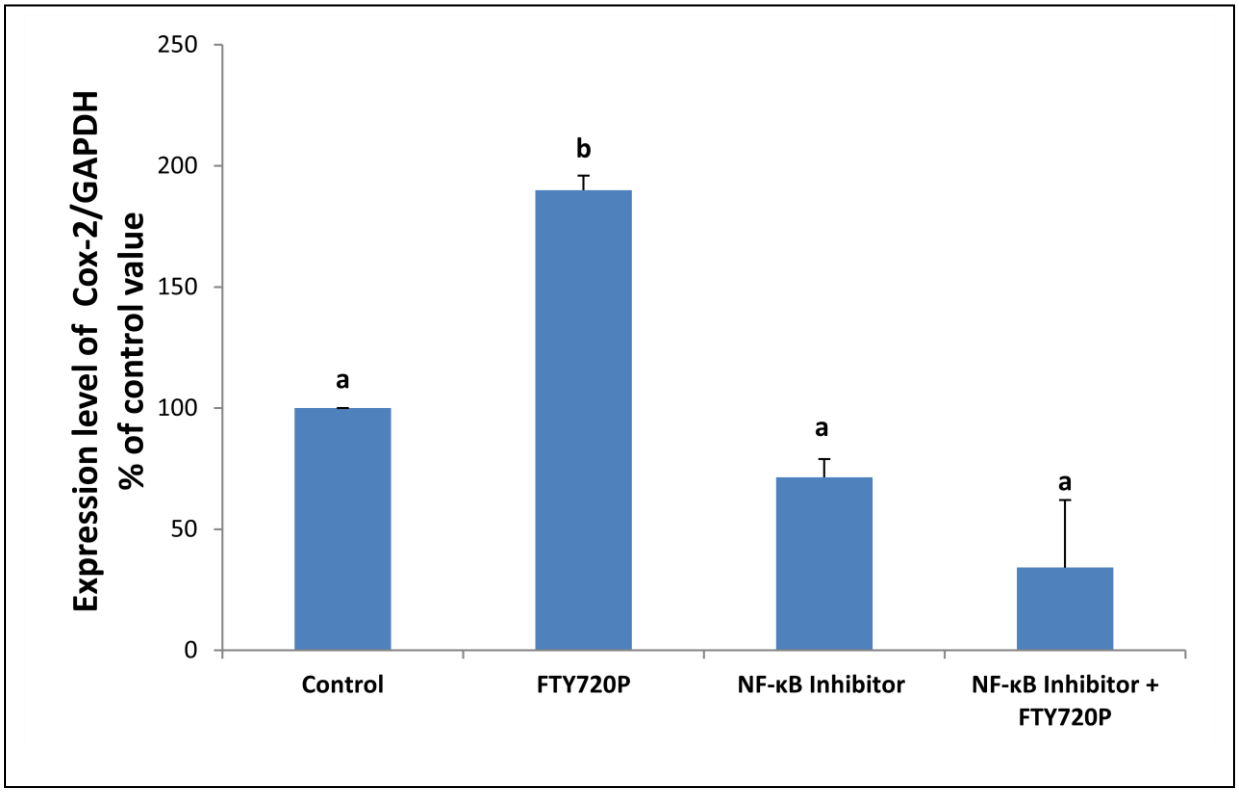

Figure 31-b: Effect of FTY720-P (7.5 nM) on COX-2 expression relative to the control at 2 hours in the presence of NF-κB inhibitor (15 µM) and EP2 receptor specific antagonist (PF-04418948 :1 µM). Values are reported as arbitrary densitometry units and normalized to GAPDH. Values are means ± SEM. N=3. Bars not sharing any common letter are statistically significant from each othes at $p < 0.05$.

b) NF-κB acts downstream of PKA and upstream of NOS

The stimulatory effect of the PKA activator (dbcAMP) (q= 8.504, P<0.01) disappeared in the presence of NF-κB inhibitor. While the stimulatory effect of SNAP (q= 10.924, P<0.001) persisted when cells where treated with the NF-κB inhibitor (Fig-32), thus indicating that NF-κB acts downstream of PKA and upstream of NO.

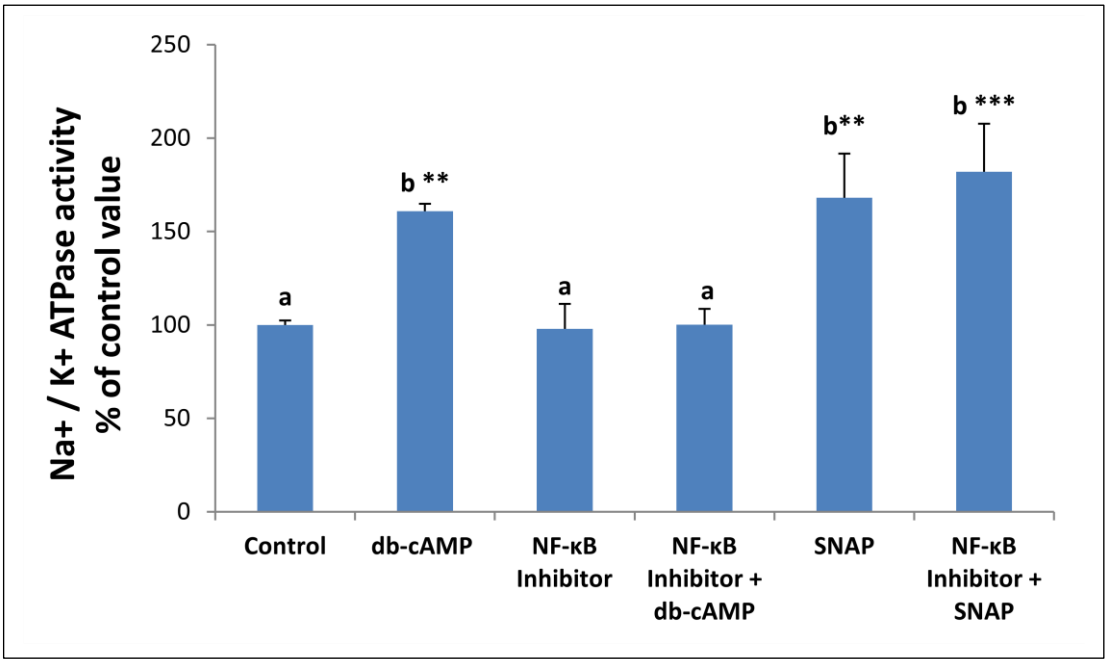

Figure 32: NF-κB acts downstream of PKA and upstream of NOS. In the presence of NF-κB inhibitor (15 µM), the stimulatory effect of the PKA activator (db-cAMP: 10 µM) on Na^+/K^+ ATPase at 2 hours disappeared while the stimulatory effect of the NO donor (Glyco-SNAP-1: 2 µM) persisted. Values are means ± SEM. N=3. Bars not sharing any common letter are significantly different from each other.
**significantly different at P< 0.01
***significantly different at P< 0.001

15. NO acts through cGMP

It is known that NO leads to the activation of cGMP, so the involvement of cGMP was tested using exogenous cGMP. The cell permeable cGMP analogue, 8-bromo cGMP (0.5 mM) mimicked the stimulatory effect of PGE2 at 2hrs ($t = 33.215$; $P<0.001$) (Fig-33).

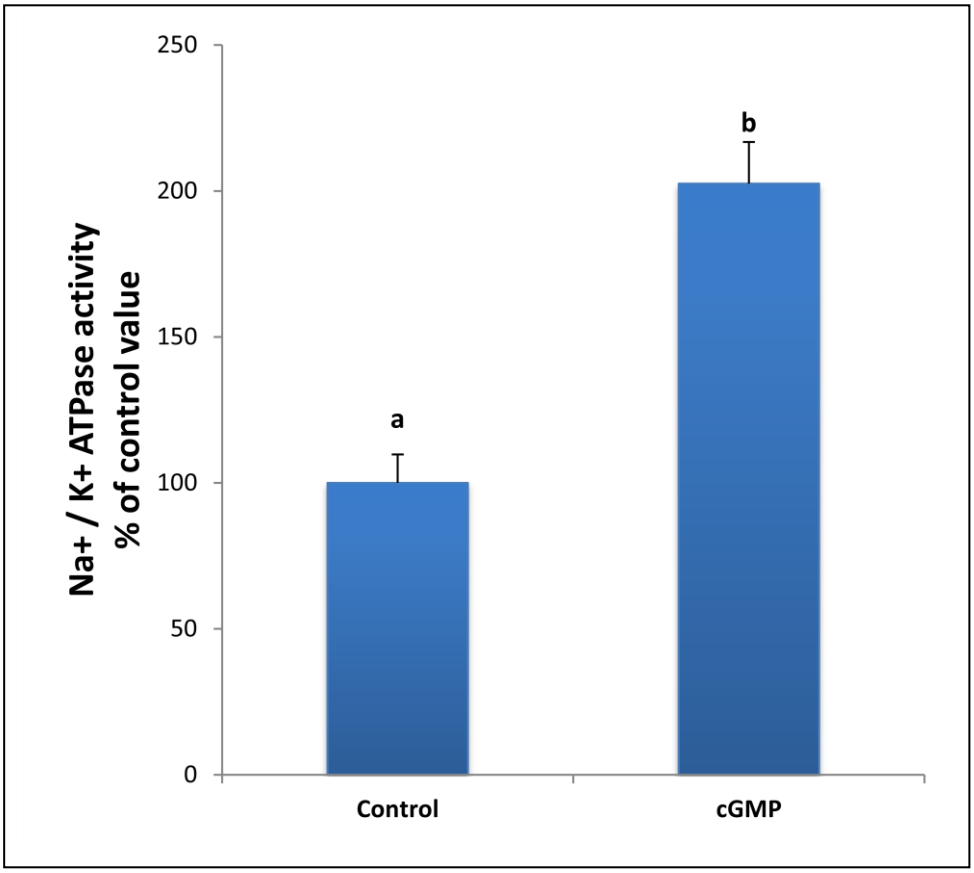

Figure 33: NO acts through cGMP. 8-bromo cGMP (0.5 mM) increased the activity of the hepatic Na^+/K^+ ATPase at 2 hours. Values are means ± SEM. N=3. Bars not sharing a common letter are statistically significant from each other at $p<0.001$. Significant differences were studied using unpaired t-test.

CHAPTER V

DISCUSSION

Many hepatic cellular processes are dependent on the sodium gradient established by Na^+/K^+ ATPase. Accordingly any alteration in the activity of the Na^+/K+ ATPase may result in impaired liver functions (Clausen MV. et al., 2017). Previous studies in our lab demonstrated opposite time-dependent effect of S1P on the Na^+/K^+ ATPase in HepG2 cells, with an inhibitory effect appearing at 15min and a stimulatory one appearing at 2hrs (Kreydiyyeh et al., 2014). S1P analogue, FTY720-P was shown to exert a similar inhibition on the pump at 15min at a concentration of 7.5nM. FTY720-P's effect at 2 hours was not however investigated, and is the focus of the present study.

HepG2 cells treated with FTY720-P (7.5nM) for 2 hours showed a significant increase (2.5 folds) in Na^+/K^+ ATPase activity. Because FTY720-P was recently approved by FDA as an oral treatment for relapsing forms of multiple sclerosis (Brinkmann et al., 2010), we want to study it further because we are concerned that its alteration of the Na^+/K^+ ATPase activity may lead to undesirable side effects. Thus, delineating the signaling pathway involved helps in understanding what is happening and may allow us decrease the negative side effects by inhibiting specific mediators.

S1P acts through five non similar receptors that are all expressed in HepG2 cells (Alam et al., 2016). The first aim of the present study was to determine the type of receptor mediating the effect of FTY720-P. Blocking S1PR2 with its specific antagonist JTE-013 did not alter the stimulatory effect of FTY720-P and Cym5520, a S1PR2 specific agonist had no effect on the ATPase. It was concluded that FTY720-P does not act through S1PR2.

The involvement of S1PR3 was next investigated. The stimulatory effect of the drug was abolished completely in the presence of the antagonist CAY1104, and the S1PR3 specific agonist, Cym5541, exerted a similar stimulatory effect to that of FTY720-P on the ATPase. Results suggest that FTY720-P acts mainly via S1PR3, since the activity of the pump went back to control values in presence of the antagonist. Had another type of receptor been activated, then a partial inhibition would have still been observed. Consequently the involvement of other receptors was not pursued.

S1PR3 is coupled to $G_q\alpha$, $G_i\alpha$, and $G_{12/13}\alpha$ (Kihara et al., 2008). Gi inhibits adenylate cyclase, decreases the level of cAMP and consequently inhibits PKA. The stimulatory effect of FTY720-P disappeared when PKA was inhibited with RpcAMP, while dbcAMP, a PKA activator, stimulated the ATPase as FTY720-P did. Accordingly, the involvement of Gi in the drug's effect was ruled out since FTY720-P exerts its effect by activating PKA and not by inhibiting it.

$G_q\alpha$ protein activates Protein Kinase C (PKC) (Jeong-Hun Kang, 2014), a kinase which exhibits a modulatory effect on the Na^+/K^+ ATPase (Singh et al., 2012; El-Zein et al., 2015; Serhan et al., 2011). This is why the involvement of PKC in the signaling pathway was next investigated. The stimulatory effect of FTY720-P was lost completely in the presence of calphostin, a PKC inhibitor, while PMA, a PKC activator, significantly increased the activity of the ATPase. The results suggest that PKC mediates the stimulatory effect of FTY720-P on the ATPase with Gqα being the only activated G protein linked to S1PR3, since the activity of the pump was restored to control value when FTY720-P was applied in presence of a PKC inhibitor. Had, another type of G protein been involved, then a partial activation in the presence of calphostin would still have been observed.

The modulatory effect of PKC on the activity of the ATPase has been reported in many works, and varied from stimulation to inhibition. PKC was implicated in the arachidonic acid inhibition of the Na^+/K^+ ATPase in sheep pulmonary artery (Singh et al, 2012). The insulin-like growth factor I (IGF-I) stimulatory effect on the Na^+/K^+ ATPase in the vascular smooth muscle cells (VSMC) was reported to be mediated by PKC and PI3K (Li et al., 1999). In proximal tubules, activation of PKC by the sequential activation of phospholipase D, and Ca^{2+}-insensitive phospholipase A_2 was also behind the stimulatory effect of angiotensin II on the Na^+/K^+ ATPase (Souza et al., 2010). In addition, PKC was shown to be involved in the inhibitory effect of FTY720-P on hepatic Na+/K+ ATPase at 15 min (Alam et al., 2016).

The literature reports a role for PGE_2 in the regulation of Na^+/K^+ ATPase activity (Cohen et al., 1993; Wald et al., 1990; Libano-Soares et al., 2011; Zeidel et al., 1991). In the rat colon (Markossian et al., 2005), liver (Kreydiyyeh et al, 2007) and cardiac myocytes (Skayain & Kreydiyyeh, 2006) PGE2 mediated the effect of TNF-α on the Na^+/K^+ ATPase. It mediated also the effect of FTY720-P on the Na^+/K^+ ATPase in HepG2 cells at 15 min (Alam et al, 2016). So the next step in our study was to investigate the involvement of COX-2/PGE_2 synthesis in the signaling pathway. The stimulatory effect of FTY720-P did not appear when PGE2 synthesis was blocked with indomecathin, indicating that PGE2 is a mediator of FTY720P action. Further confirmation of the role of PGE2 came from the stimulation of the ATPase by exogenous PGE2 and from the results of the western blot analysis which showed a higher FTY720-P induced expression of COX-2, an enzyme needed for PGE2 synthesis.

PGE2 binds and activates four G-protein coupled receptors called EP1, EP2, EP3, and EP4 (Sugimoto et al., 2007). Determination of the type of EP receptor through which PGE2 acts was necessary to unravel the signaling pathway. EP2 and EP4 are coupled to

Gs, EP1 is linked to Gq, and EP3 to Gi. FTY720-P did not alter the ATPase activity when EP2 receptors were blocked with PF-04418948, while butaprost, a specific EP2 receptor agonist, mimicked the stimulatory effect of PGE2 on the ATPase. Since the effect of PGE2 completely disappeared in presence of PF-04418948, the involvement of other EP receptors was ruled out. In support of this conclusion is the loss of the stimulatory effect of exogenous PGE2 when PKA was inhibited with RpcAMP since EP2 is coupled to Gs which increases cAMP levels and activates PKA. Activating PKA with dbcAMP stimulated the Na^+/K^+ ATPase even when PGE2 synthesis was inhibited with indomethacin confirming further the involvement of EP2 receptors and PKA. Inhibiting PKA with RpcAMP did not exert any effect on the ATPase, eliminating thus the involvement of EP3 receptors which are coupled to Gi. Had PGE2 been acting via EP3, then a similar effect to that of PGE2 would have been observed with RpcAMP. Again, the stimulatory effect of PGE2 persisted in presence of calphostin, a PKC inhibitor ruling out an involvement of EP1 receptors which are coupled to Gq and which activate PKC. All these results are in support of the conclusion that PGE2 stimulates the Na^+/K^+ ATPase by acting via EP2. In MDCK cells, EP2 receptors were also reported to be involved in the regulation of the Na^+/K^+ ATPase by prostaglandins (Matlhagela K. et al., 2006).

FTY720-P thus stimulates the Na^+/K^+ ATPase by binding to S1PR3 which are coupled to Gq, promoting PGE2 production. The latter exerts its effect via EP2 receptors which are coupled to Gs leading to PKA activation. PKA is a recognized modulator of the Na^+/K^+ ATPase. When activated by dopamine in kidney tubules it reduced the ATPase activity (Satoh et al., 1992; Satoh et al., 1993), while it increased it in skeletal muscle when activated by epinephrine (Ewart et al., 1995).

Although PKC was found not to be implicated in the effect of PGE2 on the Na^+/K^+ ATPase, inhibiting PKC with calphostin abolished the effect of FTY720-P indicating that

PKC is present along the pathway but upstream of PGE2. In fact when PGE2 synthesis was blocked with indomethacin, the stimulatory effect of PMA, an activator of PKC, disappeared. Again, inhibiting PKC with calphostin reduced the FTY720-P induced increase in the expression of COX-2 and brought it back to control levels indicating that PKC increases COX-2 expression and PGE2 synthesis.

The activation of COX-2/PGE2 pathway by PKC is not something new; it has been demonstrated previously by Iitaka et al, (2015) that PKC is involved in the production of PGE2 that mediates TNF-α induced (Claudin 1) expression in human lung carcinoma cells. In primary midbrain astrocytes also, amyloid β peptide (25–35) activated PKC, leading to COX-2 activation and PGE2 release (Hüll et al., 2006). PKC was shown similarly to regulate COX-2 expression in articular chondrocytes (Kim et al., 2003) and to increase PGE2 production during adenosine-5'-triphosphate induced lung inflammation (Lee, I. et al, 2014).

The literature reports ERK as a target of PKC (Tsao et al, 2013; Chen, W. et al., 2018; Li et al, 2015). Moreover, the Na^+/K^+ ATPase contains several phosphorylation sites that are targeted by several protein kinases including ERK (Al-Khalili et al., 2004). Whether ERK is also a mediator of FTY720-P effect is a question that needed to be answered. The inhibition of ERK with PD98059 abolished completely the stimulatory effect of FTY720-P and PMA, but not that of PGE2, implying that ERK is upstream of PGE2. Western blot analysis showed a higher FTY720-P induced expression of phospho-ERK that disappeared when PKC was inhibited with calphostin inferring that ERK is downstream PKC.

Our results are in line with the reported involvement of ERK in PGE2 synthesis. In human dermal fibroblasts, palmitate induced COX-2 expression via ERK (Oh et al,

2013). Similarly neuronal death triggered by TDP-43-depleted microglia was due to an ERK‘s induced increase in PGE2 synthesis (Xia et al, 2015).

ERK activation by PKC has also been shown in many works. $P2X_7$ receptor-mediated neuronal differentiation of neural progenitor cells was shown to be mediated by a PKC-dependent ERK phosphorylation (Tsao et al, 2013). Hyperalgesia priming is mediated via activation of the spinal PKC/ERK signal pathway (Chen, W. et al., 2018). In HEK cells expressing PAC1 receptors, PKC signaling was shown to mediate PACAP-induced ERK activation (May et al., 2014). β-Adrenergic stimulation activated also protein kinase Cε which induced ERK phosphorylation and cardiomyocyte hypertrophy (Li et al, 2015). In addition, Tsirimonaki et al, (2013) showed that PKCε also activates ERK1/2 in Human Nucleus Pulposus Cells.

ERK is also a modulator of the Na^+/K^+ ATPase. In human skeletal muscle cells, ERK1/2 mediates the stimulatory effect of insulin on the Na^+/K^+ ATPase by phosphorylation of the α-subunit (Al-Khalili et al, 2004). Previous work in our lab demonstrated ERK as a mediator of the inhibitory effect of FTY720-P on hepatic Na^+/K^+ ATPase at 15 min (Alam et al., 2017).

It was concluded that FTY720P binds to S1PR3, activates PKC which in turn activates ERK leading to PGE2 synthesis. PGE2 binds to EP2 receptors and activates PKA.

The activity of the Na^+/K^+ pump is also regulated by nitric oxide in various cells, like cultured intestinal epithelial cells, cardiac cells, rat skeletal muscle cells, rat proximal trachea, renal cells and liver cells (Xu D. et al., 1999; Pavlovic D. et al., 2013; Juel C., 2015; Bailey K. L. et al, 2011; Elias, M. D. et al, 1999; Muriel, P., & Sandoval, G., 2000). Previous work in our lab identified also NO as a downstream mediator of PGE2 (Alam,

N. A., & Kreydiyyeh, S. I., 2017) in HepG2 cells. So, NO seemed to be a potential mediator in the signaling cascade underlying FTY70-P's effect. Cells treated with the NO donor Glyco-SNAP-1 for 2 hours, showed a significant increase in their Na^+/K^+ ATPase activity even when PGE2 synthesis was blocked with indomethacin, whilst the stimulatory effect of PGE2 disappeared in presence of carboxy-PTIO, a scavenger of NO, suggesting that PGE2 acts via NO, in addition to PKA.

NF-κB is a transcription factor known to increase the expression of iNOS and COX-2, two enzymes responsible for the synthesis of NO and PGE2 respectively. Accordingly, inhibiting the transcription factor abrogated the effect of both FTY720-P and PGE2. NF-κB in the un-stimulated cell is sequestered to the cytosol by IκB, an inhibitory protein to which it is complexed. In the stimulated cell, IκB is phosphorylate by IκB kinase leading to its ubiquination and degradation by the proteasome (Oeckinghaus, A., & Ghosh, S., 2009). NF-κB then translocates to the nucleus and activates the transcription of specific genes. A decrease in the expression of IκB is thus an indicator of NF-κB activation. The expression level of IκB decreased in cells treated with PGE2 as well as with FTY720-P even when the EP2 receptors through which PGE2 acts were blocked. The results suggest that NF-κB acts upstream and downstream PGE2. Upstream it promotes COX-2 synthesis and downstream it promotes NOS synthesis.

FTY720-P did not reduce the expression of IκB when PKC and ERK were inhibited indicating that NFκB is downstream of PKC and ERK. Moreover, the FTY720-P-induced increase in COX-2 expression was not observed when NF-κB was inhibited. Thus, the results indicate that NF-κB acts downstream PKC and ERK and upstream PGE2 leading to an increase in COX-2 expression and PGE2 release. PGE2 then binds to EP2 receptors and activates PKA.

The activated PKA then induces NO production since the stimulatory effect of db-cAMP was not manifested in presence of carboxy-PTIO, while PKA inhibition with Rp-cAMP, did not affect the stimulatory effect of Glyco-SNAP-1 on the ATPase. Such an effect of PKA on NO synthesis has been reported in other works. PKA was shown to induce vasorelaxation via an enhanced eNOS activity and an increase in endothelial NO release (García-Morales, V. et al, 2014). Also, in anesthetized male rats, cAMP/PKA dependent nitric oxide release was behind the inhibitory effect of cumestrolon carotid sinus baroreceptor activity (Liu, H. et al, 2015).

The guanylyl cyclase/cGMP/PKG pathway is the canonical signaling pathway of NO (Denninger, J. W., & Marletta, M. A. et al, 1999). Many studies reported regulation of the activity of the Na^+/K^+ ATPase via NO and its downstream signaling pathway involving cGMP and PKG in a cell type depending manner (Balon et al., 1994). Liang and Knox (1999) reported an inhibition of the Na^+/K^+ ATPase in a renal tubule cell line by NO, acting via cGMP. Age-related variations in the levels of cGMP and PKG activity were responsible however, for the stimulation of cerebral Na^+/K^+ pump in rat cerebellum (Scavone et al., 2005). In addition to previous results, NO was found to act *via* cGMP since the cell permeable cGMP analogue, 8-bromo cGMP mimicked the stimulatory effect of PGE2.

In fact iNOS synthesis was shown to be controlled by NF-κB in various cells including leukemia cells (Park, J. et al, 2014), rat alveolar macrophages (Hammermann et al, 2000), and mouse hepatocytes (Hatano, et al, 2001). The promoter of the murine gene coding for iNOS (inducible nitric oxide synthase) contains two putative NF-κB binding sites involved in the induction of the gene (Grilli et al, 1993; Xi, Q. et al, 1999).

Since NF-κB is suspected to induce iNOS expression and NO production, the last step in delineating the signaling pathway underlying FTY720-P effect was to confirm that NF-κB is upstream NO. The stimulatory effect of (db-cAMP) disappeared completely while the stimulatory effect of Glyco-SNAP-1 persisted when NF-κB was inhibited. Results suggest that NF-κB acts downstream PKA and enhances NO synthesis via an increase in the expression of iNOS.

CHAPTER VI

CONCLUSION

The biochemical signaling pathway underlying the stimulatory effect of FTY70-P on the hepatic Na^+/K^+ ATPase was elucidated. When FTY720P is applied for 2 hours to HepG2 cells, it binds to S1PR3 and activates sequentially PKC, ERK, and NF-κB, leading to a greater expression level of COX-2 and more PGE2 synthesis. Prostaglandin E2 then binds to EP2 receptor and activates PKA / NF-κB / NOS / cGMP pathway resulting in a stimulation of on the Na^+/K^+ ATPase.

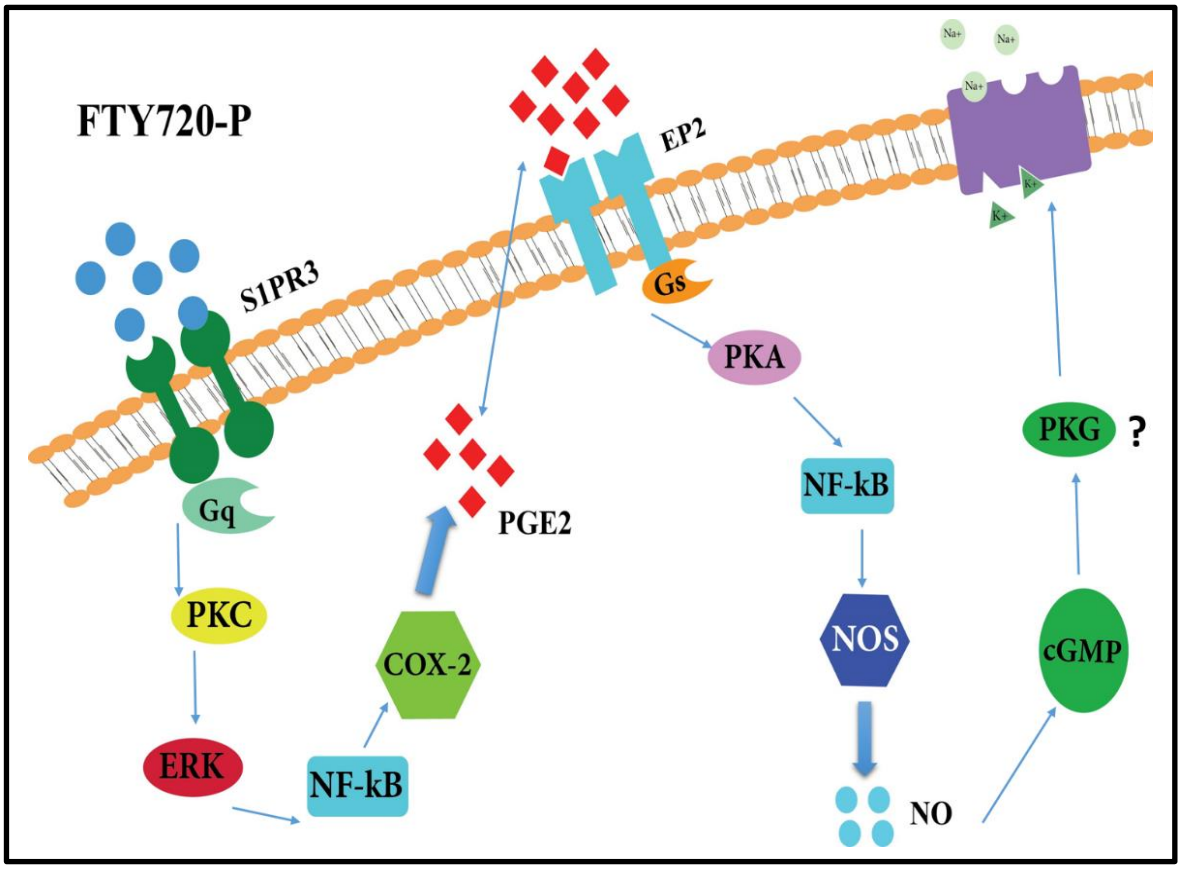

Figure 34: Signaling pathway mediating the stimulatory effect of FTY720P on hepatic Na+/K+ ATPase

CHAPTER VII

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