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# Regulation of Na-K-ATPase by arachidonic acid metabolites in intestinal epithelial cells

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# REGULATION OF Na-K-ATPase BY ARACHIDONIC ACID METABOLITES IN INTESTINAL EPITHELIAL CELLS

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By
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## APPROVAL OF THESIS

We, the faculty supervising the work of Niraj Nepal, affirm that the dissertation, *Regulation of Na-K-ATPase by Arachidonic acid metabolites in intestinal epithelial cells,* meets the high academic standards for original scholarship and creative work established by the Biomedical Sciences program and the Graduate college of Marshall University. This work also conforms to the editorial standards of our discipline and the Graduate College of Marshall University. With our signatures, we approve the manuscript for publication.

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## DEDICATION

I dedicate this thesis to my family, my wife, Kavita, and my parents for their constant support and unconditional love. I love you all dearly.

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## ABSTRACT

The integral membrane protein sodium potassium adenosine triphosphate (Na-K-ATPase), has been extensively studied in various animal cell models. Na-K-ATPase is a multifunctional enzyme which plays a significant role in cellular physiology by regulating electrochemical potential, cell volume, pH, cytosolic calcium, and many nutrient and electrolyte transport processes. As such, it has been implicated in the pathogenesis of inflammatory bowel diseases (IBD) and has been shown to play a vital role in the malabsorption of nutrients and electrolytes. In this context, a significant amount of research has been done over the last two decades to understand the mechanisms of alteration of various nutrient absorptive processes in IBD. Though it has been well acknowledged that Na-K-ATPase plays an essential role in the alteration of nutrient absorptive mechanisms during inflammation of the intestine, the molecular mechanisms responsible for the modulation of Na-K-ATPase in IBD was not known.

To study the regulation of Na-K-ATPase, we used an *in vitro* model (rat intestinal epithelial cell line, IEC-18 cells) of intestinal epithelial cells that matured from the crypt to villus cells, very similar to that seen in the mammalian system. During the maturation process, these cells show an increase in Na-K-ATPase activity to support the absorptive capacity of the villus cells. The increased activity of Na-K-ATPase was found to be likely due to phosphorylation of the α1 subunit of Na-K-ATPase rather than altered transcription or trafficking of the enzyme. Numerous inflammatory mediators are produced during IBD; therefore, it is beneficial to uncover the role of individual inflammatory mediators in regulating transport processes regulated by Na-K-ATPase. We found that the inflammatory mediator leukotriene D4 (LTD4) stimulates the activity of Na-K-ATPase through a Ca-activated PKC pathway in crypt-like cells, while

prostaglandin E2 (PGE2) reduces the activity of Na-K-ATPase through cAMP-activated PKA pathway in villus-like IEC-18 cells.

# CHAPTER 1: Na-K-ATPase

## 1.1 GENERAL INTRODUCTION

Over the last 60 years, since the discovery of Na-K-ATPase by Dr. Skou in 1957, the significance of Na-K-ATPase has increased tremendously (Skou, 1957). The goal of this introductory section is to provide some basic information on Na-K-ATPase before delving into its complexity. This membrane-bound protein, which belongs to the large family of P-type-ATPases, transports three sodium ions ($Na^+$) out and two potassium ions ($K^+$) into all animal cells. Since transportation of these ions is against their gradients, the required energy is generated by catalyzing ATP. Conventionally, Na-K-ATPase is considered as a pumping protein that primarily maintains electrochemical potential, cell volume, cytosolic pH, and cytosolic $Ca^{2+}$ level (Jorgensen, Hakansson, & Karlish, 2003; Skou, 1990; Skou & Esmann, 1992). Additionally, the pumping function also provides the essential gradient to drive secondary transport processes for transportation of other ions, nutrients, and neurotransmitters across the plasma membrane (Jorgensen et al., 2003; Skou, 1990; Skou & Esmann, 1992). However, within the last two decades, it has been demonstrated that in addition to the pumping function, Na-K-ATPase also has a signaling role regulating various signaling pathways involved in cell proliferation, cell cycle, fibrosis, and cell adhesion (Contreras, Shoshani, Flores-Maldonado, Lazaro, & Cereijido, 1999; Nguyen, Wallace, & Blanco, 2007; Shapiro & Tian, 2011). Since Na-K-ATPase has been demonstrated as an essential protein involved in cellular physiology, the abnormal function of Na-K-ATPase results in the pathological states or diseases such as hyperkalemia, cataracts (Babula, Masarik, Adam, Provaznik, & Kizek, 2013; Delamere & Tamiya, 2009; Suhail, 2010), hypertension (Lingrel, 2010; Matchkov & Krivoi, 2016; Therien & Blostein, 2000), and diabetes (Suhail, 2010).

## 1.2 STRUCTURE

Na-K-ATPase is a multimeric protein consisting of three different subunits α, β, and γ (Figure 1). There are tissue-specific isoforms of these subunits, and the combination of these isoforms yields the functional Na-K-ATPase (Jewell, Shamraj, & Lingrel, 1992). Each subunit has distinct mRNA and is synthesized independently (Geering, 1988).

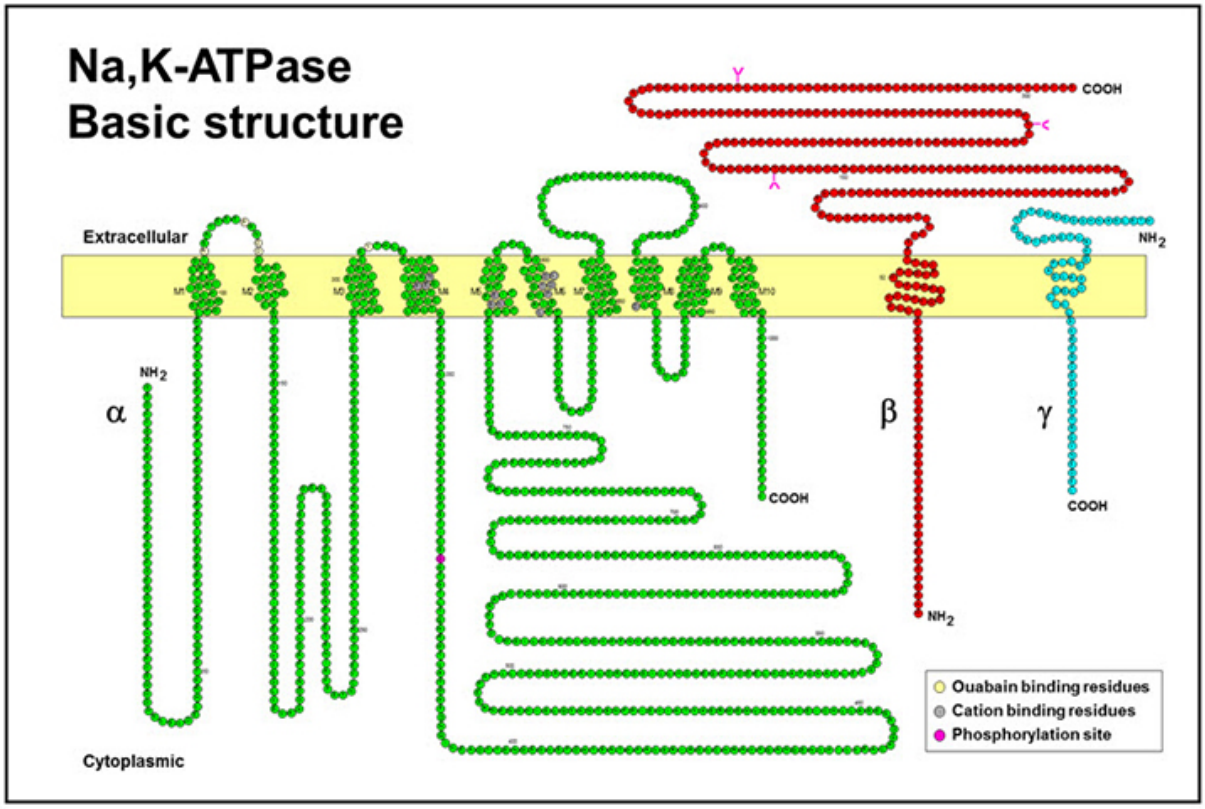


**Figure 1: Schematic representation of Na-K-ATPase subunits in plasma membrane.** (Figure adapted after the permission from Blanco *et al.* (Blanco & Mercer, 1998b)).

### 1.2.1. The Alpha (α) Subunit

The mass of the α subunit is ~112 kDa, having binding sites for ions, ATP, and cardiac glycosides. The α subunit, also known as the catalytic subunit of Na-K-ATPase, cleaves ATP to generate the energy required for the pumping of ions. This subunit consists of ten transmembrane α-helical segments and several cytoplasmic loops, in which both the N- and C-termini of the

chains reside inside the cytosol (Fambrough et al., 1994). The transmembrane domain is primarily the site of cation binding, specifically by transmembrane helices M4, M5, M6, and M8. However, helices M7, M9, and M10 anchor the α subunit to the membrane (Shinoda, Ogawa, Cornelius, & Toyoshima, 2009). Cytoplasmic regions are primarily known for ATP binding and are divided into three different domains: the A-actuator domain acts as a piston during the pumping activity of the Na-K-ATPase while passing through E1 and E2 stages, the P-phosphorylation domain phosphorylates the enzyme, and the N-nucleotide binding domain binds the nucleotide as well as proteins including Src.

**1.2.2. The Beta (β) Subunit**

The β subunit by itself does not play a role in pumping of ions but helps newly synthesized α subunit to fold correctly and integrate into the plasma membrane (Gatto, McLoud, & Kaplan, 2001; Geering, 2001). Additionally, the β subunit modulates the function of Na-K-ATPase by changing the cation binding affinity (Laughery, Todd, & Kaplan, 2003). It has also been reported that this ~55 kDa subunit plays the role in forming cell-cell junctions (Geering, 2008).

Unlike α subunit, this subunit is a highly glycosylated protein, having two to four glycosylation sites. The number of glycosylation sites is specific to its isoform (Toyoshima, Kanai, & Cornelius, 2011). It has also been reported that these glycosylation sites regulate α-β dimer formation. The β subunit consists of three domains: the N-terminal cytoplasmic domain, one helical transmembrane domain, and the extracellular domain that interacts with the α-subunit (Toyoshima et al., 2011). Since the β subunit plays a role in stabilization of the α subunit and cation binding of the holoenzyme, abnormalities (mutation or deletion) of the β subunit have

created deleterious effects like hearing or sight loss in Drosophila, motor disabilities in mice, and polycystic kidney disease in humans (Wilson et al., 2000).

### 1.2.3. FXYD (γ) subunit

The FXYD protein family is not required for the Na-K-ATPase function, but there are numerous pieces of evidence that this subunit plays an active role in the regulation of Na-K-ATPase (Beguin et al., 1997; Jones, Davies, & Kidder, 1997). This protein subunit is found to explicitly modulate the kinetics of Na-K-ATPase by regulating the affinity of ions and ATP for the enzyme (Csupor, Csorba, & Hohmann, 2016; Klodos & Ottolenghi, 1975). The FXYD subunit also consists of three domains: the cytoplasmic domain that is primarily a phosphorylation site for different kinases (PKA and PKC), the helical transmembrane domain, and the N-terminal extracellular domain (Dey, Rahaman, Chakraborti, & Chakraborti, 2013). It has already been established that the extracellular domain interacts with Na-K-ATPase, thereby regulating its function. However, it is still unclear how the FXYD proteins change the binding capacities of $Na^+$ and $K^+$ and affect their kinetic properties.

## 1.3. ISOFORMS OF Na-K-ATPase SUBUNITS

There are several isoforms of each subunit. To date, there exist four isoforms of the α subunit, three isoforms of the β subunit, and seven isoforms of FXYD subunit, expressed differentially in mammals in a tissue-specific manner, as summarized in Table 1.

| Subunit Isoform | Tissue | References |
|---|---|---|
| **α1** | Kidney, Lung, Liver, Heart, Skeletal muscle, Thymus, Brain, Intestine, and Pineal gland | (Fowles, Green, & Ouyang, 2004; He et al., 2001; Hundal, Marette, Ramlal, Liu, & Klip, 1993; Saha et al., 2015; Shyjan, Cena, Klein, & Levenson, 1990; Shyjan & Levenson, 1989; Sun & Ball, 1992; Sweadner, McGrail, & Khaw, 1992; Thompson & McDonough, 1996; Urayama, Shutt, & Sweadner, 1989) |
| **α2** | Skeletal muscle, Cardio myocytes and Brain | (Fowles et al., 2004; He et al., 2001) |
| **α3** | Brain and pineal gland | (Shyjan et al., 1990; Shyjan & Levenson, 1989; Urayama et al., 1989) |
| **α4** | Testis / reproductive cells | (Blanco, Sanchez, Melton, Tourtellotte, & Mercer, 2000; Shamraj & Lingrel, 1994; Woo, James, & Lingrel, 2000) |
| **β1** | Kidney, Lung, Liver, Heart, Skeletal muscle, Intestine and Brain | (He et al., 2001; Hundal et al., 1993; Saha et al., 2015; Shyjan et al., 1990; Shyjan & Levenson, 1989; Sun & Ball, 1992; Sweadner et al., 1992; Thompson & McDonough, 1996; Urayama et al., 1989; Woo et al., 2000) |
| **β2** | Skeletal muscle, Thymus, Pineal gland and Brain | (Blanco et al., 2000; Fowles et al., 2004; He et al., 2001; Hundal et al., 1993; Shamraj & Lingrel, 1994; Shyjan et al., 1990; Sun & Ball, 1992; Thompson & McDonough, 1996; Woo et al., 2000) |
| **β3** | Lung, Liver, Skeletal muscle and Testis | (Blanco et al., 2000; Fowles et al., 2004; He et al., 2001; Hundal et al., 1993; Shamraj & Lingrel, 1994; Shyjan et al., 1990; Sun & Ball, 1992; Thompson & McDonough, 1996; Woo et al., 2000) |
| **FXYD1** | Heart and Skeletal muscle | (Bell et al., 2008; Geering, 2006; Lubarski, Pihakaski-Maunsbach, Karlish, Maunsbach, & Garty, 2005; Sweadner & Rael, 2000) |
| **FXYD2** | Kidney | (Geering, 2006; Jones et al., 2005; Lubarski et al., 2005; Sweadner & Rael, 2000) |
| **FXYD3** | Intestine, Stomach | (Geering, 2006; Lubarski et al., 2005; Sweadner & Rael, 2000) |
| **FXYD4** | Kidney | (Geering, 2006; Goldschmidt et al., 2004; Lubarski et al., 2005; Sweadner & Rael, 2000) |
| **FXYD5** | Testis, Intestine | (Geering, 2006; Lubarski et al., 2005; Sweadner & Rael, 2000) |
| **FXYD6** | Brain | (Geering, 2006; Lubarski et al., 2005; Sweadner & Rael, 2000) |
| **FXYD7** | Brain | (Geering, 2006; Lubarski et al., 2005; Sweadner & Rael, 2000) |

**Table 1: Tissue distribution of Na-K-ATPase subunit isoforms.**

## 1.4. PUMPING MECHANISM OF Na-K-ATPase

The current understanding of how the pumping mechanism of Na-K-ATPase works is mostly based on the earlier studies. In the 1960s, Post and Albers postulated the principle mechanism for the pumping activity of Na-K-ATPase, which has been the framework for numerous investigations until now (Albers, 1967; Post, Sen, & Rosenthal, 1965). According to Post and Albers, Na-K-ATPase exists in two conformation states, $E_1$ and $E_2$. These states are influenced by interaction with $Na^+$, $K^+$, ATP, and other cardiac glycosides, such as Ouabain, and they lead to the formation of different intermediates, as shown in Figure 2.

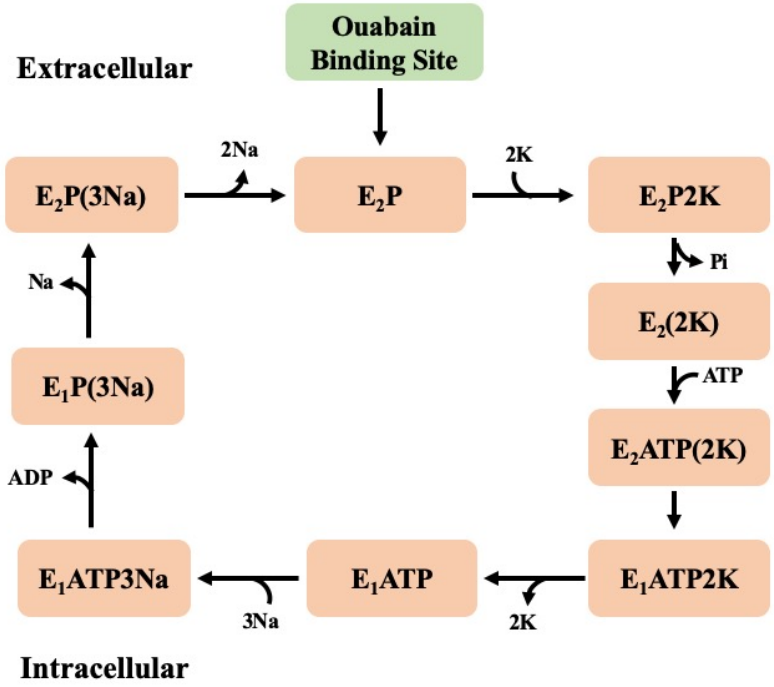


**Figure 2: Post-Albers' model for the Na-K-ATPase pump cycle.** Figure reproduced from (Kaplan, 2002).

The pumping mechanism of Na-K-ATPase can be explained in various steps. First, when the enzyme is in $E_1$ conformation, it binds to $3Na^+$ and ATP with high affinity in the cytoplasmic domain of the enzyme, forming $E_1$ATP3Na. Binding of $3Na^+$ to this orientation leads to the phosphorylation of the enzyme, which in turn forms $E_1$P-ADP3Na. The occluded ADP is

detached from the $E_1$ state, and now the binding site of Na reorients itself to the extracellular side, forming the $E_2$ state ($E_2P(3Na)$). Three $Na^+$ are released extracellularly, leaving the $E_2$ state with a phosphate ($E_2P$). In the $E_2P$ conformation, Na-K-ATPase binds two $K^+$ with high affinity, forming $E_2P2K$. The $K^+$ binding induces the dephosphorylation of the enzyme by releasing Pi intracellularly, forming the better binding of two $K^+$. At this state, ($E_2(2K)$) attaches ATP with low affinity intracellularly and reorients the $K^+$ binding site to the cytoplasmic domain forming $E_1ATP2K$. The binding of ATP induces $E_1$ conformation and triggers the release of $K^+$ into the cells. Thus, releasing two $K^+$ into the cells, Na-K-ATPase returns to the same conformation $E_1ATP$, which is reactivated to pursue the next cycle. Inhibitory molecules such as cardiotonic steroids (Ouabain) can bind to Na-K-ATPase on its extracellular site ($E_2P$ conformation) irreversibly.

Despite the robust evidence that most of the steps in Post and Albers scheme are crucial to the Na-K-ATPase pumping activity, there are controversies regarding the sub-steps of the reaction cycle (Kaplan, 2002) and whether the functional unit of the enzyme is an αβ-protomer or an oligomer (Dey et al., 2010; Repke & Schon, 1973; Vilsen, Andersen, Petersen, & Jorgensen, 1987).

## 1.5. Na-K-ATPase AS A SIGNALING MOLECULE

Any modulation in the Na-K-ATPase activity by extrinsic or intrinsic factors alters the physiological state of cells. Hence, the inhibition of the Na-K-ATPase activity leads to changes in intracellular ion homeostasis primarily by increasing intracellular sodium $[Na^+]_i$. This buildup of $[Na^+]_i$ leads to changes in intracellular pH via $Na^+/H^+$ exchanger, and intracellular calcium $[Ca^{2+}]_i$ via $Na^+/Ca^{2+}$ exchanger (Blaustein, 1993). Such alterations in ions' dynamics in cells affect the cellular physiology inducing various signaling processes.

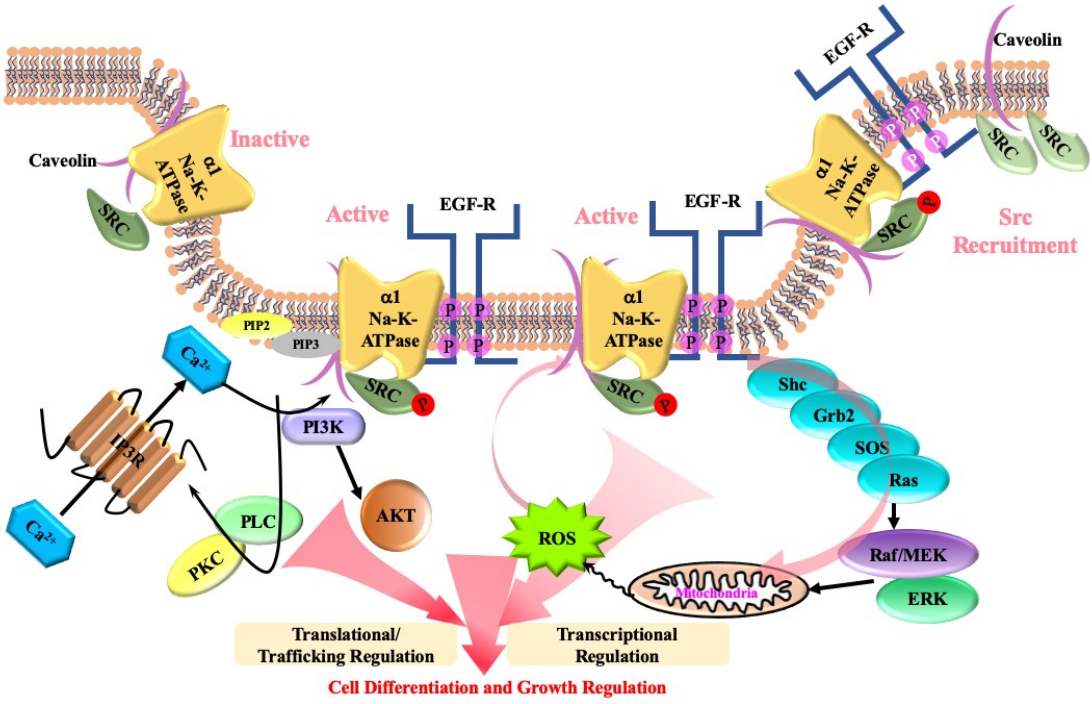


**Figure 3: Schematic representation of Na-K-ATPase-mediated signaling through protein interaction.** EGFR- Epidermal growth factor receptor, PLC- Phospholipase C; PKC- Protein kinase. (Figure reproduced from (Cui & Xie, 2017)).

Low doses of ouabain lead to the activation of classical signaling pathways such as ERK (Extracellular signal-regulated kinases) and Src pathways. It has been reported that Na-K-ATPase has been linked with Src protein, forming the Na-K-ATPase-Src complex (Abram & Courtneidge, 2000; Brown & Cooper, 1996). Src, a non-receptor tyrosine kinase, phosphorylates various tyrosine-mediated downstream signaling pathways. One of the classical pathways

activated by Src tyrosine kinase is the phosphorylation of epidermal growth factor receptors, which in turn activates downstream signals such as the activation of the Ras/Raf/MEK/ERK pathway, and the production of reactive oxygen species (ROS) (Boscher & Nabi, 2012; Wu et al., 2013; Yuan et al., 2005). These pathways indeed affect the proliferation, differentiation, migration, and metabolism of cells resulting in various pathophysiological alterations.

Moreover, Na-K-ATPase has also been associated with various other proteins like PI3K (Phosphoinositide 3-kinase), PLC (Phospholipase C), IP3R (Inositol triphosphate receptor), and caveolin-1. Interaction of such proteins with Na-K-ATPase creates a series of signal transduction pathways, which are collectively named as 'signalosome' in caveolae (Blanco, Sanchez, & Mercer, 1995; Jewell & Lingrel, 1991)

## 1.6. REGULATION OF Na-K-ATPase

Na-K-ATPase, a regulatable enzyme, is considered to be regulated both short-term and long-term depending upon the stimulus. Short-term regulation typically involves either modulation in kinetic properties of the enzyme or alteration in the trafficking of Na-K-ATPase from the cytoplasm to the plasma membrane (Therien & Blostein, 2000). The long-term regulation of Na-K-ATPase, on the other hand, generally affects the synthesis and degradation of Na-K-ATPase (Therien & Blostein, 2000). There is a considerable amount of investigation indicating that Na-K-ATPase is regulated by various intrinsic and extrinsic factors. Various extrinsic factors like inflammatory mediators, hormones, neurotransmitters, and cardiotonic steroids modulate Na-K-ATPase activity. Also, Na-K-ATPase is intrinsically regulated through phosphorylation and dephosphorylation reactions by kinases and phosphatases respectively, which are in turn stimulated by extrinsic factors and intracellular second messengers (Aperia, 1995; Bertorello & Katz, 1993).

The phosphorylation-mediated regulation of Na-K-ATPase is primarily carried out by protein kinase C (PKC), protein kinase A (PKA), and protein tyrosine kinases (PTKs). These protein kinases directly or indirectly regulate the activity of Na-K-ATPase. The direct regulation of Na-K-ATPase activity is through phosphorylation of its subunits ($\alpha$ and $\gamma$), whereas indirect regulation of Na-K-ATPase involves the signaling of various pathways that affect the transcription factor associated with regulating gene expression of the Na-K-ATPase.

### 1.6.1. PKC-mediated Na-K-ATPase regulation

There are contradictory reports suggesting that the activation of PKC results in the stimulation or inhibition of Na-K-ATPase activity depending upon cell type and experimental conditions. One study reported that dopamine reduced Na absorption by inhibiting Na-K-ATPase

activity in proximal tubule cells and the reduction in activity was mediated by PKC (Aperia, 1995; Bertorello & Katz, 1993). According to a different study, the same hormone increased Na-K-ATPase activity in pulmonary epithelial cells and the stimulation was also through PKC (Barnard et al., 1999; Bertorello, Ridge, Chibalin, Katz, & Sznajder, 1999). Additionally, another study done by Efendiev *et al.* suggests that the ultimate activity of Na-K-ATPase is PKC isoform-dependent (Efendiev, Bertorello, & Pedemonte, 1999). Therefore, the effect of PKC on Na-K-ATPase activity has been suggested to be cell- and tissue-specific.

Studies performed by Pedemonte *et al.* and Feraille *et al.* suggest that stimulation of PKC by PMA (phorbol 12-myristate 13-acetate) stimulated the activity of the Na-K-ATPase (Feraille et al., 1995; Pedemonte, Pressley, Cinelli, & Lokhandwala, 1997; Pedemonte, Pressley, Lokhandwala, & Cinelli, 1997). The increase in the activity of Na-K-ATPase was due to the translocation of Na-K-ATPase to the membrane. Another study by Efendiev *et al* reported that mutation in $Ser^{16}$ (analogous to $Ser^{11}$-after posttranslational modification) and $Ser^{23}$ (analogous to $Ser^{18}$-after posttranslational modification) of the α subunit was unable to produce the PMA-mediated stimulation of Na-K-ATPase, suggesting the N terminus $Ser^{11}$ and $Ser^{18}$ are the phosphorylation sites for PKC (Efendiev et al., 2000). In contrast, dopamine inhibited the Na-K-ATPase in renal proximal tubule cells and opossum kidney cells. The reduction of enzyme activity was mediated through PKC phosphorylation of the α subunit at $Ser^{18}$ causing Na-K-ATPase at the plasma membrane to translocate into the cytoplasmic compartment (Chibalin, Katz, Berggren, & Bertorello, 1997; Chibalin et al., 1999; Chibalin, Pedemonte, et al., 1998; Chibalin, Zierath, Katz, Berggren, & Bertorello, 1998).

On the other hand, various transcription factors also regulate the activity of Na-K-ATPase. These factors are specific to the subunits and isoforms of Na-K-ATPase (Li &

Langhans, 2015). One of the studies done by Galuska *et al.* reported that stimulation of renal tubular cells with C-peptide increases the activity of Na-K-ATPase. Increase in enzyme activity was due to increased expression of α1 subunit by activation of transcription factor ZEB through PKC and MAPK (Microtubule associated protein kinase) pathways (Galuska et al., 2011).

### 1.6.2. PKA-mediated Na-K-ATPase regulation

Similar to PKC-mediated regulation of Na-K-ATPase, there are also conflicting reports suggesting that there is inhibition (Bertorello, Aperia, Walaas, Nairn, & Greengard, 1991), activation (Cornelius & Logvinenko, 1996), or no effect on Na-K-ATPase activity (Cornelius & Logvinenko, 1996; Feschenko & Sweadner, 1994) by PKA. A possible justification to these contradictions is phosphorylation of different isoforms of the α subunit by PKA. This justification was further corroborated by the study conducted in insect cells (S*f*-9), where all isoforms of the α subunit are expressed. In this study, an endogenous activation of PKA in SF-9 cells activates α3β1 isoenzyme but inhibits α1β1 and α2β1 isoenzyme (Blanco, Sanchez, & Mercer, 1998). Unlike PKC phosphorylation, PKA targets phosphorylation of the serine residue present in the C terminus of α subunit, specifically $Ser^{943}$ (Beguin et al., 1994; Feschenko & Sweadner, 1994; Fisone et al., 1994).

### 1.6.3. PKG-mediated Na-K-ATPase regulation

Similar to the PKA- and PKC-mediated regulation, protein kinase G (PKG) also regulates Na-K-ATPase activity in a tissue- and isoform-specific manner. It has been reported that there is a cyclic guanosine monophosphate (cGMP)-mediated decrease in Na-K-ATPase in the kidney (McKee, Scavone, & Nathanson, 1994; Scavone, Scanlon, McKee, & Nathanson, 1995). The reduced enzyme activity is primarily due to decreased expression of the α1 subunit. However, in

another study, there is a marked increase of Na-K-ATPase in Purkinje neurons, due to increased expression of Na-K-ATPase α3 subunit (Nathanson, Scavone, Scanlon, & McKee, 1995).

### 1.6.4. Tyrosine kinase-mediated regulation of Na-K-ATPase

Besides activation of the tyrosine kinase signaling cascade as discussed above, tyrosine-protein kinase (Sarcoma (Src)) also phosphorylates the tyrosine residue at $Tyr^{10}$ (analogous to Tyr5-after posttranslational modification) of the α subunit, resulting in an increase in enzyme activity (Chibalin et al., 1999). Studies on skeletal muscle stimulation by insulin (Chibalin et al., 2001), and in kidney proximal tubules where dopamine-like receptor are stimulated (Narkar, Hussain, & Lokhandwala, 2002) have shown increased tyrosine phosphorylation resulting in increased Na-K-ATPase activity. Other studies have reported that an increased tyrosine phosphorylation of Na-K-ATPase α1, by inhibiting protein tyrosine phosphatase (PTP) on human and pig renal cells, reduces the activity of Na-K-ATPase (El-Beialy et al., 2010)

### 1.6.5. FXYD-mediated regulation of Na-K-ATPase

FXYD is a signal transmembrane protein composed of an extracellular -N terminus and a cytoplasmic-C terminus (Dey et al., 2013). A specific domain at the N-terminus interacts with Na-K-ATPase and hence regulates the activity of Na-K-ATPase, whereas the -C terminus consists of phosphorylation sites (Dey et al., 2013). To date, there are at least 12 different members of FXYD expressed differentially in a cell- and tissue-specific manner. FXYD family proteins are expressed in a wide array of tissues including kidney, colon, pancreas, heart, and skeletal muscle (J. Y. Cheung et al., 2010). Also, due to the presence of phosphorylation sites in the C-terminus, FXYD proteins are subject to phosphorylation by different protein kinases (Cornelius & Mahmmoud, 2007; Cortes, Ribeiro, Barrabin, Alves-Ferreira, & Fontes, 2011;

Mounsey et al., 2000). Moreover, phosphorylation of FXYDs by kinases modulate the activity of Na-K-ATPase.

### 1.6.6. Transcriptional regulation of Na-K-ATPase

The Na-K-ATPase expression is modulated by various factors including hormones, growth factors, lipids, transcription factors, and extracellular stimuli. These factors modulate the expression by transcriptionally regulating the subunits of Na-K-ATPase. In the current study, the focus is on the regulation of α1 and β1 subunit.

#### 1.6.6.1. Transcriptional regulation of Na-K-ATPase α1 subunit

*ATP1A1* is the gene responsible for transcribing the α1 subunit. It contains a TATA box (M. M. Shull, Pugh, & Lingrel, 1990), two mineralocorticoid/glucocorticoid response element (MRE/GRE) sites (Kolla, Robertson, & Litwack, 1999), binding sites for AP-1, AP-2, AP-3 (M. M. Shull et al., 1990), SP1 and CTF/NF-1 binding site (M. M. Shull et al., 1990). Additionally, ATF/CRE binding site is also found in the promoter region of *ATP1A1* (Kobayashi & Kawakami, 1995).

Specificity protein (SP) consists of various transcription factors: Sp1, Sp2, Sp3, and Sp4. Among these SP transcription factors, Sp1, Sp3, and Sp4 had been shown to increase transcription of the α1 subunit (Johar, Priya, & Wong-Riley, 2012; M. M. Shull et al., 1990). Another transcription factor is cAMP response element binding protein family (CREB), Phosphorylation of CREB by cAMP has been shown to increase transcription of the α1 subunit (Dagenais et al., 2001). Similarly, phosphorylation of activating transcription factor 1 (ATF-1) also transcriptionally upregulate the α1 subunit (Kobayashi, Shimomura, Hagiwara, & Kawakami, 1997). The zinc finger E-Box Binding Homeobox 1 (ZEB1/AREB6) also bind to the

promoter region of *ATP1A1* and stimulates the transcription of α1 subunit in a tissue-specific manner (Watanabe, Kawakami, Hirayama, & Nagano, 1993).

#### 1.6.6.2. Transcriptional regulation of Na-K-ATPase β1 subunit

The β1 subunit is encoded by the gene *ATP1B1* and has been widely studied among all other subunits. The *ATP1B1* gene consists various transcription factors binding sites: three MRE/GRE half-sites (Derfoul, Robertson, Lingrel, Hall, & Litwack, 1998), four E-boxes (Espineda, Chang, Twiss, Rajasekaran, & Rajasekaran, 2004), one NF-1 (Espineda et al., 2004), three prostaglandin response elements (PGRE) (Matlhagela, Borsick, Rajkhowa, & Taub, 2005; Matlhagela & Taub, 2006b), a hypoxia response element (HRE) (Mony, Lee, Harper, Barwe, & Langhans, 2013), Smad binding domain (SBD) (Mony et al., 2013), three thyroid response elements (TRE) (Feng, Orlowski, & Lingrel, 1993), three progesterone element (PRE) half sites (Cochrane et al., 2012), several Sp1 binding sites (Matlhagela et al., 2005; Matlhagela & Taub, 2006b), and a Cebpb binding sites (Deng et al., 2013). Moreover, the TATA box of *ATP1B1* also consist of calcium and serum response elements (Liu & Gick, 1992).

Similar to the α1 subunit, the β1 subunit is also transcriptionally upregulated by Sp1, Sp3, and Sp4 in murine neurons by binding to their promoter regions (Johar et al., 2012). Cebpb also increases the β1 subunit in human epithelial cells by binding to the CCAT box (Deng et al., 2013). Beside transcriptional upregulation of the β1 subunit, it is transcriptionally repressed by Snail protein by binding to the E-box on the promoter region (Espineda et al., 2004). Another transcription factor that represses the β1 subunit is hypoxic-inducible factor-1(HIF-1), which suppress the transcription of the β1 subunit by binding to a HRE (Hypoxia response element) (Mony et al., 2013).

# CHAPTER 2: THE GASTROINTESTINAL TRACT AND ROLE OF EICOSANOIDS

## 2.1. THE GASTROINTESTINAL TRACT

Every day the human gastrointestinal (GI) tract processes 8 to 10 liters of fluid which is taken orally or secreted inside the intestine every day. Because of the robust absorption mechanism in the GI tract it only eliminates 100 to 200 mL of fluid waste each day (Kiela & Ghishan, 2016). Along with fluid secretion and absorption, the GI tract also extracts nutrients, vitamins, and minerals that are ingested as food material, and provides a barrier against harmful pathogens and toxins (Kiela & Ghishan, 2016). The process of absorption, secretion and defense mechanism go hand-in-hand in normal homeostatic conditions. The multiplicity of GI activity is only possible due to the unique tissue, cellular, and molecular architecture of the small and large intestine (Figure 4), as well as in combination with other regulatory mechanisms. Regulation of GI activity includes the complex crosstalk between endocrine and paracrine hormones, neurotransmitters, immunomodulators, and luminal factors (Kumral & Zfass, 2018; Track, 1980).

### 2.1.1. Intestinal architecture

The structure of the small intestine gives an advantage for proper absorption of nutrients, electrolytes, and fluids. The cylindrical small intestine consists of special structural features such as circular folds (plicae circulares), villus-crypt architecture, and microvilli, which amplify the absorption process by increasing the surface area about 600-fold (Hilton, 1902; Walton, Mishkind, Riddle, Tabin, & Gumucio, 2018). However, the large intestine, which generally lacks these structural features, is only efficient to reabsorb fluid and some fatty acids.

The small intestine has the ability to multitask, for which it needs a variety of cells, including enterocytes (nutrient absorption), goblet cells (mucus secretion), Paneth cells (defensin

production against antimicrobial agents) and enteroendocrine cells (Barker & Clevers, 2010). All these cell lineages are derived from the stem cells which lie in the pit known as the crypt. Stem cells divide into rapidly proliferating transit-amplifying cells, which subsequently arise from the crypt and start to differentiate and begin to express differentiation markers for three lineages: enterocytes, enteroendocrine and goblet cells (Barker & Clevers, 2010). These cells are fully differentiated as they reach the crypt-villus junction. These cells move along the crypt-villus axis as they grow and mature and eventually undergo apoptosis and slough off from the tip of the villus (Barker, van de Wetering, & Clevers, 2008; van der Flier & Clevers, 2009). The whole process from the origin of the cell to its death takes about 3 to 5 days (Tesori, Puglisi, Lattanzi, Gasbarrini, & Gasbarrini, 2013).

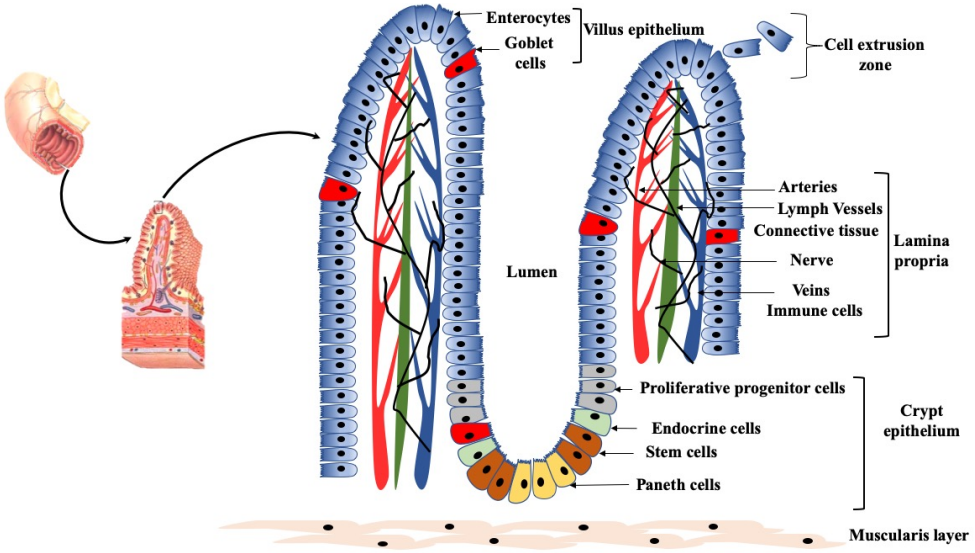


**Figure 4: Schematic diagram of small intestine architect including crypt and villus cells.**

Some stem cells differentiate into Paneth cells that migrate to the bottom of the crypts. However, the intestinal lining mostly consists of enterocytes that help to absorb nutrients from the lumen. These enterocytes, while growing from immature crypt cells to fully mature

absorptive villus cells, have various morphological and physiological alterations. Therefore, depending on the position of enterocytes along the crypt-villus axis, there is a differential expression of transport proteins. Most of the transporters (Na-nutrient, NHE3 (Na-hydrogen exchanger) and SLC26A6) are accrued while moving towards the villus, whereas transporters like CFTR (Cystic fibrosis transmembrane conductance regulator) exhibit a greater density in the base of the crypt. However, some of the transporters are expressed equally throughout the crypt-villus axis (Field, 2003; S. K. Singh, Binder, Boron, & Geibel, 1995).

### 2.1.2. Epithelial cell model

The monolayer of enterocytes in the small intestine provides a semipermeable barrier and separates two distinct compartments, outer mucosal (lumen) and inner serosal (blood-side) (Schneeberger, Roth, Nieuwenhuis, & Middendorp, 2018). All enterocytes from the small intestine to the colon (colonocytes) share a common topography. Enterocytes are polarized epithelial cells with morphologically and molecularly distinct apical (brush border or luminal) and basolateral (serosal) membranes (Figure 5). Enterocytes are adhered together from the lateral side by junctional proteins (such as tight junctions, adheren junctions, and desmosomes proteins) (Anderson & Van Itallie, 1995; Giepmans, 2004; Marchiando, Graham, & Turner, 2010). Tight junction permeability is relatively leaky towards the small intestine, whereas very tight towards the colon (Field, 2003).

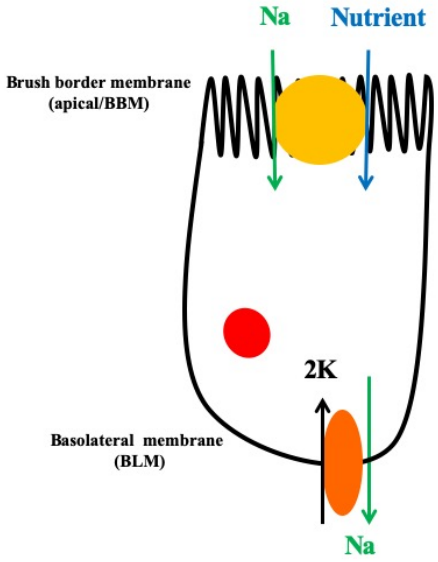


**Figure 5: Typical small intestine epithelial cell.**

Enterocytes acquire distinct polarized characteristics specifically by inserting transporters into the brush border or basolateral membrane. Intracellular trafficking processes, cytoskeletal and protein-sorting signals interact synergistically to direct *de novo* proteins to either the brush border or basolateral membrane (Apodaca, 2001; Massey-Harroche, 2000). Generally, glycosylphosphatidylinositol (GPI) anchors (e.g., alkaline phosphatase) are associated with lipid rafts, and the GPI anchor targets newly synthesized proteins to the apical membrane (Alberts, Johnson, & Lewis, 2007). Specific membrane-sorting signals (amino acid sequences) in the cytoplasmic tail direct proteins to the basolateral membrane. Proteins lacking the membrane sorting signal and GPI anchor are arbitrarily inserted into either the brush border or basolateral membrane. Finally, the intracellular trafficking regulation ensures delivery of the right protein to the right membrane (Schneeberger et al., 2018). All these processes mentioned above are major factors that contribute to the development of polarized epithelial cells and thus support nutrient and electrolyte transport. There is variation in the intestinal transport system, along the cephalocaudal length of intestine and along the crypt-villus axis within the specific segment.

### 2.1.3. Na-dependent transporters in the intestine

Various transporters present in the membrane of the enterocytes facilitate its absorptive capacity. These transporters might be present on any side of the enterocytes. Some of them are coupled transporters, which transport two substrates at a time; whereas others are not, as they transport only a single substrate. Among coupled transporters, we are mostly focused here on Na-dependent transporters that are present in the brush border membrane of enterocytes and help in the absorption of nutrients and electrolytes. Some of the Na-dependent transporters present in the brush border membrane of enterocytes are Na-amino acids [Na-glutamine (B0AT1), Na-alanine (NAcT)], Na-glucose (SGLT1), and Na-bile acid. All these Na-dependent transporters

perform perfectly only when there is an optimum $Na^+$ gradient maintained between the lumen and enterocytes. Therefore, Na-K-ATPase, a basolateral membrane protein maintains the $Na^+$ gradient that is necessary to drive Na-dependent transporters.

## 2.2. EICOSANOID BIOSYNTHESIS

### 2.2.1. Arachidonic acid synthesis

The lipolytic enzyme known as phospholipase A2 (PLA2) catalyzes the hydrolysis of cell membrane phospholipids into free fatty acids and a lysophospholipid. PLA2 is a member of a superfamily of enzymes consisting of various classifications. PLA2 has been broadly classified

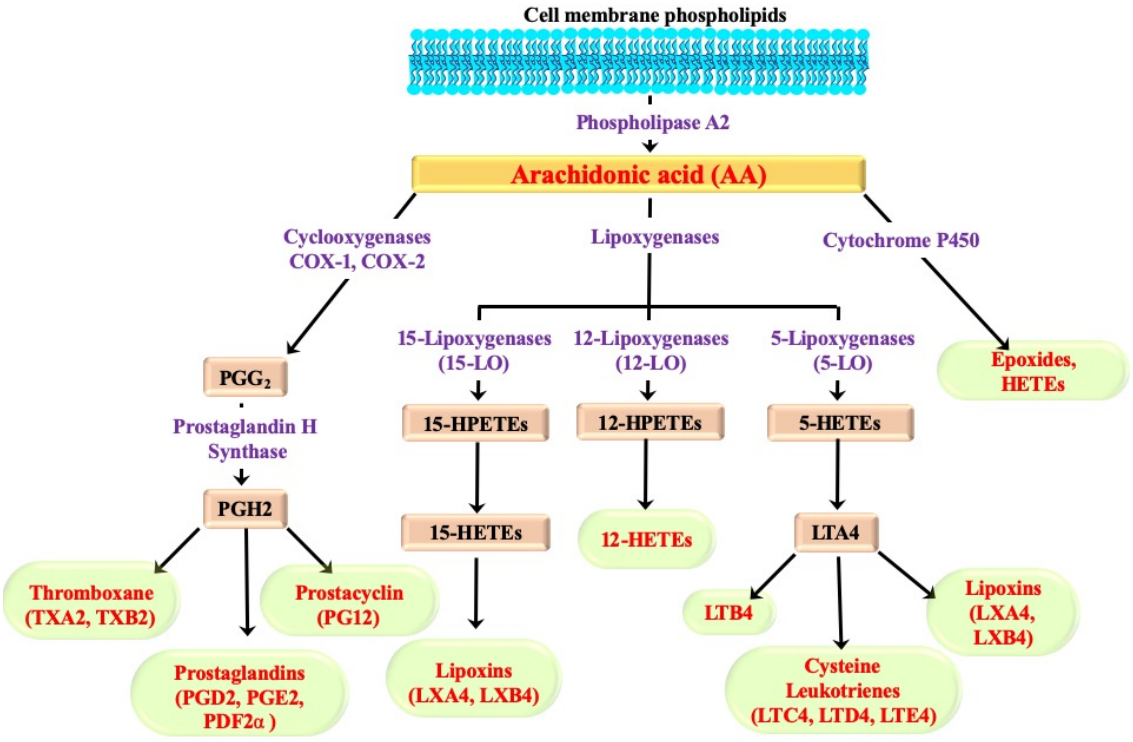


**Figure 6: Major pathways of arachidonic acid metabolism.** Figure reproduced from (Harizi, Corcuff, & Gualde, 2008).

into three types: secretory (sPLA2), cytosolic $Ca^{2+}$-dependent (cPLA2), and cytosolic $Ca^{2+}$-independent (iPLA2) (Balsinde, Winstead, & Dennis, 2002). This systematic classification has been based on various properties such as their amino acid sequences (Dennis, 1994; Six &

Dennis, 2000), and specificity to the substrate and catalytic function (Balsinde et al., 2002). In response to various stimuli such as cAMP, intracellular $Ca^{2+}$ and other responses, PLA2 releases 20-carbon free fatty acid derivative known as arachidonic acid (AA) from the phospholipid, as shown in Figure 6. Different eicosanoids products, including prostaglandins (PGs), leukotrienes (LTs) and lipoxins are generated from this free-formed AA (Smith, DeWitt, & Garavito, 2000).

### 2.2.2. Arachidonic acid metabolism

Released arachidonic acids are metabolized into various eicosanoids products primarily through three major oxidative pathways: cyclooxygenase, lipoxygenase, and cytochrome $P_{450}$ pathways, which are explained below (Figure 6) (Moskowitz, Shapiro, Schook, & Puszkin, 1983).

#### 2.2.2.1. Prostaglandin biosynthesis

Different kinds of PGs are produced by the enzyme cyclooxygenase, thus named the cyclooxygenase pathway. Cyclooxygenase exists into two isoforms; cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) (Mohajer & Ma, 2000). These enzymes catalyze the oxidation of AA into PGG2 by inserting two oxygen molecules. The reduction of PGG2 converts it into PGH2, which is further converted to other eicosanoids including prostaglandins (PGE2, PGD2, PGF2), thromboxanes (TXA2, TXB2) and prostacyclin (PGI2) (DuBois, Eberhart, & Williams, 1996). COX-1, known as constitutive cyclooxygenase, basically maintains the mucosal integrity by performing under the basic homeostatic conditions. However, COX-2 is expressed in response to extrinsic or intrinsic stimuli; therefore the enzyme is named as inducible cyclooxygenase (DuBois et al., 1996). COX-2 has been observed as a key player to initiate PGs involved inflammation, resulting in swelling and pain (Mitchell & Evans, 1998), as well as

participating in housekeeping functions (DuBois et al., 1996). These COX enzymes are the target of various nonsteroidal inflammatory drugs (NSAIDs) for reducing inflammation.

#### 2.2.2.2. Lipoxygenase biosynthesis

Lipoxygenase enzyme catalyzes the conversion of AA to various hydroperoxyeicosateraenoic acids (HPETEs) and hydroxy eicosatetraenoic acids **(**HETEs) by inserting one molecule of oxygen into AA (Lewis, Austen, & Soberman, 1990). 5-HETEs are converted to LTA4 and subsequently converted to cysteinyl LTs (LTC4, LTD4, LTE4), LTB4, and lipoxins (LXA4, LXB4). Similarly, 15-HPETEs are also converted to lipoxins (LXA4, LXB4) (Harizi & Gualde, 2005).

#### 2.2.2.3. Cytochrome $P_{450}$ pathways

The cytochrome $P_{450}$ epoxygenase pathway catalyzes the formation of epoxides and HETEs by inserting a single atom of oxygen into AA (Capdevila et al., 1992).

## 2.3. EICOSANOIDS IN CELLULAR SIGNALING

Eicosanoids produce various physiological alterations by binding to membrane receptors. Binding with different receptors can result in various changes, including increase or decrease in cytosolic secondary messengers (cAMP, $Ca^{2+}$), stimulation of specific protein kinases, and changes in membrane potential, subsequently modulating the physiological state (Harizi et al., 2008) (Figure 7). Here, we will focus on PG and LT signaling because of their importance in the small intestine and its pathophysiological state.

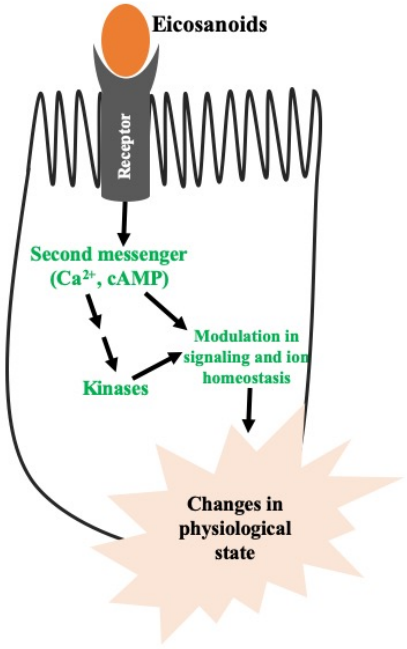


**Figure 7: Canonical pathway for eicosanoids signaling**

### 2.3.1. Prostaglandin receptor signaling

PGs are secreted endogenously or during pathophysiological conditions, and mediate their action by binding to specific membrane surface G-protein-coupled receptors (GPCRs) (Dore, 2011; Liu, Qu, & Yan, 2015). To date, there exist at least nine prostanoid receptors in mice and humans, as well as other splice variants (Narumiya & FitzGerald, 2001). Among these nine receptors, four of the receptors ($EP_1$, $EP_2$, $EP_3$, and $EP_4$) bind to PGE2, two ($DP_1$ and $DP_2$) bind to PGD2, FP specifically binds to PGF2α, and two bind to prostacyclin and thromboxane (Hirai et al., 2001; Monneret, Gravel, Diamond, Rokach, & Powell, 2001). These receptors are further classified into four different categories based on the specific isoform of G protein involved in signaling. The first group, $DP_1$, $EP_2$, and $EP_4$ receptors exert signaling through $G_s$-mediated intracellular cAMP. $EP_2$ and $EP_1$ receptors are a second group that signal through $G_q$-

mediated intracellular $Ca^{2+}$. $EP_3$ falls into the third category, which regulates adenyl cyclase activity through activation of $G_i$ and $G_s$ proteins. Unlike other receptors, the last group DP2 is a member of a chemoattractant receptor family (Harizi et al., 2008; Hirai et al., 2001; Monneret et al., 2001). Prostanoid receptors are mostly localized to the plasma membrane; however some are found at the nuclear membrane as well (Bhattacharya et al., 1998; Bos, Richel, Ritsema, Peppelenbosch, & Versteeg, 2004).

### 2.3.2. Leukotriene receptor signaling

LTs mediate signaling through four different kinds of GPCR. The first, BLT1, known as a high-affinity LTB4 receptor, mediates signaling through cGMP via guanylyl cyclase. It also mediates signaling through $G_q$ and $G_i$ (Yokomizo, Izumi, Chang, Takuwa, & Shimizu, 1997; Yokomizo, Kato, Terawaki, Izumi, & Shimizu, 2000). The second, BLT2, reported as a low-affinity LTB4 receptor, signals through $G_q$. The other two subtypes, CysLT1 and CysLT2, are cysteinyl-LT receptors that are targeted by LTC4 and LTD4. CysLT1 is activated explicitly by LTD4, whereas CysLT2 couples with both LTC4 and LTD4 (Kanaoka & Boyce, 2004).

## 2.4. ROLE OF EICOSANOIDS IN THE SMALL INTESTINE

### 2.4.1. Eicosanoids in intestinal secretion

Large volumes of fluid are secreted into the lumen of the small intestine during digestion. However, these fluids are reabsorbed back into the body. Fluids are secreted either by an increase in luminal osmotic pressure or by PG-induced secretion. The mechanism by which a PG-induced intestinal fluid secretion is mediated is through intracellular secondary messengers such as cAMP, $Ca^{2+}$, and acetylcholine. These secondary messengers induce intestinal secretion by stimulating chloride and bicarbonate secretion and inhibiting sodium and chloride absorption (Diener, Bridges, Knobloch, & Rummel, 1988a, 1988b; Powell, 1986; Racusen & Binder, 1980; Rask-Madsen, Bukhave, & Lauritsen, 1988).

Various *in vitro* and *in vivo* studies reported that PG stimulates intestinal secretion (Diener et al., 1988a, 1988b; Rask-Madsen et al., 1988). In rats, when PGE2 was administered orally, it induced fluid secretion (Ruwart, Klepper, & Rush, 1979). Similarly, when PGE2 and PGF2 were administered intravenously, they produced a secretory effect (Milton-Thompson, Cummings, Newman, Billings, & Misiewicz, 1975). Like in other studies in which PG perfusion was performed in different segments of animals (Canine jejunum, Rat jejunum and Cat ileum), it yielded fluid secretion (Gaginella, 1990). The fluid secreted was rich in chloride but low in calcium and sodium. In contrast, when PG synthesis was reduced by blocking COX with a nonspecific COX inhibitor (indomethacin), it resulted in decreased fluid secretion (Ruwart et al., 1979). This gives us the premise that reduction of PG endogenously may reduce the fluid secretion in the small intestine.

As shown in Figure 7, secondary messengers when activated induce protein kinases, which subsequently induce the phosphorylation of specific substrate proteins present in the

cytoplasm or cell membrane. These activated substrate proteins urge apical membrane anionic channels to open, resulting in secretion of chloride and bicarbonate anions into the lumen of the small intestine (Diener et al., 1988a; Rask-Madsen et al., 1988). Thus, the secretion of chloride ions creates an electrochemical gradient between serosal and mucosal layers, where the serosal layer lacks anions and the mucosal layer is rich in anions. The electrochemical gradient thus produced withdraws positively charged $Na^+$ and water into the lumen through a paracellular route, resulting in net fluid secretion in the lumen (Gaginella, 1990; Robert, Nezamis, Lancaster, Hanchar, & Klepper, 1976; Turnberg, 1991).

Additionally, PG-mediated fluid secretion is also regulated through central and peripheral nervous systems. PG induces the myenteric ganglia and cholinergic nerve terminal to produce acetylcholine, which activates muscarinic receptors on enterocytes to increase the influx of $Ca^{2+}$ into the cytoplasm. Thus, this increased $Ca^{2+}$ stimulates $Cl^-$ secretion in the lumen (Ehrenpreis, 1981; Keast, 1987).

**2.4.2. Eicosanoids in epithelial barrier**

The monolayer of intestinal epithelial cells forms a cellular barrier that prevents harmful pathogens, toxins, and immunogenic substances from reaching the subepithelial tissue. The epithelial layer consists of a lipid bilayer at the apical membrane and tight junction (TJ) proteins at the lateral surface (Figure 8), which obstruct such harmful molecules from reaching into the systemic circulation (Anderson & Van Itallie, 1995; Madara, 1989). Although enterocytes form the structural barrier, they also transport all molecules through two major pathways: transcellular pathway - transport of molecules through the apical plasma membrane, and paracellular pathway- transport of molecules laterally through TJ barrier (Ma, 1997). However, hydrophilic

molecules which do not have specific transporter on membrane permeate through TJs rather than lipid bilayers.

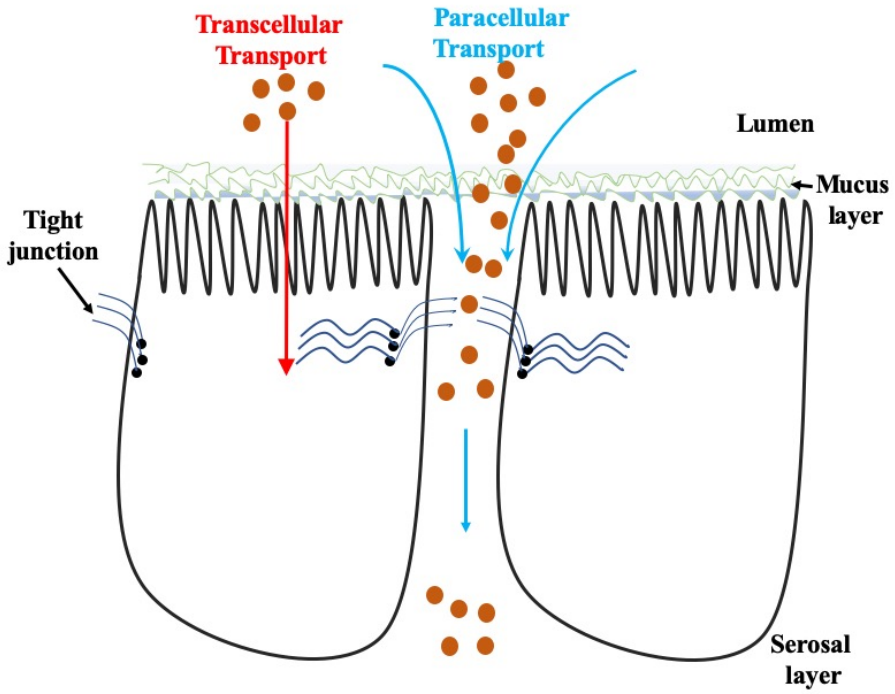


**Figure 8: Pathways for transportation of molecules in small intestinal epithelial cells.**

Therefore, the permeability of hydrophilic molecules is solely dependent on the relative tightness of the TJ barrier (Ma, 1997; Madara, 1989). Thus, enterocytes in normal conditions have a restrictive permeability and are referred to as non-leaky enterocytes, whereas enterocytes with an increase in permeability during pathophysiological conditions are known as leaky enterocytes.

Given this background, PGs also play a vital role in growth, maintenance and fortification of the intestinal epithelial barrier. In one study, it has been reported that there is an increase of PGE2 along with the maturation of the intestinal epithelial barrier (Gerstle, Seaton, Kauffman, & Colony, 1994). In the same study, inhibition of PGE2 in mature animals (rats) by a

COX-inhibitor (indomethacin) caused an increase in epithelial permeability (Gerstle et al., 1994). Similarly, in another *in vitro* study, intestinal ischemia produced a marked increase in epithelial permeability and reduced epithelial resistance in porcine ileum (Blikslager, Roberts, Rhoads, & Argenzio, 1997). However, ischemic porcine ileum recovered back to normal after the exposure to PGI2 and PGE2 (Blikslager et al., 1997). Moreover, cAMP and $Ca^{2+}$ ionophores A23187 also restored epithelial resistance, thus concluding that PG restoration reverts injured intestinal epithelial back to normal. Consistent with the studies referenced above, various NSAIDs (indomethacin, aspirin, and ibuprofen) have been shown to increase intestinal permeability and hence decrease intestinal epithelial barrier function in humans (Bjarnason, Fehilly, Smethurst, Menzies, & Levi, 1991; Bjarnason & Thjodleifsson, 1999).

**2.4.3. Eicosanoids in mucosal protection**

Mucosa provides an additional layer of defense against varying temperature, pH, osmolarity, cytotoxic substances and bacterial products dispersed in the lumen (J. L. Wallace, 2008). Therefore, mucosal integrity and health are essential for protection against invasion of noxious agents into the serosal layer. Numerous studies have reported that PG treatment has a protective effect on mucosal damage (Robert, 1976, 1981). PGs are involved in mucus synthesis and secretion, mucosal bicarbonate secretion, and fluid secretion. All of these processes help in developing mucosal health and integrity by diluting noxious substances present in the intestinal lumen (Leung, Miller, Reedy, & Guth, 1989; Lugea, Salas, Guarner, Azpiroz, & Malagelada, 1992).

Another important role of gastroduodenal mucosa is to secrete bicarbonate ions ($HCO_3^-$) into the lumen to neutralize the acidic pH of the lumen from gastric acid which can reduce the luminal pH to 1.5-2 (Bukhave, Rask-Madsen, Hogan, Koss, & Isenberg, 1990; Takeuchi, Yagi,

Kato, & Ukawa, 1997). $HCO_3^-$ secretion is regulated by endogenous PGs and the nervous system. Moreover, Bukhave *et al.* have reported that patients with duodenal ulcers have reduced $HCO_3^-$ secretion and reduced endogenous PGs compared to normal patients, indicating that endogenous PGs have a role in $HCO_3^-$ secretion (Bukhave et al., 1990).

Additionally, endogenous PGs have also been involved in the proliferation of crypt cells. In this regard, NSAIDs have shown to reduce villus height, crypt depth, and the number of enterocytes (Uribe, Alam, & Midtvedt, 1992).

**2.4.4. Eicosanoids in endothelial barrier**

Endothelial cells are also an important source of eicosanoids. Endothelial cells have all major pathways involved in the production of eicosanoids, including PGI2, PGE2, PGF2$\alpha$, thromboxane $A_2$, LTs and hydroxy fatty acids (Schror, 1985). Eicosanoids produced by endothelial cells have been shown to regulate vascular tone, cell locomotion, platelet activation and cell proliferation (Schror, 1985). One of the deleterious effects of NSAID- or ethanol-induced GI mucosal injury has been due to damaged vasculature, resulting in increased vascular permeability. This increased vascular permeability eventually leads to the formation of interstitial edema, compression of blood vessels, thrombosis, ischemia, and stasis of blood vessels (Szabo, 1987). In concordance with previous studies, PG treatment to NSAID-induced GI injury improved vasculature and mucosal blood flow (L. Y. Cheung, 1980; Guth, 1986; Szabo, 1987; Szabo & Goldberg, 1990). Therefore, PGs have been shown to protect GI injury from NSAIDs by fortifying the endothelial barrier function.

**2.4.5. Eicosanoids in intestinal muscle motility**

Like other organ systems in which PGs stimulate the contraction of smooth muscles, PGs are also involved in contraction of the small intestine, specifically initiation and propagation of

migrating motor complex (MMC) in the intestine (Burakoff, Nastos, & Won, 1990; Karim & Filshie, 1970). It has been reported that when human duodenum was exposed to PGE2, there was a delay in initiation of MMC as well; PGE2 prevented propagation of initiated MMC (Tollstrom, Hellstrom, Johansson, & Pernow, 1988). However, PGF2α has been found to induce motility in the small intestine (Tollstrom et al., 1988).

The opposite effect of PGs in small intestinal motility is explained by various muscle layers involved in contraction, specifically longitudinal and circular smooth muscle. PGE2 has been shown to stimulate contraction of longitudinal smooth muscles, whereas it relaxes the circular smooth muscles (Bennett & Fleshler, 1970; Waller, 1973). However, PGF2α has been reported to stimulate both longitudinal and circular smooth muscle layers (Bennett & Fleshler, 1970; Waller, 1973).

## 2.5. ROLE OF EICOSANOIDS IN INFLAMMATORY BOWEL DISEASE (IBD)

Besides being involved in maintaining normal homeostatic intestinal physiology, eicosanoids also play a crucial role during pathophysiological conditions. During pathophysiological conditions, eicosanoids might alter intestinal motility, mucosal growth and development, endothelial functions, and transport processes (electrolyte and nutrient) involved in intestinal secretion, and absorption. However, in this section we will focus on the enterocyte transport process in regard to inflammatory bowel disease (IBD). IBD is known as inflammation of the GI tract and includes ulcerative colitis and Crohn's disease. Ulcerative colitis is primarily the inflammation of the colon whereas Crohn's disease is the inflammation of the small intestine and/or colon.

### 2.5.1. Role of Prostaglandin

As mentioned before, due to the diverse role of PGs in the intestine, many elaborative studies are devoted to the understanding of the role PGs play in IBD. It has been reported that there is increased production of cyclooxygenase products during IBD: about ~ 100% increase of PGE2 and thromboxane and ~ 50% increase in prostacyclin (Ligumsky et al., 1981; Rachmilewitz, Ligumsky, Haimovitz, & Treves, 1982; Sharon & Stenson, 1984). During intestinal pathological conditions like Crohn's disease or ulcerative colitis, there is excessive production of PGs causing diarrhea (Hawkey, Karmeli, & Rachmilewitz, 1983; Hawkey et al., 1998). The cause of diarrhea may be due to the following two mechanisms: 1) PG-mediated secretion of intestinal fluid, and 2) PG-induced stimulation of small intestinal motility. The increase in PGs level during Crohn's ileitis is due to up-regulation of COX-2 but not COX-1 expression (Hendel & Nielsen, 1997; Singer et al., 1998). Singer *et al*. has reported that COX-1 expression is limited to crypt cells of the small intestine during normal and inflamed condition;

whereas, COX-2 expression was elevated in Crohn's ileitis and limited to villus epithelium. Furthermore, COX-2 was also seen to be markedly increased in mononuclear cells in Crohn's ileitis. However, the increased level of COX-2 was relatively higher in epithelial cells compared to mononuclear cells present in lamina propria (Singer et al., 1998). These findings suggest that COX-2 induced PG is involved in the induction of intestinal inflammation. However, in clinical studies, inhibiting COX with NSAIDs did not recover the patient from IBD, rather aggravated the situation (Bjarnason & Peters, 1989; Kaufmann & Taubin, 1987; Rampton & Sladen, 1981).

**2.5.2. Role of Leukotrienes**

The role of LTs was observed much later than the role of PGs in IBD. Actually, the role of LTs in the context of IBD gathered attention after Musch *et al.* reported that LOX product stimulates colonic secretion (Musch, Miller, Field, & Siegel, 1982). Biopsy samples of active IBD patients showed a remarkable rise in the lipoxygenase products 5-HETE and LTB4 (Sharon & Stenson, 1984), and the mechanism behind the increase of LTB4 was due to reduced activity of ω-hydroxylase and increased activity of 5-LOX (Ikehata et al., 1995). Peskar *et al.* also reported there was a significant increase in peptide LTs (LTC4, LTD4, LTE4) in IBD patients (Peskar, Dreyling, Peskar, May, & Goebell, 1986). The IBD patients treated with NSAIDs showed more aggravating conditions compared to non-treated patients (Hudson, Balsitis, Everitt, & Hawkey, 1993). The mechanism behind the aggravating situation was due to NSAIDs shunting AAs to a remarkable increase in LTB4 production (Hudson et al., 1993).

In several investigations with animal models of IBD, there has been success in treating IBD with inhibitors of LT (J. L. Wallace et al., 2011). However, when IBD patients were treated with the selective inhibitor of LTB4 (MK-591), it did not produce a significant improvement in the clinical activity of the patients (Roberts et al., 1997). Likewise, when patients were treated with a

5-LOX inhibitor (zileuton), the IBD group had the same remission rate compared to the placebo group (Hawkey, Dube, Rountree, Linnen, & Lancaster, 1997). All these studies indicate that there might be a possibility that LT might play a role in IBD, but the mechanisms need to be explored further.

### 2.5.3. Role of eicosanoids in Intestinal epithelial transporters

The small intestine is the major site of nutrient and electrolyte absorption. However, ample evidence exists to show that Na-coupled co-transporters including Na-glutamine (System B0 amino acid transporter 1 (B0AT1)), Sodium-alanine co-transporter (NAcT), Soidum-Glucose transporter 1 (SGLT1) and Na-neutral amino acid co-transporter are reduced in absorptive villus cells during *in vivo* or *in vitro* IBD-related studies (Table 2). The mechanism of reduced co-transporter activity is confined to the specific co-transporters, either due to a decrease in protein expression or a decrease in affinity ($1/K_m$) (Table 2). Unlike villus cells, the Na-glutamine (System N transporter 2 (SN2)) co-transporter in crypt cells is stimulated during IBD, and the mechanism is an increase in affinity of the co-transporter rather than the change in expression of the protein (Table 2). In all of these studies, at the intact cell level, there is a positive correlation between Na-dependent transporter and Na-K-ATPase, indicating that the reduced or increased activity of the Na-dependent co-transporter is in part due to the activity of Na-K-ATPase.

Moreover, there is a differential effect on isoforms of Na/H exchanger during IBD. There are mixed reports with increased or decreased activity of NHE1 (performs housekeeping function), whereas NHE3 (performs the absorptive function) appeared to have reduced activity during IBD (Magalhaes, Cabral, Soares-da-Silva, & Magro, 2016).

#### 2.5.3.1 Role of eicosanoids in Na-K-ATPase

Widespread studies in humans, animals, and cell lines provide robust evidence suggesting that there is decreased Na-K-ATPase during IBD or when cells are exposed to IBD-associated proinflammatory factors (Table 3 & 4). The mechanism is mostly due to reduced levels of α1 and β1 isoforms, resulting in a decrease in the number of functional pumps in the membrane. However, there are alternative mechanisms responsible for the reduction of Na-K-ATPase activity: 1) stimulation of the PKC-STAT1 phosphorylation downstream signaling pathway without changing the level of Na-K-ATPase protein, and 2) lack of Na-K-ATPase to properly adhere to membranes due to downregulation and depolarization of ankyrin protein (Table 3 & 4).

| Model/ Cell Types | Related Methods | Major Findings | Mechanisms | References |
|---|---|---|---|---|
| Rabbit IBD<br>(Villus cells) | Radioactive uptake,<br>Inorganic phosphate (P*i*) measurement<br>WB | ↓ Na-bile acid (ASBT)/ ↓ Na-K-ATPase in IBD<br>**Whereas, Glucocorticoid (Methylprednisolone) treatment reversed the IBD effect.** | ASBT - ↓ Vmax / ↑ Km<br>↓ Protein expression in IBD<br>ASBT - ↑ Vmax / ↓ Km<br>↑ Protein expression in treated | (Coon, Kekuda, Saha, & Sundaram, 2010) |
| Rabbit treated with cNOS inhibitor (L-Name) | Radioactive uptake,<br>P*i* measurement<br>WB, qRT-PCR | ↓ SGLT-1(Na-glucose)/ ↑ Na-K-ATPase / ⇔NAcT (Na-neutral amino acid) | SGLT1 mechanism- ↓Km<br>⇔mRNA expression<br>⇔Protein synthesis | (Coon, Kim, Shao, & Sundaram, 2005) |
| Rabbit IBD<br>(Villus cells) | Radioactive uptake,<br>P*i* measurement, WB | ↓ B0AT1(Na-glutamine)/ ↓Na-K-ATPase in IBD<br>**Whereas, Glucocorticoid (Methylprednisolone) treatment reversed the IBD effect.** | B0AT1- ↓ Vmax / ⇔ Km<br>↓ Protein expression in IBD<br>B0AT1- ↑Vmax / ⇔ Km<br>↑ Protein expression in treated | (Arthur, Saha, Sundaram, Kekuda, & Sundaram, 2012; Saha, Arthur, Kekuda, & Sundaram, 2012) |
| Rabbit IBD<br>(Crypt cells) | Radioactive uptake,<br>P*i* measurement, WB | ↑ SN2 (Na-glutamine)/ ↑ Na-K-ATPase in IBD<br>**Whereas, Glucocorticoid (Methylprednisolone) treatment reversed the IBD effect.** | ⇔ Vmax, ↓ Km and ⇔Protein expression in IBD<br>⇔ Vmax, ↑ Km and ⇔Protein expression in treated | |
| Rabbit IBD (Crypt cells)<br>Treated with COX and LOX inhibitors | Radioactive uptake,<br>P*i* measurement, WB | ATK-PLA2 inhibitor-Reversed IBD effect<br>PRX- COX inhibitor-Did not reverse<br>MK886- LOX inhibitor-Reversed<br>**LOX pathway mediated stimulation of $SN_2$ in crypt cells during IBD** | ↓ $SN_2$ / ↓ Na-K-ATPase in treated animal. Because of ⇔Vmax, ↑ Km and ⇔Protein expression of $SN_2$ | (S. Singh, Arthur, & Sundaram, 2018) |
| Rabbit IBD (Villus cells)<br>Treated with COX and LOX inhibitors | Radioactive uptake,<br>P*i* measurement, WB | ATK-PLA2 inhibitor-Reversed IBD effect<br>PRX- COX inhibitor- Reversed IBD effect<br>MK886- LOX inhibitor- Did not reverse<br>**COX pathway mediated reduction of** B0AT1 **in crypt cells during IBD** | ↑ B0AT1, ↑Na-K-ATPase activity in treated animal. Because of ↑ Vmax, ⇔ Km and ↑ protein expression | (Arthur, Singh, & Sundaram, 2018) |
| Rabbit IBD | Radioactive uptake,<br>P*i* measurement, WB | ↓ NAcT (Na-alanine) / ↓Na-K-ATPase in IBD<br>**Whereas, Glucocorticoid (Methylprednisolone) treatment reversed the IBD effect.** | ↑ NAcT activity in treated animal. Because of ↓Km and ⇔mRNA level, | (Sundaram, Wisel, & Coon, 2007) |
| IEC-18 cells<br>Treated with LTD4 (1 uM) | Radioactive uptake,<br>P*i* measurement, WB | ↓ NAcT (Na-alanine) / ⇔Na-K-ATPase | NAcT- ⇔$V_{max}$, ↑ Km and<br>⇔ protein expression | (Talukder, Kekuda, Saha, & Sundaram, 2008) |
| IEC-6<br>Treated with PGE2 (1 nM) | Radioactive uptake,<br>P*i* measurement, WB | ↓SGLT-1 / ⇔Na-K-ATPase | SGLT-1- ↓ Vmax and decrease in protein synthesis | (Talukder, Griffin, Jaima, Boyd, & Wright, 2014) |

**Table 2: Studies on small intestinal epithelial Na-dependent transporters (Na-bile acid, Na-glucose, Na-glutamine and Na-K-ATPase) in animal IBD models or animal cells.** WB- Western blot, COX- cyclooxygenase, LOX- Lipoxygenase, ATK-Arachidonyl Trifluoromethyl Ketone, PRX- piroxicam, Affinity of transporter ($1/K_m$), PGE2- Prostaglandin E2, LTD4- Leukotriene D4, IEC- Intestinal epithelial cell (Rat ileum). ⇔-no change, ↓- down regulation in activity and expression, ↑ up-regulation in in activity and expression.

| Model/ Cell Types | Related Methods | Major Findings (Function) | Mechanisms | References |
|---|---|---|---|---|
| Colonic UC biopsies | N/A | ↓Na-K-ATPase was lower in UC biopsies compared to normal | N/A | (Rachmilewitz, Karmeli, & Sharon, 1984) |
| Colonic biopsies from healthy patients | Inorganic phosphate release (P*i*) | ⇔ Na-K-ATPase<br>↑HETEs, LTB4, ROS | N/A | (Allgayer, Kruis, & Erdmann, 1988) |
| Colonic UC or CD biopsies | Coupled optical assay of Na-K-ATPase activity | ↓Na-K-ATPase | N/A | (Allgayer, Kruis, Paumgartner, et al., 1988) |
| Rectal UC or CD | P*i* measurement | ↓Na-K-ATPase in patients with UC with severe rectal inflammation<br>⇔ Na-K-ATPase in patients with mild UC or no rectal inflammation | | (Ejderhamn, Finkel, & Strandvik, 1989) |
| Distal colon inflamed mucosa of UC or CD | *Isc* measurement (activity) under mystatin and [$Na^+$] variation | ↓Na-K-ATPase | ↓ number of pumps | (Sandle et al., 1990) |
| Sigmoid biopsies of UC, CD or normal patients | Coupled optical assay (activity) and $^3H$ Ouabain binding assay | Glucocorticoids reduced diarrheal symptoms in IBD and shows anti-inflammatory effects | ↑ Na-K-ATPase | (Scheurlen, Allgayer, Hardt, & Kruis, 1998) |
| Sigmoid and proximal rectal UC biopsies | WB, IHC, NB | ↓ α1 isoform protein level<br>⇔ α1 isoform mRNA level | | |
| Mucosa of sigmoid and proximal rectal biopsies of UC | WB-WC, IHC, NB | ↓ α1 and β1 isoform protein level<br>⇔ α1 and β1 isoform mRNA level | | (Greig, Boot-Handford, Mani, & Sandle, 2004) |
| Inflamed ileal and sigmoid mucosa of UC or CD biopsies | WB-WC, IHC | ↓ α1 isoform protein level | | (Sullivan et al., 2009) |
| Caco-2 Cell line | WB-PM, P*i* measurement | IFN-γ reduces ↓Na-K-ATPase in time and concentration dependent manner | ⇔ Protein level<br>PKC-STAT1 phosphorylation<br>RAF-1, MEK, ERK2 and p38 pathways involved | (Magro, Fraga, Ribeiro, & Soares-da-Silva, 2004) |
| Caco-2 Cell line | WB-PM, P*i* measurement | TNF-α reduces ↓Na-K-ATPase through PGE2 | ↓Protein level | (Markossian & Kreydiyyeh, 2005) |
| Caco-2 Cell line | WB-PM, P*i* measurement | Long term treatment of IFN-γ ↓Na-K-ATPase, Whereas TGF-β ⇔ Na-K-ATPase | | (Magro, Fraga, Ribeiro, & Soares-da-Silva, 2005) |

**Table 3: Studies on Na-K-ATPase in human IBD or human intestinal epithelial cells/tissues.** UC-Ulcerative colitis, CD- Crohn's disease, IBD-inflammatory bowel disease, WB-WC-Western blot in whole cell, WB-PM-Western blot in plasma membrane, IHC-immunohistochemistry, NB- northern blotting, HETEs- hydroxyeicosatetraenoic acid, LTB4- Leukotriene B4, PGE2 -Prostaglandin E2, *Isc*- Short-circuit current. ⇔-no change, ↓- down regulation in activity and expression, ↑ up-regulation in activity and expression.

| Model/ Cell Types | Related Methods | Major Findings (Function) | Mechanisms | References |
|---|---|---|---|---|
| Ileal enterocytes isolated from TNBS treated rats | N/A | ↓Na-K-ATPase compared to normal | ↓ α1 and β1 isoform protein and mRNA levels | (Wild & Thomson, 1995) |
| Jejunal and ileal mucosa of healthy murine | Inorganic phosphate release (P*i*) | IFN-γ - ↓Na-K-ATPase | Due to nitric oxide-dependent manner | (Yoo et al., 2000) |
| Isolated Jejunal enterocytes of healthy rat | WB-PM, radiolabeled ouabain binding and P*i*-*measurement* | IL-1β - ↓Na-K-ATPase | ↓ α1 isoform protein level | (Kreydiyyeh & Al-Sadi, 2002a) |
| Isolated colonocytes of healthy rats | WB-PM, and P*i*-*measurement* | IL-1β - ↓Na-K-ATPase | ↓ α1 isoform protein level | (Kreydiyyeh & Al-Sadi, 2002b) |
| Isolated colonic surface and crypt cells of healthy rats | WB-PM, and P*i*-*measurement* | TNF-α reduces ↓Na-K-ATPase through PGE2 | ↓ α1 isoform protein level | (Markossian & Kreydiyyeh, 2005) |
| Mucosa of colon, jejunum and terminal ileum of TNBS-treated rats | WB-PM, and P*i*-*measurement* | ↓Na-K-ATPase on proximal colon<br>↑ Na-K-ATPase on distal colon | ↓ α1 isoform protein level in proximal colon | (Magro, Fraga, & Soares-da-Silva, 2005) |
| Colonic crypts of healthy rats | *Isc* activity | $NH_2Cl$ -↑ Na-K-ATPase | N/A | (Schultheiss, Lan Kocks, & Diener, 2005) |
| Colonic mucosa of $NHE3^{-/-}$ mice | RT-qPCR | ↑ α1 isoform mRNA level | | (Laubitz et al., 2008) |
| Colonic mucosa of DSS- and TNBS-treated rats | WB-WC, IHC | ⇔ α1 isoform protein level in colitis model | | (Sullivan et al., 2009) |
| Colon and ileum of DSS-treated mice | WB, RT-qPCR | ↓ α1 isoform protein level<br>⇔ α1 isoform mRNA level | | (Hirota & McKay, 2009) |
| Villus cells from rabbit IBD model | WB-PM, WB-WC, IHC, RT-qPCR and P*i*-*measurement* | ⇔ α1 and β1 isoform protein mRNA level in whole cell, but<br>↓ α1 and β1 isoform protein mRNA level in plasma membrane | Downregulation and depolarization of ankyrin protein | (Saha et al., 2015) |

**Table 4: Studies on Na-K-ATPase in animal IBD or animal intestinal epithelial cells/tissues.** IBD-inflammatory bowel disease, WB-WC-Western blot in whole cell, WB-PM-Western blot in plasma membrane, IHC-immunohistochemistry, IFN-γ- interferon gamma, IL-Interleukin, and *Isc*- Short-circuit current. ⇔-no change, ↓- down regulation in activity and expression, ↑ up-regulation in activity and expression.

# CHAPTER 3: PHOSPHORYLATION OF Na-K-ATPase α1 SUBUNIT REGULATES ITS ACTIVITY DURING GROWTH AND MATURATION OF INTESTINAL EPITHELIAL CELLS

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## 3.1. ABSTRACT

The most important function of the mammalian intestinal cells is the absorption of nutrients, electrolytes, and water. In the mammalian small intestine, nutrients are primarily absorbed via Na-nutrient co-transport processes on the brush border membrane (BBM) of primarily absorptive villus cells. The necessary favorable transcellular Na gradient for the proper functioning of Na-dependent nutrient co-transporters is provided by Na-K-ATPase on the basolateral membrane of enterocytes. In the intestine, as a primarily secretory crypt cell matures to a primarily absorptive villus cell, it acquires all the BBM Na-solute co-transport processes that are dependent on Na-K-ATPase. Indeed, as cells mature from crypt to villus, Na-K-ATPase doubles, to accommodate for the increased BBM Na-dependent nutrient absorption. However, the mechanism of increase of Na-K-ATPase during growth and maturation of intestinal cells from the crypt to villus is not known**.** Therefore, this study aimed to determine the mechanisms involved in the functional transition of Na-K-ATPase during the maturation of crypt to villus cells. Rat intestinal epithelial cell (IEC-18 cell line) is an excellent model of in vitro crypt (0 day) to villus (4 days) maturation. Na-K-ATPase activity, as measured by inorganic phosphate release and $^{86}Rb^{+}$ uptake, gradually increased as the cells matured *in vitro* from day 0 through day 4 of post-confluence. mRNA abundance and Western blot studies showed no change in the levels of Na-K-ATPase subunits α1 and β1 from 0 to 4 days post-confluence. However, Na-K-ATPase α1 phosphorylation levels on serine and tyrosine, but not threonine, residues gradually increased as cells matured *in vitro* from day 0 to 4. These data indicate that as enterocytes mature from crypt-like to villus-like in culture, the functional activity of Na-K-ATPase increases secondary to altered affinity to accommodate the functional preference of the intestinal cell type without

changing its membrane expression levels. This altered affinity is likely due to phosphorylation of the α1 subunit, specifically at serine and tyrosine residues.

## 3.2. INTRODUCTION

An essential function of the mammalian small intestine is nutrient absorption (Gerbe, Legraverend, & Jay, 2012). While the intestinal epithelium is composed of multiple specialized cells types including goblet cells, enteroendocrine cells, Paneth cells, and enterocytes (de Santa Barbara, van den Brink, & Roberts, 2003), only enterocytes are responsible for nutrient absorption from the intestinal lumen. The enterocytes are comprised of undifferentiated crypt cells, which proliferate and differentiate to mature villus cells (Babyastsky & Podolsky, 1999). Nutrient, electrolyte and fluid absorption primarily occur by the villus cells while the crypt cells are thought to be primarily secretory.

During the differentiation process, enterocytes acquire more transporters on both sides and are physiologically able to absorb more nutrients compared to undifferentiated crypt cells. Among the acquired transporters, Na-K-ATPase, a basolateral membrane (BLM) transporter, plays an important role in regulating ionic homeostasis, cell volume and maintaining membrane potential (Kay, 2017). Na-K-ATPase transports three $Na^+$ out of the cell in exchange for two $K^+$ into the cell, thus maintaining a high level of intracellular $K^+$ and low intracellular $Na^+$ concentration (Kaplan, 2002). This establishes a $Na^+$ gradient that is responsible for driving other secondary transport processes across the brush border membrane (BBM). Transport of different ions ($H^+$, $Ca^{2+}$, $Cl^-$, $PO_4^{2-}$, $SO_4^{2-}$), nutrients like glucose, amino acids and vitamins, certain nucleic acids, bile acids, and neurotransmitters across the plasma membrane are all dependent on Na-K-ATPase activity (Skou, 1990; Skou & Esmann, 1992). Previous studies have shown that Na-nutrient co-transport mechanisms in the BBM, namely Na-glucose (SGLT1), Na-alanine (NAcT), Na-glutamine (B0AT1), Na-bile acid (Apical sodium bile acid transporter (ASBT)) and Na-adenosine (Divalent metal transporter 1 (DMT1)) that depend on the BLM Na-K-ATPase for

their optimal activity, are inhibited. Further, their inhibition in villus cells in a rabbit model of inflammatory bowel disease (IBD) (Ejderhamn et al., 1989; Rachmilewitz et al., 1984; Sundaram & West, 1997) is at least in part secondary to altered Na extruding capacity of the cell from diminished Na-K-ATPase activity. Therefore, Na-K-ATPase plays a crucial role in nutrient absorption and maintenance of intestinal health.

The Na-K-ATPase enzyme consists of various subunits: alpha (α), beta (β) and gamma (γ) subunits (Kaplan, 2002). The α and β subunits are ubiquitously present and are important for the activity of Na-K-ATPase (G. E. Shull, Lane, & Lingrel, 1986; G. E. Shull, Schwartz, & Lingrel, 1985), while the γ-subunit is an optional subunit and its expression is restricted to certain tissues (Beguin et al., 1997). Of these subunits, α subunit does the catalytic function of the transporter (G. E. Shull et al., 1985), whereas other subunits (β and γ) are responsible for the regulatory function of the α subunit (Arystarkhova, Wetzel, Asinovski, & Sweadner, 1999; Geering, 2001). The α subunit is a transmembrane protein having intracellular $Na^+$, ATP and phosphate binding sites, and extracellular $K^+$ binding sites (Kaplan, 2002). The β subunit is important for the proper folding of the α subunit and its translocation to the plasma membrane (Ackermann & Geering, 1990; McDonough, Geering, & Farley, 1990). At least four isoforms of the α subunit (α1, α2, α3, and α4) and three isoforms of the β (β1, β2, and β3) subunit are known. These isoforms are expressed in a tissue-specific manner (Blanco, Melton, Sanchez, & Mercer, 1999; Kaplan, 2002; Mercer, 1993). The combination of different isoforms of the α and β subunits make up a series of Na-K-ATPase isoenzymes (Blanco & Mercer, 1998b). Each isoenzyme has different functional properties and they are expressed differentially in tissue- and cell specific-manners (Jewell et al., 1992). Of these isoforms, α1 and β1 are ubiquitously present in epithelial cells and are also present in the mucosa of the intestine (Saha et al., 2015).

Na-K-ATPase activity is tailored to change according to the physiological requirements of the cell. There are several mechanisms that regulate Na-K-ATPase activities. For example, availability of the substrate ($Na^+$, $K^+$ and ATP), the amount of the enzyme at the plasma membrane, which can be modified by changes in the rate of synthesis or degradation of the individual Na pump polypeptide, and movement of the pump from cytoplasmic vesicles to the plasma membrane by exo/endocytotic vesicular transport (Blanco et al., 1998). Besides these mechanisms, Na-K-ATPase activities at the cell surface are directly regulated by direct phosphorylation and dephosphorylation by protein kinases and protein phosphatases (Bertorello & Katz, 1993; Ewart & Klip, 1995; McDonough & Farley, 1993). Alterations in the phosphorylation levels of the α and regulatory γ subunits are known to change the affinity and therefore the activity of the transporter.

As mentioned above, published studies using *in vivo* models have shown that the Na-K-ATPase functional activity varies depending on the cell type and function: rabbit villus cells that have the primary function of sodium-dependent nutrient absorption have twice the amount of Na-K-ATPase function compared to the crypt cells that have minimal absorptive capacity (S. Singh et al., 2015). However, the mechanism(s) underlying the alteration of Na-K-ATPase function during maturation of crypt to villus cell is not known at this time. Therefore, the aim of this study is to explore the mechanism(s) underlying the alteration of Na-K-ATPase activity during maturation of intestinal epithelial cells.

## 3.3. MATERIALS AND METHODS

### 3.3.1. Cell culture

Rat small intestinal epithelial cells (IEC-18, American Type Culture Collection), between passages 5 and 20, were used for all of the experiments. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% (vol/vol) fetal bovine serum, 100 U/l human insulin, 0.25 mM β-hydroxybutyric acid and 100 U/ml of penicillin and streptomycin. These cells were cultured in a humidified atmosphere of 10% $CO_2$ at 37°C. The cells were fed with fresh medium every other day. When the cells reached 100% confluence, it was considered as 0 day, and cells were grown until 4 days post-confluence. Experiments were conducted in 0-day, 1-day, 2-day, 3-day, and 4-day post-confluent cells.

### 3.3.2. Alkaline phosphatase activity (ALP) assay

The activities of ALP were determined using an enzyme assay kit, according to the manufacturer's protocol (Abcam#83369, Alkaline phosphatase assay kit, colorimetric). ALP activity was calculated as picomole/min/ml from the cells that were harvested between 0 and 4-day post-confluence.

### 3.3.3. Na-dependent glucose uptake studies

To determine the Na-dependent glucose transport in IEC-18 cells, glucose uptake studies were performed on cells grown on transwell inserts (0.4 μ , Thermoscientific #140620) in 24 well plates. Cells were rinsed once with warm wash buffer (130 mM NaCl, 5mM KCl, 1mM $MgSO_4$, 2 mM $CaCl_2$, 20 mM HEPES and pH adjusted to 7.4) and incubated with the same buffer for 10 mins. After the incubation, wash buffer was removed from the wells and replaced with a reaction mixture containing a trace amount of [$^3$H] OMG in Na-HEPES buffer with and without phlorizin (SGLT1 inhibitor). The reaction was stopped after two minutes with cold wash

buffer (130 mM NaCl, 5mM KCl, 1mM $MgSO_4$, 2 mM $CaCl_2$, 20 mM HEPES, 10mM D-Glucose and pH adjusted to 7.4), and the cells were then washed twice with the same buffer. Cells were lysed by incubating them with 1M NaOH for 20 mins at 70°C. The lysed cells from each well were then mixed with 5 ml of Ecoscint A (National Diagnostics). The vials were kept in darkness overnight and radioactivity retained by the cells was determined in a Beckman Coulter 6500 scintillation counter.

### 3.3.4. Crude plasma membrane isolation

Plasma membrane (crude) was prepared from cells according to the method of Havrankova *et al*. (Havrankova, Roth, & Brownstein, 1978). Briefly, 100 mg cells were homogenized in 5ml of 1mM $NaHCO_3$ and centrifuged at 600 x g for 30 min. The supernatant was collected and centrifuged for 30 min at 20,000 x g. The membrane pellets were washed twice with 1mM $NaHCO_3$. The final pellets were resuspended in 0.04 M Tri-HCl buffer (pH 7.4) containing 0.1% BSA. All the procedures mentioned above were carried out at 4ºC.

### 3.3.5. Na-K-ATPase activity assay

Na-K-ATPase activity was measured as *Pi* liberated in cellular homogenates and crude plasma membrane fractions from the cells that were harvested 0-4 days post-confluence according to the protocol of Forbush *et al.* (Forbush, 1983). Enzyme-specific activity was expressed as nanomoles of $P_i$ released per milligram protein per minute.

### 3.3.6. Determination of Na-K-ATPase activity and kinetics by $^{86}Rb^+$ uptake

Uptake studies were performed in cells grown on transwell inserts in 24 well plates. IEC-18 cells were plated with approximately $1\times10^5$ cells. Uptake studies for Na-K-ATPase were done using radioactive Rubidium ($^{86}Rb^+$, Perkin Elmer). Cells were incubated for 1hr in serum-free DMEM media at the aforementioned conditions. The cells were subsequently washed with

serum-free media and incubated for 10 min at 37°C with 20 μM monensin (in serum-free DMEM) added to both the apical and basolateral sides. The cells were then washed with serum-free DMEM. Uptake of $^{86}Rb^{+}$ was started by incubating the cells for 15 mins in a reaction mixture (Serum-free DMEM) containing ~1 μCi/ of $^{86}Rb^{+}$ well added to the basolateral side of the cells in the presence and absence of ouabain (1mM).

For Na-K-ATPase kinetics, after incubation with serum-free DMEM for 1hr, the cells were subsequently washed with an incubation buffer (130 mM NaCl, 5mM KCl, 1mM $MgSO_4$, 2 mM $CaCl_2$ and 20 mM HEPES) and incubated for 10 min at 37°C in the incubation buffer with 20μM monensin on both sides of the membrane. Then cells were washed with a wash buffer (130 mM NaCl, 1mM $MgSO_4$, 2 mM $CaCl_2$, 20 mM HEPES and 5 mM mannitol) on both sides. Na-K-ATPase kinetic studies were then performed in cells by incubating them for 30 secs with reaction mixtures (130 mM tetramethylammonium chloride, 1mM $MgSO_4$, 2 mM $CaCl_2$, 20 mM HEPES, 5 mM mannitol and $^{86}Rb^{+}$ (~1 μCi/ well)) containing different concentrations (0.05-2 mM) of RbCl added to the basolateral side of the membrane in the presence and absence of ouabain (1 mM).

For both uptake and kinetics experiments, the reaction was then stopped by the addition of ice-cold $MgCl_2$. The cells were then washed three times with $MgCl_2$ and lysed with 800 μl of 1N NaOH by incubating them for 30 min at 70°C. The lysed cells from each well were then mixed with 5 ml of Ecoscint A (National Diagnostics). The vials were kept in darkness overnight and radioactivity retained by the cells was determined in a Beckman Coulter 6500 scintillation counter. The absolute Na-K-ATPase activity was calculated by subtracting $^{86}Rb^{+}$ uptake in the presence of ouabain from that in the absence of ouabain.

### 3.3.7. RNA isolation and qRT-PCR

RNA was isolated from 0-4 days post-confluent cells by using the RNeasy mini kit obtained from Qiagen. The real-time quantitative PCR (qRT-PCR) was performed using an isolated total RNA by a two-step method. First, total RNA was used to synthesize complementary deoxyribonucleic acid (cDNA) using SuperScript III (Invitrogen Life Technologies). Then, newly synthesized cDNA was used as a template to perform real-time PCR using TaqMan universal PCR master mix from Applied Biosystems (Foster City, CA) according to the manufacturer's protocol. Rat-specific Na-K-ATPase α1 and Na-K-ATPase β1 primers and probes were used for the qRT-PCR studies. In addition, the rat-specific β-actin primer was used to normalize the expression levels of mRNA in the samples.

### 3.3.8. Immunoprecipitation

Immunoprecipitation (IP) assays were conducted in order to investigate the phosphorylation level of Na-K-ATPase α1 present in the plasma membrane. Cells were first rinsed with PBS, scraped and collected in PBS, and pelleted by brief low-speed centrifugation (800 rpm). The collected pellet was further used for plasma membrane isolation as mentioned above. The prepared plasma membrane fraction was sonicated and dissolved in 200 ul of lysis buffer (T-per Buffer, Thermo scientific) with protease inhibitors (Thermo Scientific). Plasma membrane protein was further diluted to 1 μg/μl in a microcentrifuge tube with T-per buffer and placed on a rocking shaker at 4°C with 2 μg of Anti-Na-K-ATPase α1 antibody overnight. Antibody-protein complexes formed were captured by adding 50 μl of washed protein G agarose bead slurry (Thermo Scientific, Catalog # 16-266) with rotation at 4°C for 2 hours. The agarose beads with the protein-antibody complex were collected by centrifugation (14,000 x g) and washed three times with ice-cold lysis buffer. These agarose beads were resuspended in 2X

sample loading buffer and incubated at 37°C for 15 minutes. The beads in sample loading buffer were vortexed, and the supernatant was used for Western blot analysis.

### 3.3.9. Western blot analysis

Western blot analysis was performed in the whole cell lysate and plasma membrane preparations. An equal amount of protein (20 μg) was denatured in sample buffer (Laemmli sample buffer, BIO-RAD #3100010639) and separated by electrophoresis on a 8%-12% gradient gel. Proteins on the gel were transferred to a polyvinylidene fluoride membrane which was blocked with 5% dry milk or BSA in TBS (20 mM Tris pH 7.5, 150 mM NaCl) with 0.1% Tween-20 and then incubated with one of the following primary antibodies, overnight at 4°C: Na-K-ATPase α1 (Millipore, # 05-369); Na-K-ATPase β1 (Abcam, ab2873); p-Serine (Abcam, ab9332); p-Threonine (Abcam, ab179530); p-Tyrosine (Abcam, ab9337); p-Na-K-ATPase α1-$Ser^{23}$ (Cell Signaling Technology, #4006) and p-Na-K-ATPase α1-$Ser^{16}$ (Cell Signaling Technology, #4020). The membrane was washed three times with TBST followed by TBS. The membrane was then incubated with secondary antibody for 1hr. Enhanced chemiluminescence (ECL) western blotting detection reagent (GE Healthcare Bio-Sciences, Piscataway, NJ) was used to detect the immobilized protein. The chemiluminescence was detected using a FluorChem M instrument (Alpha Innotech, San Leandro, CA) and the intensity of the bands were analyzed by its software. β-actin was used to normalize the expression levels in cellular homogenate.

### 3.3.10. Protein determination

Total protein was measured by the Lowry method using the Bio-Rad protein assay kit (Hercules, CA). BSA was used as a standard.

### 3.3.11. Statistical analysis

All groups presented have at least n=3 per group repeated with a different passage. The values are presented as mean ± SEM, and significant values of $P<0.05$ were taken to indicate statistical significance. All of the data were analyzed using one-way or two-way analysis of variance (ANOVA) using GraphPad Prism software (San Diego, CA).

## 3.4. RESULTS

### 3.4.1. Alkaline phosphatase (ALP) levels during cell growth

To confirm the cell growth and maturation in IEC-18 cells, alkaline phosphatase activity was measured from cells collected from 0-day to 4-day post confluence (Figure 9). The highest level of alkaline phosphatase activity was observed at 4-day post-confluence (1536±44.16 picomole/min·ml), whereas the lowest level activity (1092±5.432 picomole/min·ml) was observed at 0-day post-confluence. This trend of increasing alkaline phosphatase activity demonstrates that cells mature as they grow from 0-day post confluence (crypt-like) to 4-day post confluence (villus-like).

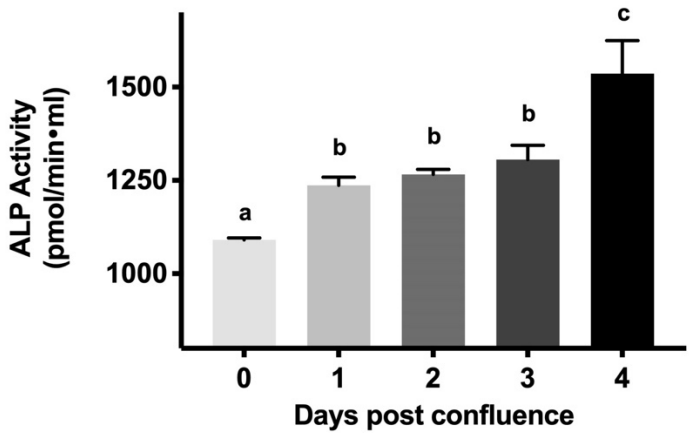


**Figure 9. Alkaline phosphatase (ALP) activity to validate cell maturation as IEC-18 cells grow.** Values are represented as means ± SEM, n=4. Values not sharing common superscripted letters are significantly different at $P < 0.001$.

### 3.4.2. Na-dependent glucose uptake during cell growth

As enterocytes mature from crypt to villus, physiological alterations are accompanied by the appearance of different transporters in the BBM. Specifically, the Na-glucose co-transporter SGLT1 appears as enterocytes mature from crypt to villus. Similar to *in vivo* observations (Hwang, Hirayama, & Wright, 1991), we also found that Na-dependent glucose uptake increased

almost three-fold as cells matured from crypt-like to villus-like. Figure 10 shows that minimal SGLT1 activity (92.53±13.35 picomole/mg protein·min) was at 0-day ost-confluence. There was a steady and robust increase in SGLT1 activity from 0 to 4-day post-confluence in IEC-18 cells (376± 57.71). This phenomenon of increasing SGLT1 activity may be due to increasing cellular maturation, cellular polarity and/or an increase in the number of transporters itself and is comparable to what is seen in vivo during crypt to villus maturation (Coon et al., 2005).

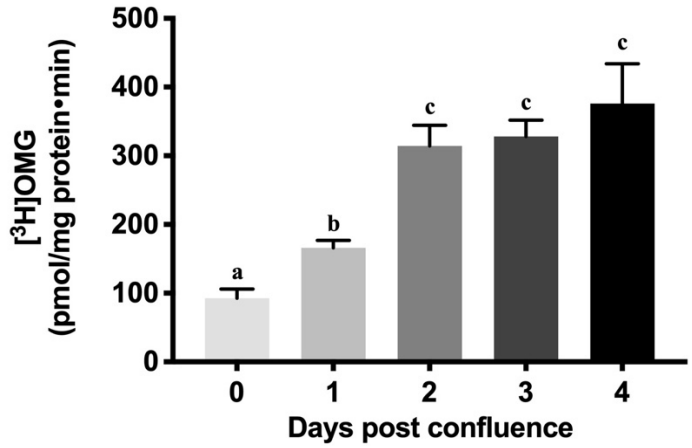


**Figure 10. Increase in Na-dependent glucose uptake as IEC-18 cells mature.** Uptake was performed in the presence and absence of phlorizin (1 mM) in reaction medium containing [$^3$H]-OMG tracer. Values are represented as means ± SEM, n=6 independent experiments. Values not sharing common superscripted letters are significantly different at $P < 0.001$.

### 3.4.3. Na-K-ATPase activity levels during cell growth

As enterocytes mature, along with the appearance of BBM transporters, BLM Na-K-ATPase activity increases to provide the favorable Na gradient necessary to absorb nutrients. Similar to the *in vivo* observation in rabbit intestine (S. Singh et al., 2015), Na-K-ATPase activity, as determined by inorganic phosphate *(Pi*) release increased gradually in IEC-18 cellular homogenates from 0-4 days post-confluence (Figure 11A, 5.01±0.12 nanomole/mg protein.min in 0-day, 5.74±0.47 in 1-day, 14.48±1.07 in 2-day, 14.58±0.90 in 3-day, 13.72±0.23 in 4-day). Since Na-K-ATPase is a membrane protein, plasma membrane fraction were prepared

from IEC-18 cells, and in these preparations, Na-K-ATPase activity also increased gradually from 0-day to 4-day (Figure 11B, 49.81±02.74 nanomole/mg protein·min in 0-day, 81.36±1.775 in 1-day, 86.53±1.617 in 2-day, 102.8±4.01 in 3- day, 115.6±3.58 in 4-day). Finally, since radiolabeled rubidium ($^{86}Rb^{+}$) uptake is more a functional measure of Na-K-ATPase activity in live cells, it was measured. $^{86}Rb^{+}$ uptake also increased gradually from 0-day to 4-day (Figure 12, 649.2±6.95 nanomole/mg protein·min in 0-day, 958.6±32.52 in 1-day, 1285±22.28 in 2-day, 1335±39.27 in 3-day, 1565±8,17 in 4-day). Thus, these data indicate that as IEC-18 cells mature from crypt-like at 0-day post-confluence to villus-like at 4-day post-confluence, Na-K-ATPase activity increased comparable to in vivo crypt and villus cells.

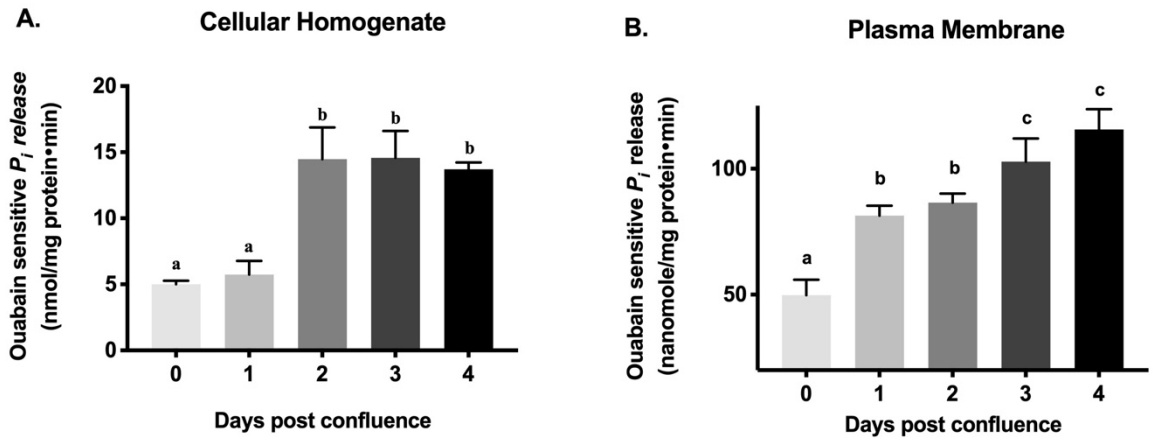


**Figure 11. Na-K-ATPase activity as measured by $P_i$ release in IEC-18 cells.** Na-K-ATPase activity was measured in the presence or absence of ouabain (1 mM). The absolute Na-K-ATPase activity presented was calculated by subtracting $P_i$ release in the presence of ouabain from that in the absence of ouabain. A. Cellular homogenates. B. Plasma membrane preparations. Values are represented as means ± SEM, n=5. Values not sharing common superscripted letters are significantly different at $P < 0.01$.

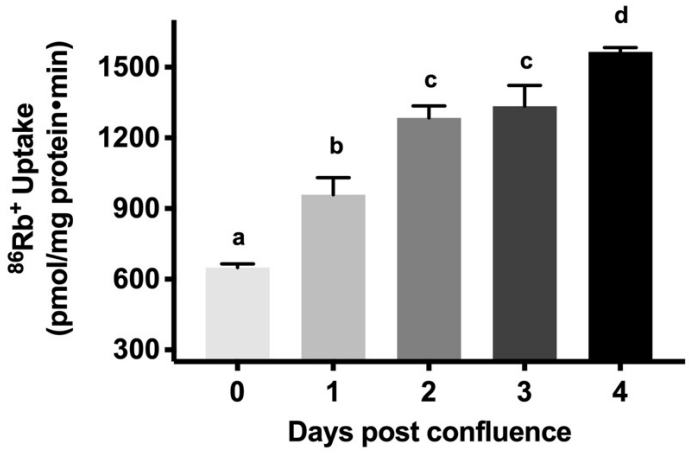


**Figure 12: Na-K-ATPase activity as measured by $^{86}Rb^+$ uptake in IEC-18 cells.** Values are represented as means ± SEM, n=5. Values not sharing common superscripted letters are significantly different at $P < 0.01$.

### 3.4.4. Kinetic Studies during cell growth

To determine the mechanism of the increase in Na-K-ATPase activity from 0-4 days post-confluence, we performed $^{86}Rb^+$ kinetics in IEC-18 cells. As the concentration of extracellular $Rb^+$ was increased, $Rb^+$ uptake was stimulated and subsequently became saturated in all conditions. Kinetic parameters showed that there was no significant change in $V_{max}$ among the groups. However, there was a significant difference in $K_m$ from 0-4 days post-confluence (Table 5). Affinity ($1/K_m$) of Na-K-ATPase increased gradually as the cells matured from 0-4 days post-confluence as shown in Table 5.

| | 0 Day | 1 Day | 2 Day | 3 Day | 4 Day |
|---|---|---|---|---|---|
| $V_{max}$ **(picomole/mg protein*30 Sec)** | 742.5±62 | 714±83.5 | 718±58.4 | 713±27.9 | 733±44.8 |
| $K_m$ **(mM)** | $0.68±0.11^a$ | $0.61±0.15^a$ | $0.39±0.08^b$ | $0.37±0.04^b$ | $0.33±0.06^b$ |

**Table 5: Kinetic parameters of Na-K-ATPase transporters in IEC-18 Cells.** Uptake of $^{86}Rb^+$ as a function of varying concentration of extracellular $Rb^+$ (Rubidium chloride (RbCl)). As extracellular $Rb^+$ concentration was increased, uptake of $^{86}Rb^+$ was stimulated and subsequently became saturated in all groups. Kinetic parameters [maximal uptake ($V_{max}$) and affinity ($1/Km$)] were obtained after the analysis of data with GraphPad Prism software. Values are represented as

mean ± SEM, n=6. Values not sharing common superscripted letters are significantly different at $P < 0.01$.

### 3.4.5. Na-K-ATPase α1 and Na-K-ATPase β1 subunit mRNA abundance during cell growth

The α subunit primarily provides Na-K-ATPase functional activity whereas the β subunit does not have pumping activity but contributes to proper transportation of the α subunit to the plasma membrane to make the entire protein fully functional. Therefore, to determine whether the change in Na-K-ATPase activity may be transcriptionally regulated, we performed qRT-PCR. There was no significant difference in the relative expression of Na-K-ATPase α1 mRNA (Figure 13A) between different groups (0-4 days). Similarly, the Na-K-ATPase β1 subunit mRNA abundance (Figure 13B) was also not statistically different between the groups (0-4 days).

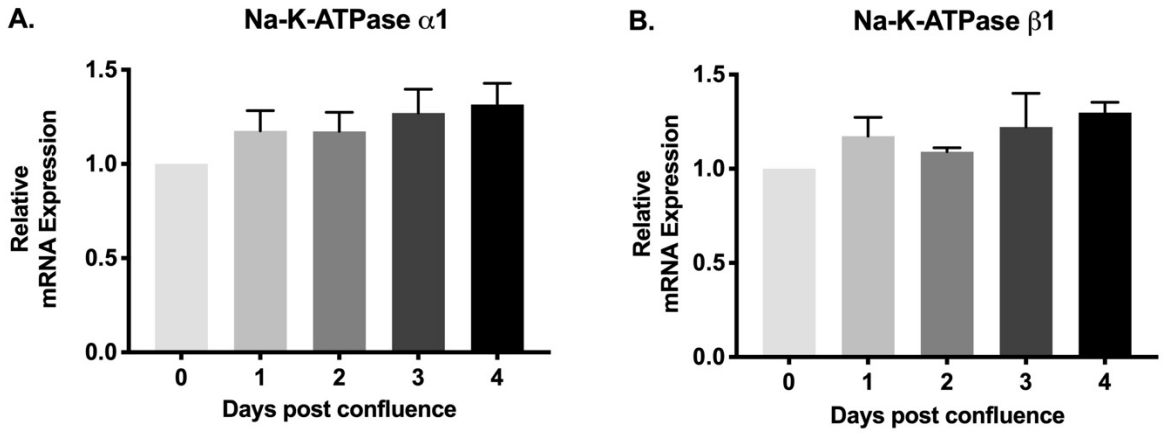


**Figure 13. qRT-PCR analysis of IEC-18 cells on different days post confluence.** Values are relative to 0-day and normalized to β-actin. A. Na-K-ATPase α1. B. Na-K-ATPase β1. Values are represented as mean ± SEM, n=4.

### 3.4.6. Na-K-ATPase α1 and Na-K-ATPase β1 subunit protein expression during cell growth

Since mRNA levels of the Na-K-ATPase α1 and Na-K-ATPase β1 subunits do not always correlate with protein expression, Western blot analysis of protein expression of the Na-K-ATPase α1 and Na-K-ATPase β1 subunits were performed. Immunoreactive protein levels of

Na-K-ATPase α1 and Na-K-ATPase β1 subunits were determined in cellular homogenates and plasma membrane fractions. Densitometric analysis of relative protein expression revealed that the level of Na-K-ATPase α1 protein expression was not statistically different in IEC-18 cells from 0-4 days post-confluence in cellular homogenates (Figure 14A) or plasma fractions (Figure 15A). Similarly, there was also no significant difference in Na-K-ATPase β1 subunit protein expression among IEC-18 cells from 0-4 days post-confluence in cellular homogenates (Figure 14B) and plasma membrane fractions (Figure 15B). Protein expression of Na-K-ATPase α1 and Na-K-ATPase β1 subunits correlates with mRNA expression of these subunits, respectively. Further, these studies are consistent with kinetic parameters determined above. Therefore, the expression of Na-K-ATPase α1 and Na-K-ATPase β1 subunits were unaltered in cellular homogenates and plasma membrane fractions in IEC-18 cells during maturation. Thus, the increase in Na-K-ATPase activity as IEC-18 cells mature from crypt-like to villus-like cells is secondary to increased affinity of the protein which may be due to altered phosphorylation of the α1 Na-K-ATPase subunit.

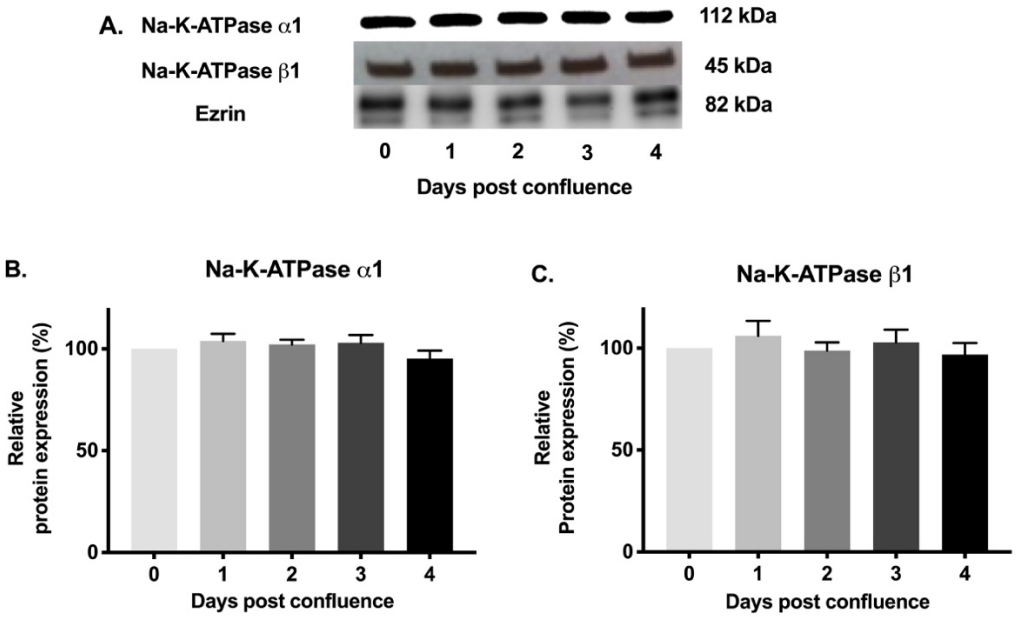


**Figure 14. Western blot analysis of different post confluence IEC-18 cells in cellular homogenates.** A. Representative blot of Na-K-ATPase α1, Na-K-ATPase β1 and internal control β-actin. Densitometric quantitation of western blots. B. Na-K-ATPase α1 and **C.** Na-K-ATPase β1. Values are relative to 0-day and normalized to β-actin. Values are represented as mean ± SEM, n=4.

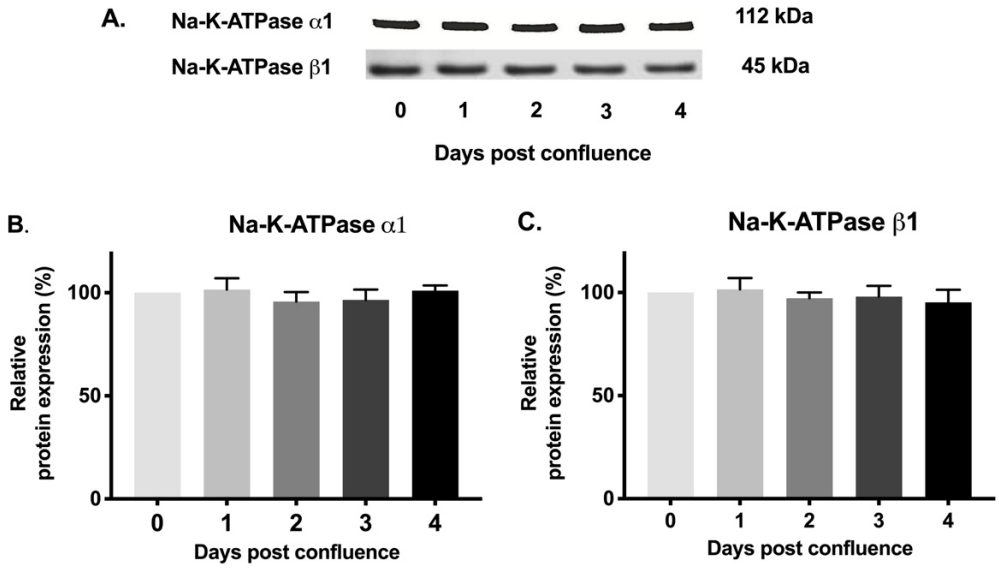


**Figure 15. Western blot analysis of different post confluence IEC-18 cells in plasma membrane fractions.** A. Representative blots of Na-K-ATPase α1 and Na-K-ATPase β1. Densitometric quantitation of western blots. B. Na-K-ATPase α1 and C. Na-K-ATPase β1. Equal amount of proteins was loaded. Values are relative to 0-day and represented as mean ± SEM, n=4.

### 3.4.7 Protein phosphorylation of Na-K-ATPase α1 subunit during cell growth

To examine whether changes in Na-K-ATPase activity during different days of post confluence is due to phosphorylation of Na-K-ATPase α1 subunit, thus affecting its affinity, immunoprecipitation studies were performed. Na-K-ATPase α1 subunit was immunoprecipitated from the plasma membrane on different days of post-confluence and probed with antibodies specific for phosphorylated amino acids (serine, threonine, and tyrosine). As shown in the representative blot and densitometric analysis in Figure 16, immunoprecipitated Na-K-ATPase α1 subunit from different days of post confluence had a gradual increase in the levels of phosphorylated tyrosine (Figure 16B; 100% in 0-day and 170±21.41 in 4-day post-confluence). Similarly, phosphorylated serine levels also increased (Figure 16C; 100% in 0-day and 179±5.08 in 4-day post confluence). However, phosphorylated threonine levels remained unchanged. To determine which specific residues might be the target of phosphorylation during maturation, proteins extracted from different days were probed with anti-p-Na-K-ATPase α1-$Ser^{23}$ and anti-p-Na-K-ATPase α1-$Ser^{16}$ antibodies, the targets of which have been previously implicated in alterations in Na-K-ATPase activity. As shown in the representative blot and densitometric analysis in Figure 17, p-Na-K-ATPase α1-$Ser^{23}$ had a gradual increase (100% in 0-day and 241.3±5.5 in 4-day post-confluence) in its phosphorylation levels during IEC-18 cell growth. These data confirmed that a change in Na-K-ATPase activity during cell growth is likely due to changes in phosphorylation of Na-K-ATPase α1 subunit, specifically at serine and tyrosine amino acid residues and possibly involving p-Na-K-ATPase α1-$Ser^{23}$ residue.

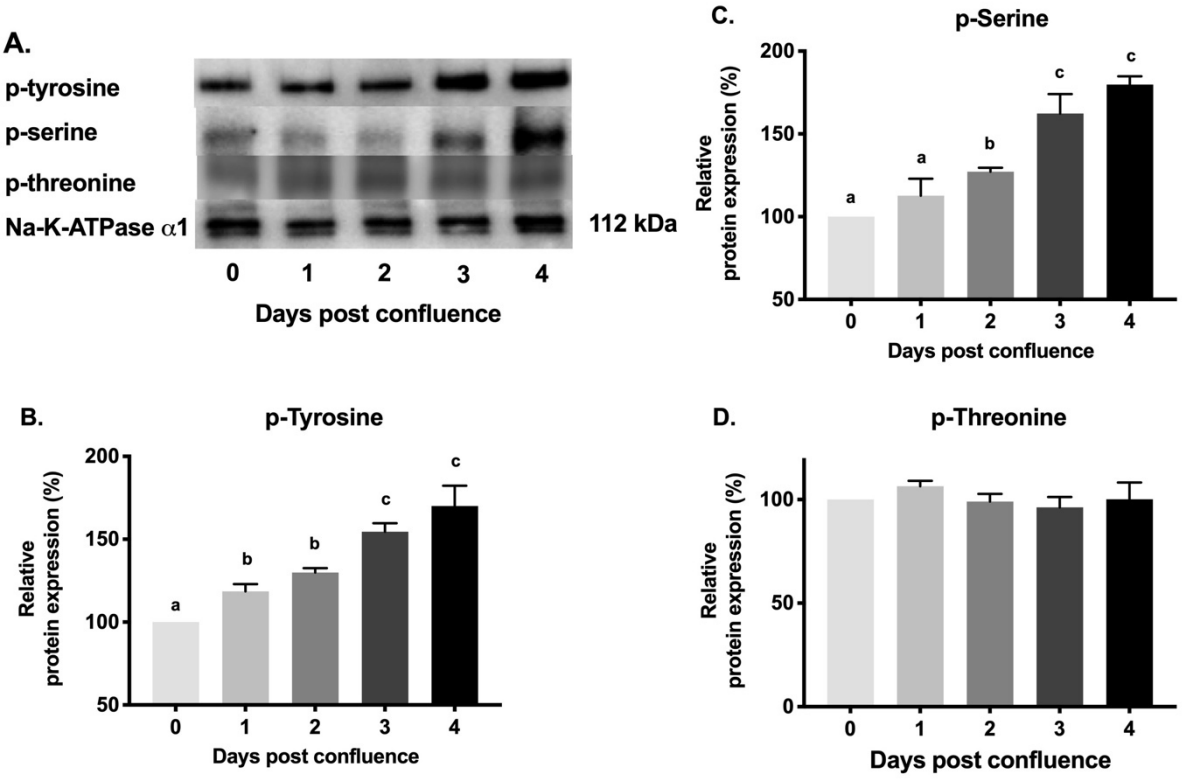


**Figure 16. Western blot analysis of phosphorylation levels of Na-K-ATPase α1 during different days post confluence IEC-18 cells in plasma membrane fractions.** A. Representative blot of p-tyrosine, p-serine, p-threonine and Na-K-ATPase α1 after immunoprecipitation with Na-K-ATPase α1. Densitometric quantitation of western blots. B. p-tyrosine. C. p-Serine. D. p-Threonine. Values are relative to 0-day and normalized to Na-K-ATPase α1. Values are represented as mean ± SEM, n=4. Values not sharing common superscripted letters are significantly different at $P < 0.05$.

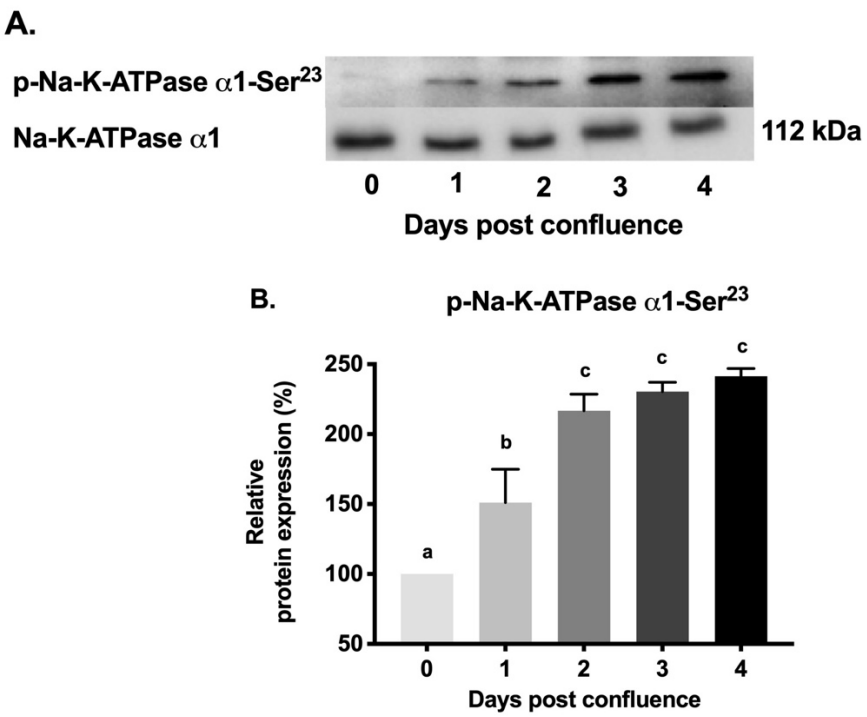


**Figure 17. Western blot analysis of Na-K-ATPase α1-Ser$^{23}$ at different days post confluence IEC-18 cells in plasma membrane fractions.** A. Representative blot of experiment done in triplicate. B. Densitometric analysis of western blots. Values are relative to 0-day and normalized to Na-K-ATPase α1. Values are represented as mean ± SEM, n=3. Values not sharing common superscripted letters are significantly different at $P < 0.01$.

## 3.5. DISCUSSION

Villus and crypt cells have been extensively investigated concerning absorption mechanisms of Na-dependent nutrient co-transporters at the level of the co-transporters in the BBM. At the cellular level, all of these Na-dependent co-transport processes may be regulated secondary to alterations in BLM Na-K-ATPase since it provides the necessary Na gradient for these secondary active transport processes. However, the Na-K-ATPase located in the BLM that drives these nutrient transporters has not been previously studied concerning its mechanism of adaption to support the increasing nutritional absorptive capacity during the growth and maturation of intestinal epithelial cells. Therefore, the present study was undertaken to understand the mechanism of Na-K-ATPase regulation during the maturation of intestinal epithelial cells from the crypt to villus.

During the maturation of intestinal cells from the crypt to villus, significant morphological and physiological changes occur. Morphological changes include changes in composition and membrane surface area. Along with these changes, physiologically mature cells acquire more transporters and therefore, their capacity to absorb nutrients increases. A study of the crypt-villus axis of piglets has shown that there is upregulation of proteins associated with glycolysis/gluconeogenesis, fatty acid metabolism, amino acid metabolism, and the citrate cycle as cells mature from crypt to villus (Yang, Wang, Xiong, & Yin, 2016). Additionally, it has been reported that alkaline phosphatase (ALP), aminopeptidase, sucrose, lactase, and Na-K-ATPase activities increased along the crypt-villus axis in jejunum and ileum of neonatal pigs (Fan, Stoll, Jiang, & Burrin, 2001). Similar to these results, our study has shown that there is an increase in ALP and SGLT1 activities as IEC-18 cells mature from crypt-like to villus-like cells in vitro.

There are conflicting reports in the literature addressing the levels of Na-K-ATPase activity during the maturation of cells (Blostein, Drapeau, Benderoff, & Weigensberg, 1983; Brugnara & de Franceschi, 1993; Furukawa, Bilezikian, & Loeb, 1981). For example, in one study a reduction in activity was found to be due to the shedding of Na-K-ATPase into exosomes or due to degradation (Mairbaurl, Schulz, & Hoffman, 2000). This study has shown that there is an increase in Na-K-ATPase activity as IEC-18 cells mature from crypt-like to villus-like cells.

The increase or decrease in activity may be the result of various mechanisms: firstly, by increasing or decreasing the subunits α and β respectively (Erichsen, Zuo, Curtis, Rarey, & Hultcrantz, 1996; Vasarhelyi et al., 2000; Wang & Celsi, 1993; Wang, Yasui, & Celsi, 1994). The increased Na-K-ATPase activity could be, therefore, due to an increase in these subunits by increased mRNA and protein synthesis. Secondly, the translocation of α and β subunits from the cytoplasm to the plasma membrane (Bertorello & Katz, 1993; McDonough & Farley, 1993; Pedemont & Bertorello, 2001). The increased translocation of α and β subunit to the plasma membrane could, therefore, increase the Na-K-ATPase activity. Among different isoforms of α and β subunits, α1 and β1 isoforms are expressed ubiquitously and are observed to perform a housekeeping role in most epithelial cell types, including intestinal epithelial cells (Rajasekaran, Barwe, & Rajasekaran, 2005; Saha et al., 2015). During the development and maturation of erythrocytes, Na-K-ATPase enzyme activities were found to decrease with maturation, correlating with a decrease in the α1 subunit (Rosen & Sigstrom, 1978). Similarly, in conjunction with Na-K-ATPase activity, the α1 isoform also increases during the maturation of cochlea (Curtis, ten Cate, & Rarey, 1993). However, in the present study, we did not find any significant changes in mRNA expression of the α1 isoform or its protein levels though there was an increase in Na-K-ATPase activity.

For Na-K-ATPase to be fully functional as a transporting unit in the plasma membrane, assembly of the β subunit with the α subunit is essential. The association of the β subunit with the α subunit is required for maturation, membrane insertion and transport activity of the α subunit (Tokhtaeva, Clifford, Kaplan, Sachs, & Vagin, 2012). Na-K-ATPase α and β heterodimer are selective in their subunit isoform association, where the α1 subunit mostly assembles with the β1 isoform (Tokhtaeva et al., 2012). Although there was elevated Na-K-ATPase activity, the expression level of β1 mRNA and protein in cellular homogenates remain unchanged during maturation. Therefore, the level of Na-K-ATPase α1 and β1 mRNA and protein in cellular homogenates confirmed that Na-K-ATPase is regulated post-translationally. Correspondingly, there was also no alteration in Na-K-ATPase α1 and β1 expression in the plasma membrane. Therefore, the unaltered level of Na-K-ATPase α1 and β1 protein expression in both cellular homogenate and plasma membrane also indicate that increased Na-K-ATPase activity during maturation is not likely due to altered intracellular trafficking.

The increase in Na-K-ATPase activity could be due to a change in the affinity of the Na-K-ATPase to $Na^+$, $K^+$ and ATP (Faleiros, Garcon, Lucena, McNamara, & Leone, 2018; Kristensen & Juel, 2010). In the present study, there was an increase in the affinity for $K^+$ of Na-K-ATPase during cell maturation. This change in affinity may be due to the level of the phosphorylation of the protein. Considerable evidence indicates that the Na-K-ATPase activity is regulated by phosphorylation of the Na-K-ATPase α1 subunit on serine, threonine (Beguin et al., 1994; Bertorello et al., 1991; Chibalin, Vasilets, Hennekes, Pralong, & Geering, 1992; Fisone et al., 1994) and tyrosine residues (El-Beialy et al., 2010; Feraille et al., 1999). It has been reported that there are at least 21 phosphorylation sites in rat Na-K-ATPase α1 (Soltoff, Asara, & Hedden, 2010), including 6 tyrosine, 1 threonine, and 14 serine phosphorylation sites. Protein kinases A

and C (PKA, PKC) have been reported to phosphorylate the α subunit specifically on serine and threonine residues (Beguin et al., 1994; Bertorello et al., 1991; Chibalin et al., 1992; Fisone et al., 1994) whereas various tyrosine kinases phosphorylate at tyrosine residues, thus, regulating Na-K-ATPase activity (El-Beialy et al., 2010; Feraille et al., 1999). Multiple studies have reported that the phosphorylation of the Na-K-ATPase α1 subunit on tyrosine residues causes different responses on Na-K-ATPase activity (Al-Khalili et al., 2004; El-Beialy et al., 2010; Feraille et al., 1999). While some reports have demonstrated that increased tyrosine phosphorylation of Na-K-ATPase α1 in rat kidney tubules (Feraille et al., 1999) and human skeletal muscle cells (Al-Khalili et al., 2004) increases the activity of the Na-K-ATPase, others have reported that increased tyrosine phosphorylation of Na-K-ATPase α1 in human and pig renal cells reduces the activity of Na-K-ATPase (El-Beialy et al., 2010). This differential regulation of Na-K-ATPase pump activity by tyrosine phosphorylation might be due to phosphorylation at different residues or due to the differences in both tissue and species. Our study demonstrates that there is an increase in Na-K-ATPase α1 tyrosine phosphorylation in conjunction with its increase in activity as cells mature. The different results suggest that depending on the tissue studied, and the experimental conditions, phosphorylation of serine and tyrosine leads to either activation or inhibition of Na-K-ATPase (Pedemont & Bertorello, 2001). However, in the present study, we observed that there was an increase in serine and tyrosine phosphorylation whereas there was no change in threonine phosphorylation as cells mature.

Various studies have demonstrated that $Ser^{16}$ and $Ser^{23}$ on the N terminus of Na-K-ATPase α1 are PKC phosphorylation sites (Beguin et al., 1994; Chibalin et al., 1992). $Ser^{16}$ is well conserved among mammalian α1 isoforms but is weakly phosphorylated (Pierre, Duran, Carr, & Pressley, 2002), whereas Na-K-ATPase α1-$Ser^{23}$ is the most extensively studied residue.

Furthermore, Na-K-ATPase α1-$Ser^{23}$ phosphorylation either increases or decreases the activity of Na-K-ATPase activity depending on the tissue type and experimental conditions. However, in the present study, Na-K-ATPase α1-$Ser^{23}$ phosphorylation increases as cells mature with increases in Na-K-ATPase activity.

## 3.6. CONCLUSION

In summary, this study determined that as the intestinal epithelial cells mature from secretory crypt-like (0-day) to absorptive villus-like (4-day post confluence) cells, the Na-K-ATPase activity also increases to support the increasing absorptive capacity of the enterocytes. Na-K-ATPase activity increased secondary to increases in affinity ($1/K_m$) rather than an increase in transporter numbers as cells matured from crypt to villus. Specifically, altered affinity is likely due to changes in phosphorylation of α1 subunit of Na-K-ATPase.

**ACKNOWLEDGEMENTS**

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**CONFLICTS OF INTEREST**

The authors have declared that no conflicts of interest exist.

# CHAPTER 4: MOLECULAR MECHANISM OF STIMULATION OF NA-K-ATPASE BY LEUKOTRIENES IN INTESTINAL EPITHELIAL CELLS

Unpublished Manuscript

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## 4.1. ABSTRACT

In the mammalian small intestinal epithelial cells, Na-K-ATPase provides a favorable transcellular Na gradient required for the functioning of Na-dependent nutrient transporters, thus enabling the assimilation of nutrients. Glutamine, the primary metabolite for enterocytes, is absorbed via Na-glutamine co-transporter (SN2) in intestinal crypt cells. SN2 is stimulated during chronic intestinal inflammation secondary at least in part to the stimulation of Na-K-ATPase during chronic ileitis. Immune-inflammatory mediator Leukotriene D4 (LTD4) is known to be elevated in the mucosa during chronic enteritis, but how it may regulate Na-K-ATPase is not known. Therefore, this study aims to determine the effect of LTD4 on Na-K-ATPase in intestinal epithelial cells and define its mechanism of action. In an *in vitro* model of rat intestinal epithelial cells (IEC-18 cells), grown to confluence, Na-K-ATPase activity was significantly stimulated by LTD4 (1 μm) as measured by $^{86}Rb^{+}$ uptake in cells and inorganic phosphate ($P_i$) release in the plasma membrane. Since LTD4 mediates its action via calcium-dependent protein kinase C (PKC), calcium levels were measured and were shown to be increased by LTD4. Phorbol 12-Myristate 13-Acetate (PMA), an activator of PKC, mediated stimulation of Na-K-ATPase similar to LTD4, while BAPTA-AM (calcium chelator) and Calphostin-C (PKC inhibitor) reversed the stimulation of Na-K-ATPase activity by LTD4. LTD4 caused a significant increase in mRNA and plasma membrane protein expression of Na-K-ATPase α1, and β1 subunits which were prevented by Calphostin-C. These studies demonstrate that LTD4 stimulates Na-K-ATPase. The mechanism of stimulation is secondary to the transcriptional increase of Na-K-ATPase α1 and β1 subunits in the chronically inflamed intestine. Therefore, LTD4 mediates this transcriptional stimulation of Na-K-ATPase via $Ca^{2+}$-activated PKC pathway in intestinal epithelial cells during chronic intestinal inflammation.

## 4.2. INTRODUCTION

The primary function of the mammalian small intestine is an absorption of nutrients vital for the survival of the organism. The process of nutrient absorption occurs through a monolayer of epithelial cells (innermost layer of the mucosa), called enterocytes, present in the small intestine. These enterocytes are further divided into immature enterocytes (crypt cells) and mature enterocytes (villus cells). In the mammalian small intestine, the villus cells are generally considered to be absorptive thus possessing virtually all nutrient absorptive transport pathways. In contrast, crypt cells are typically considered to be secretory and not known to have any nutrient absorptive pathways except for Na-glutamine co-transport, SN2 in the brush border membrane (BBM).

Crypt cell BBM SN2 requires a favorable Na-gradient which is established by Na-K-ATPase, an integral transmembrane protein located on the basolateral membrane (BLM) of these cells. In an animal model of chronic intestinal inflammation, resembling human inflammatory bowel disease (IBD), it has been demonstrated that SN2 is stimulated in crypt cells, at least in part secondary to the stimulation of Na-K-ATPase (Arthur et al., 2012; Saha et al., 2012). Further, it has been shown that the immune inflammatory mediator, LTD4, is increased in the mucosa during chronic intestinal inflammation. Finally, it has also been shown that inhibition of the formation of leukotrienes in this animal model of IBD, reverses the stimulation of Na-K-ATPase and subsequently SN2 in crypt cells (S. Singh et al., 2018). However, it is unknown how LTD4 may stimulate Na-K-ATPase in crypt cells during chronic intestinal inflammation.

It is well established that various physiological and pathophysiological conditions alter Na-K-ATPase through numerous mechanisms. It has been reported that there is a reduction of Na-K-ATPase activity in several diseases such as chronic neurodegenerative disorder (Chauhan,

Lee, & Siegel, 1997), cardiovascular (Ziegelhoffer et al., 2000), or renal disease (Rose & Valdes, 1994). Similarly, malabsorption of a variety of nutrients in IBD is partly due to altered Na-K-ATPase activity (Arthur et al., 2012; Coon et al., 2010; Coon et al., 2005; Saha et al., 2012; Sundaram et al., 2007). For example, in the IBD intestine it has been shown that in villus cells, BBM Na-glucose co-transport (SGLT1), Na-alanine co-transport (NAcT), Na-bile acid cotransporter (ASBT) and Na-glutamine co-transport (B0AT1) are all inhibited. All of these co-transporters are altered at the level of the co-transporter in the BBM in the IBD intestine. But at the cellular level, the down-regulation of these co-transporters is due to the down-regulation of BLM Na-K-ATPase leading to the loss of the intracellular $Na^+$ gradient. However, interestingly only in crypt cells, Na-K-ATPase activity was upregulated during chronic intestinal inflammation with concurrent stimulation of BBM Na-glutamine co-transporter SN2 (Saha et al., 2012). This new observation is made all the more critical because SN2 is the sole nutrient absorptive pathway in crypt cells in health and disease. Further, Na-K-ATPase activity and subsequently SN2 activity was reversed to normal levels by treatment with a corticosteroid, methylprednisolone (Saha et al., 2012; S. Singh et al., 2018; S. Singh et al., 2015). These studies establish that immune inflammatory mediators are responsible for altered BLM Na-K-ATPase as well as BBM SN2 in crypt cells during chronic intestinal inflammation.

Arachidonic acid metabolites (AAMs) are prominent inflammatory mediators present in the mucosa of intestinal tissue from patients with IBD (Romero et al., 2008) and ulcerative colitis (Carty, Nickols, Feakins, & Rampton, 2002). AAMs, specifically prostaglandin E2 (PGE2) and leukotriene D4 (LTD4) have been associated with the pathogenesis of several diseases including rheumatoid arthritis, asthma, psoriasis, multiple sclerosis, and IBD. It has been reported that LTD4 has also been associated with inflammatory processes (Ford-

Hutchinson, Bray, Doig, Shipley, & Smith, 1980) as well as intestinal secretion (Musch et al., 1982). LTD4 has also been involved in the stimulation of SN2 and Na-K-ATPase in IEC-18 cells (S. Singh & Sundaram, 2015). However, the molecular mechanisms responsible for the stimulation of Na-K-ATPase by LTD4 in crypt cells during IBD have not been determined.

Na-K-ATPase is dynamically regulated to change its activity according to its physiological requirement. This holoenzyme is constituted of various subunits: alpha (α), beta (β) and gamma (γ) subunits. Of these subunits, two dissimilar subunits, α and β, are required for the proper assembly and functioning of Na-K-ATPase (Li & Langhans, 2015; McDonough & Farley, 1993; Mercer, 1993). However, the γ subunit is expressed in specific tissues and is optional for the functional activity of Na-K-ATPase (Kaplan, 2002). Of these subunits, the α subunit has the catalytic function: exchange of $2K^+$ inside the cells to $3Na^+$ outside the cells as well as cleaving of ATP (Pressley, Allen, Clarke, Odebunmi, & Higham, 1996). However, the β subunit facilitates maturation of the α subunit by the formation of α/β heterodimer and transporting this enzyme to the plasma membrane (Geering, 1991). Thus, both of these subunits are required for the efficient functioning of Na-K-ATPase hence generating optimum sodium gradient inside the cell. This sodium gradient actuates secondary transport processes on BBM, specifically Na-nutrient co-transporter, thus enabling intestinal epithelial cells to absorb nutrients efficiently. At least four isoforms of the α subunit (α1, α2, α3, and α4) and three isoforms of the β subunit (β1, β2, and β3) are reported to date. These isoforms are expressed in a tissue-specific manner (Blanco et al., 1999; Kaplan, 2002; Mercer, 1993). The combination of different isoforms of α and β subunits make up a series of Na-K-ATPase isoenzymes (Blanco & Mercer, 1998a). Each isoenzyme has different functional properties and is expressed differentially in a

tissue- and cell-specific manner (Jewell et al., 1992). Of these isoforms, α1 and β1 are ubiquitously present in epithelial cells and are also present in the mucosa of the intestine.

Na-K-ATPase activity is tailored to change according to the physiological requirements of the cell. There are several mechanisms that regulate Na-K-ATPase activity-for example, the availability of the substrates ($Na^+$, $K^+$, and ATP). Also, the amount of the enzyme at the plasma membrane can be modified by changes in the rate of synthesis or degradation of the individual Na pump polypeptide and movement of the pump from the cytoplasm to the plasma membrane by exo/endocytotic vesicular transport (Blanco et al., 1998). Apart from these mechanisms, Na-K-ATPase activity at the cell surface is directly regulated by phosphorylation and dephosphorylation by protein kinases and protein phosphatases respectively (Bertorello & Katz, 1993; Ewart & Klip, 1995; McDonough & Farley, 1993). Moreover, phosphorylation of transcription factors associated with Na-K-ATPase α and β subunits also changes the expression and activity of Na-K-ATPase (Li & Langhans, 2015; Serhan & Kreydiyyeh, 2011).

Given this background, very little research has been conducted regarding the effect of LTD4 on Na-K-ATPase in crypt cells, although Na-K-ATPase is vital for the absorption of glutamine in crypt cells. Thus, this study aims to determine the molecular mechanism of stimulation of Na-K-ATPase by LTD4 in crypt cells during chronic intestinal inflammation.

## 4.3. MATERIALS AND METHODS

### 4.3.1. Reagents

All reagents were purchased from Cayman chemicals (Ann Arbor, Michigan, USA): Leukotrine D4 (LTD4) (Cat # 20310), REV 5901 (Cat #70600), Calphostin-C (Cat #15383), REV 5901 (Cat #70600), Phorbol 12-myristate 13-acetate (PMA, Cat# 10008014), BAPTA-AM (Cat# 15551), Amlodipine (Cat#14838) used in the experiments. All reagents were dissolved in DMSO except LTD4, which was dissolved in ethanol to make the stock solutions. The final working concentration of the solutions contained less than 0.5% of DMSO or ethanol. Toxicity of all the drugs was assessed, and the safe dose was used for further experiments (Supplementary Figures- Figure 39).

### 4.3.2. Cell culture

The rat small intestine cells (IEC-18) (American Type Culture Collection), between passages 5 and 20, were used for all of the experiments. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% (vol/vol) fetal bovine serum, 100 U/l human insulin, 0.25 mM β-hydroxybutyric acid, and 100 units/ml penicillin and streptomycin. These cells were cultured in a humidified atmosphere of 10% $CO_2$ at 37°C. Cells were fed with fresh DMEM every other day.

### 4.3.3. Cell viability assay

For assessing the cell viability, the MTT (3-(4,5-dimethylthaizol-2-yl)-2,5-diphenlytetrazolium bromide) assay was performed. Vybrant MTT cell proliferation kit (cat#V-13154) was purchased from Thermofisher Scientific. To perform the MTT assay, cells were seeded with equal number of cells in 96 well plate and cultured until confluent, then treated with test chemicals for desired time. Then medium was then removed from the wells and replaced

with 100 µl of fresh medium. After that, 10 µl of 12 mM MTT solution (prepared in PBS) was added and incubated at 37°C for 2 hours. The whole medium was aspirated and replaced with 100 µl of SDS-HCL solution to each well and mixed thoroughly. The cells were incubated in SDS-HCL solution overnight (~12 hrs) at 37°C. Then the solution was again mixed, and absorbance was read on the spectrophotometer at 570nm. Trypan blue exclusion assay was also performed to measure the cell viability.

**4.3.4. Crude plasma membrane preparation**

Plasma membrane (crude) was isolated from cells according to the method of Havrankova *et al*. (Havrankova et al., 1978). Subsequently, cells were mixed with 2.5-fold volume of 0.001 M $NaHCO_3$ and homogenized 3 times (10 seconds each) with homogenizer (IKA, Cat# 823707, T25 S1c). These homogenized cells were centrifuged at 600 x g for 30 min. The resultant supernatant was centrifuged for 30 min at 20,000 x g. The membrane was washed twice with 0.001 M $NaHCO_3$. The final pellet was resuspended in 0.04 M Tris-HCl buffer (pH 7.4) containing 0.1% BSA. All procedures were carried out at $4^oC$.

**4.3.5. Na-K-ATPase activity assay**

Na-K-ATPase activity was measured as *Pi* liberated (Forbush, 1983) in plasma membrane fractions from cells. Enzyme-specific activity was expressed as nanomoles of *Pi* released per milligram protein per minute.

**4.3.6. RNA isolation and qRT-PCR**

RNA was isolated from different experimental groups by using RNeasy mini kit obtained from Qiagen. Real-time quantitative PCR (qRT-PCR) was performed using isolated total RNA by a two-step method. First, total RNA was used to synthesize cDNA using SuperScript III (Invitrogen, Life Technologies) and an equal mixture of oligo (dT) primer and random hexamers.

Second, newly synthesized cDNA was used as template to perform real-time PCR using TaqMan Universal PCR master mix from Applied Biosystems (Foster city, CA) according to manufacturer's protocol. Rat specific Na-K-ATPase α1- and β1-specific primers and probe were used for the qRT-PCR studies. Rat specific β-actin primer and probe was used as housekeeping gene to normalize the expression of samples.

### 4.3.7. $^{86}Rb^{+}$ uptake for Na-K-ATPase activity

IEC-18 cells uptake studies were grown in 24 well transwell inserts (Corning Transwell # 29442, insert size 24 mm, pore size 0.4 μ). IEC-18 cells were plated with equal number of cells. Uptake studies for Na-K-ATPase were done using radioactive Rubidium ($^{86}Rb^{+}$, PerkinElmer). Cells were incubated for 1 hr in serum free DMEM (SFM). The cells were subsequently washed with SFM and incubated for 10 min at 37°C in SFM containing 20μM monensin on both sides of the membrane. Then cells were washed with SFM. Na-K-ATPase uptake studies were then performed by incubating cells for 15 min with reaction mixture [SFM and $^{86}Rb^{+}$ (~1 μCi/well)] on the basolateral side of the membrane in presence and absence of ouabain (1 mM). The reaction was stopped by addition of ice-cold $MgCl_2$, subsequently washed three times with $MgCl_2$, and the cells were lysed with 800 μl of 1N NaOH and incubation for 30 min at 70°C, which was then mixed with 5 ml of Ecoscint A (National diagnostics). The vials were kept in darkness overnight and radioactivity retained by the cells was determined in a Beckman Coulter 6500 scintillation counter.

### 4.3.8. Calcium measurement

Calcium measurement was performed using the Fluo-8 calcium flux assay kit (Abcam, cat# ab112129). Cells were seeded with equal number of cells in 96 well plate and cultured until confluent, then treated with test chemicals for desired time. Subsequently, the supernatant was

removed and replaced with 100 µl Fluo-8 dye-loading solution per well and incubated for 30 min at room temperature. Next, the fluorescence intensity was measured at Ex/Em of 490/525 nm using a fluorescence plate reader (Spectramax i3x, Molecular Devices).

**4.3.9. Immunocytochemistry (ICC) staining**

IEC-18 cells were grown on a coverslip until 4-day post-confluence. Cells were treated respectively as mentioned before. Following the treatment for 24 hours, cells were fixed with 100% methanol (chilled at -20°C) at room temperature for 5 min. After cells were permeabilized with PBST (PBS + 0.5% Tween 20) for 10 min, cells were blocked with 3% BSA in PBST for 30 min. Then the cells were incubated for an hour at room temperature with primary antibody Zonula occludens (ZO-1) (Anti-Rabbit; Invitrogen, cat# 40-2200), Na-K-ATPase (Anti-mouse; Millipore, cat# 05-369). The secondary antibody (Alexa Fluor; Invitrogen) was added, and the cells were incubated at 37°C for 1h. Cells were mounted with DAPI mounting medium and sealed with nail polish to prevent cells from drying. An EVOS microscope was used to capture images, and ImageJ was used for analysis.

**4.3.10. Western blot analysis**

Western blot analysis was performed on plasma membrane fractions prepared from different samples as described above. Equal amounts of protein were denatured in sample buffer (10 mM Tris-HCl pH 7,12% glycerol, 2% SDS, 0.01% bromophenol blue, and freshly added 1mM Dithiothreitol) and separated by electrophoresis on an 8% polyacrylamide gel. Proteins on the gel were transferred to a polyvinylidene fluoride membrane which was blocked with 5% milk or BSA in TBS (20 mM Tris pH 7.5, 150 mM NaCl) with 0.1% Tween-20 and then incubated with primary antibody against Na-K-ATPase α1 (Millipore, cat# 05-369) or Na-K-ATPase β1 (Abcam, cat# ab2873) overnight at 4°C. Membranes were washed three times each with TBS and

TBST, followed by incubation with secondary antibody for 1 hr. Membranes were washed again three times each with TBS and TBST. An ECL western blotting detection reagent (GE healthcare Bio-Sciences, Piscataway, NJ) was used to detect the immobilized protein. The chemiluminescence was detected using FluorChemM instrument (Alpha Innotech, San Leandro, CA) and analyzed with its software. Ezrin antibody (Abcam, cat#ab4069) was used to normalize the expression levels of proteins in plasma membrane.

**4.3.11. Protein determination**

Total protein was measured by the Lowry method using the Bio-Rad DCTM Protein Assay Kit (Hercules, CA, USA). Different concentrations of BSA were used as a standard. Samples were diluted to 200 µl with water. The diluted sample was mixed with 250 µl of DC Protein Assay reagent A (cat# 500-0113) and incubated for 2 minutes. Subsequently, the sample was incubated for another 15 minutes after the addition of 2 ml DC Protein Assay reagent B (cat# 500-0114). Finally, the sample was read at OD750nm using a spectrophotometer.

**4.3.12. Statistical analysis**

All groups presented have at least n=4 per group, repeated with different passages. The values are presented as mean ± SEM, and p-values of $<0.05$ were taken to indicate statistical significance. All of the data were analyzed using t- test, one-way or two-way analysis of variance (ANOVA) using Graphpad prism software (San Diego, CA).

## 4.4. RESULTS

### 4.4.1. Stimulation of Na-K-ATPase by LTD4

It has been shown that Na-K-ATPase activity in crypt cells, as well as LTD4 in the mucosa is elevated in the chronically inflamed rabbit intestine (S. Singh et al., 2018). IEC-18 cells grown till confluence, at 0-day post confluence physiologically behave like crypt cells (e.g., has SN2 but not SGLT1 or NHE3). Therefore, in order to determine whether the LTD4 is responsible for the stimulation of Na-K-ATPase, IEC-18 cells were treated with various concentrations of LTD4 and the activity of Na-K-ATPase was measured by $^{86}Rb^{+}$ uptake. Our observations showed that Na-K-ATPase was significantly stimulated at 1µm of LTD4 (674.2±45.19 picomole/mg protein/min) compared to control (334.8±73.8). However, lower amounts of LTD4 did not show any stimulation (0.1µm-399.4±59.46, 0.5µm-380.3±42.93p) and higher amounts of LTD4 than 1 µm also did not show any additional stimulation of Na-K-ATPase (Figure 18A) The stimulation of Na-K-ATPase activity in plasma membrane by 1µm of LTD4 was further corroborated with Na-K-ATPase assay $P_i$ release (0µm-5.96±0.66, 1µm-10.96±0.45 nanomole/mg protein/min; Figure 18B).

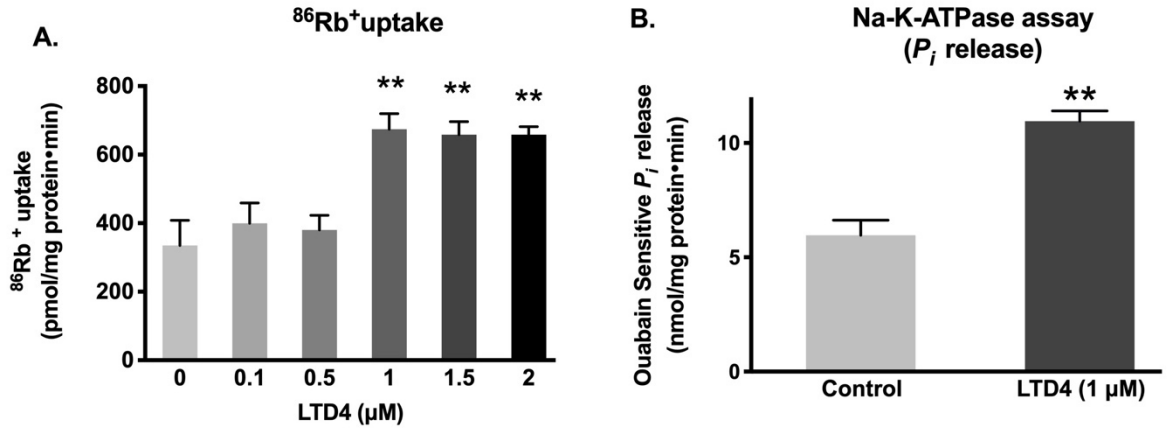


**Figure 18. Effect of LTD4 exposure for 24 hours on Na-K-ATPase activity in IEC-18 cells.** **A.** Measurement of Na-K-ATPase activity by $^{86}Rb^{+}$ uptake (n=6). **B**. Measurement of Na-K-

ATPase activity by $P_i$ release in plasma membrane (n=4). Values are represented as means ± SEM. **, P < 0.01 vs 0 µM or control.

### 4.4.2. Effect of LTD4 exposure on cell viability

To ensure that LTD4 did not negatively affect the viability of IEC-18 cells, MTT and trypan blue assays were performed. Cell viability was not significantly affected by LTD4 (Figure 19A) below 2 µm, thus for all experiments, a concentration of 1 µm was subsequently used. This was also validated with the trypan blue exclusion assay (Figure 19B).

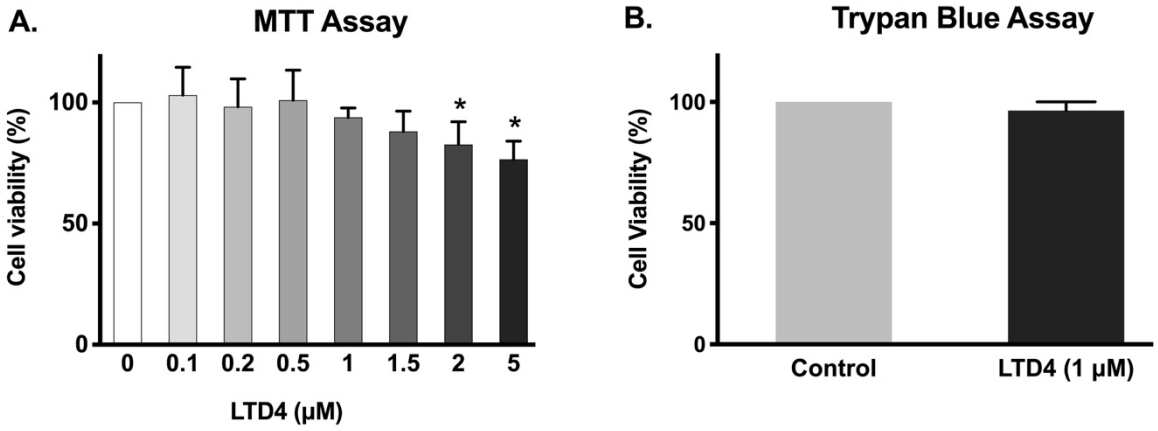


**Figure 19. Effect of LTD4 exposure for 24 hours on cell viability. A.** Measurement of cell Viability by MTT assay. **B.** Measurement of cell viability by trypan blue assay. Values are represented as means ± SEM, n=6. *, P < 0.05 vs 0 µM.

### 4.4.3. Leukotriene receptor antagonist inhibited LTD4 effect on Na-K-ATPase

To determine whether the effect of $LTD_4$ is mediated through its receptor, the effect of the LTD4 receptor antagonist, REV5901, was studied in IEC-18 cells. REV5901 is a competitive antagonist of peptidoleukotrienes and specifically blocks both receptors cysteinyl leukotriene receptors 1 and 2, CysLT1 and CysLT2 (Van Inwegen et al., 1987). It is known that IEC-18 cells have LTD4 receptors (CysLT1 and CysLT2) (Talukder et al., 2008) and these receptors are inhibited by the specific LTD4 receptor inhibitor REV5901 (5 uM) (Talukder et al., 2008). After the pretreatment with REV5901, cells were treated with or without LTD4 for 24 hours, followed

by a rubidium uptake assay. While LTD4 alone produced a significant increase in rubidium uptake, neither REV5901 alone nor REV5901+LTD4 affected rubidium uptake compared to the control, as seen in Figure 20. These data indicate that the stimulatory effect of LTD4 is mediated through its receptor activation in IEC-18 cells.

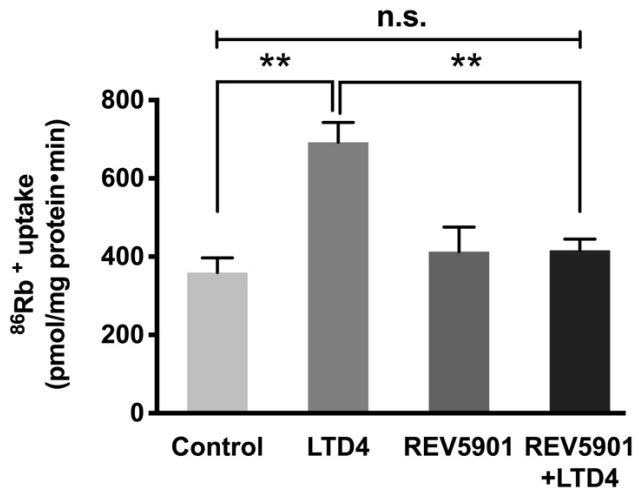


**Figure 20. Effect of LTD4 receptor inhibitor REV5901 (5 µM) on Na-K-ATPase activity in IEC-18 cells.** Measurement of Na-K-ATPase activity by $^{86}Rb^+$ uptake. Values are represented as means ± SEM, n=6. **, $P < 0.01$; n.s., not significant.

### 4.4.4. Effect of LTD4 on intracellular calcium $[Ca^{2+}]_i$

$Ca^{2+}$ is a second messenger and plays an essential role in signal transduction by either activation through ion channels or by G-protein coupled receptors (Clapham, 2007). This cation is vital for many physiological processes, including muscle contraction, neuronal excitability, cellular motility, cell growth and apoptosis (Berridge, Lipp, & Bootman, 2000; Rash, Ackman, & Rakic, 2016). Therefore, we looked for the effect of LTD4 on $[Ca^{2+}]_i$ levels. Calcium channels were blocked with amlodipine in IEC-18 cells and then cells treated with LTD4. The $[Ca^{2+}]_i$ levels increased in cells treated with LTD4 as shown in Figure 21. Intracellular $Ca^{2+}$ increased almost 2-fold within 5 minutes and peaked at 10 minutes (~ 3-fold) with LTD4. Levels reduced gradually at later time points (30 minutes: ~2.3 fold, 60 minutes: ~1.8 fold, 180 minutes: ~1.3

fold) but were still significantly higher than control. These data indicate that LTD4 increases intracellular calcium.

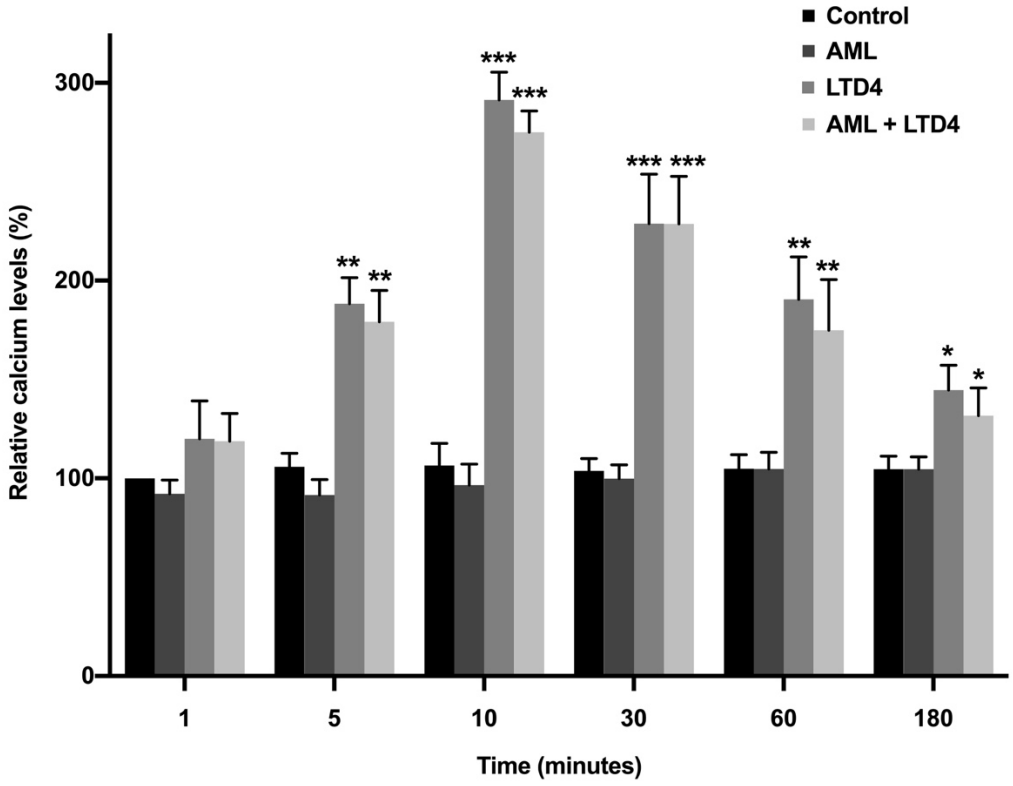


**Figure 21. Level of calcium at different time points during LTD4 treatment.** Measurement of $Ca^{2+}$ by Fluo-8 calcium flux assay. Values are relative to 1 min control and are represented as means ± SEM, n=8. *, P< 0.05, **, P< 0.01 and ***, P<0.001 vs Control. Control and Amlodipine (AML, calcium channel blocker) did not change significantly at different time points.

### 4.4.5. Effect of calcium chelator on Na-K-ATPase activity in IEC-18 cells

Since LTD4 treatment elevated the $Ca^{2+}$ level as shown in Figure 21, to determine whether elevated intracellular $Ca^{2+}$ is responsible for the stimulation of Na-K-ATPase, we used the Bapta-AM. IEC-18 cells were pretreated with calcium chelator (1 μM) Bapta-AM followed by measuring $^{86}Rb^{+}$ uptake. Na-K-ATPase was found to be stimulated with LTD4 whereas Batpa-AM treatment inhibited LTD4 mediated stimulation of Na-K-ATPase as shown in Figure

22 (Control-381.6±50.54, LTD4-726.3±92.26, Bapta-AM-344±25.6, Bapta-AM+ LTD4-326.5±46.8 picomole/mg protein/min). Bapta-AM alone had no effect (Figure 22).

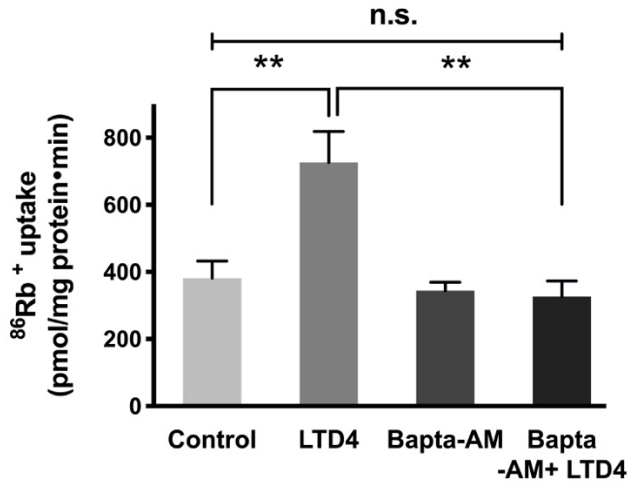


**Figure 22. Effect of calcium chelator BAPTA-AM (1μM) on Na-K-ATPase activity in IEC-18 cells.** Measurement of Na-K-ATPase activity by $^{86}Rb^{+}$ uptake. Values are represented as means ± SEM, n=6. **, $P < 0.01$; n.s., not significant.

### 4.4.6. Effect of inhibition and activation of the PKC pathway on Na-K-ATPase activity in IEC-18 cells

Previous studies have shown that PKC phosphorylates Na-K-ATPase subunits which subsequently affects the activity of Na-K-ATPase (Galuska et al., 2011). To see whether PKC mediates the LTD4 effect, the cells were pretreated with 0.1 μM calphostin-C (PKC inhibitor) for an hour. After pretreatment, cells were treated with LTD4 for 24 hours, subsequently followed by $^{86}Rb^{+}$ uptake. Pretreatment with calphostin inhibited the LTD4-mediated stimulation of Na-K-ATPase (Control-317.3±42.8, LTD4-710.3±61.17, Cal-C-400.8±30.14, Cal-C+ LTD4-452±28.31 picomole/mg protein/min; Figure 23A). Similarly, the PKC activator PMA also stimulated Na-K-ATPase comparable to LTD4 (Control-360.9±53.12, LTD4-636.9±65.51, PMA-659.3±39.74; Figure 23B). These data indicate that PKC mediates LTD4 stimulation of Na-K-ATPase in IEC-18 cells.

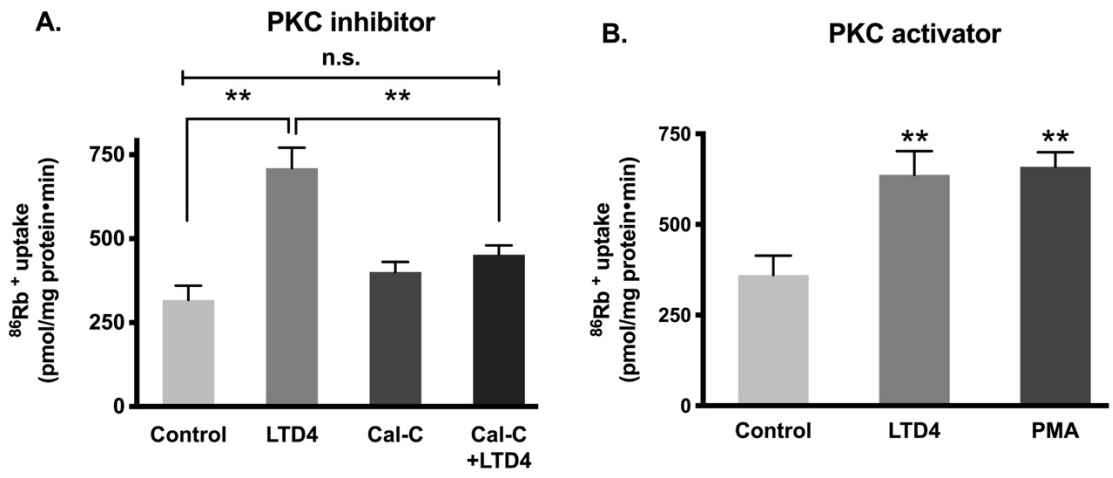


**Figure 23. Effect of PKC inhibitor and activator on Na-K-ATPase activity in IEC-18 cells.** A. PKC inhibitor calphostin-C (0.1 μM). B. PKC activator PMA (1 μM). Measurement of Na-K-ATPase activity by $^{86}Rb+$ uptake. Values are represented as means ± SEM, n=6. **, $P < 0.01$ vs Control; n.s., not significant.

### 4.4.7. Na-K-ATPase α1 and Na-K-ATPase β1 subunit mRNA abundance during LTD4 treatment

Na-K-ATPase functional activity is primarily due to its α subunit whereas the β subunit does not have pumping activity but provides proper transportation of the α subunit to plasma membrane making it fully functional. Therefore, to determine whether the change in Na-K-ATPase activity is transcriptionally regulated, we performed qRT-PCR. There was a significant increase in mRNA level of Na-K-ATPase α1 and Na-K-ATPase β1 subunits when exposed with LTD4 by 160% and 180% respectively (Figure 24A and B). However, the Na-K-ATPase α1 and Na-K-ATPase β1 subunits mRNA level remain unaltered compared to control when cells were treated with Cal-C+ LTD4 (Figure 24A and B). This data indicated that Na-K-ATPase α1 and Na-K-ATPase β1 subunits were transcriptionally regulated when treated with LTD4. Additionally, the Na-K-ATPase α1 and Na-K-ATPase β1 subunits mRNA abundance returned back to control levels when treated with Cal-C+LTD4, state that PKC play an important role in LTD4 mediated regulation of Na-K-ATPase.

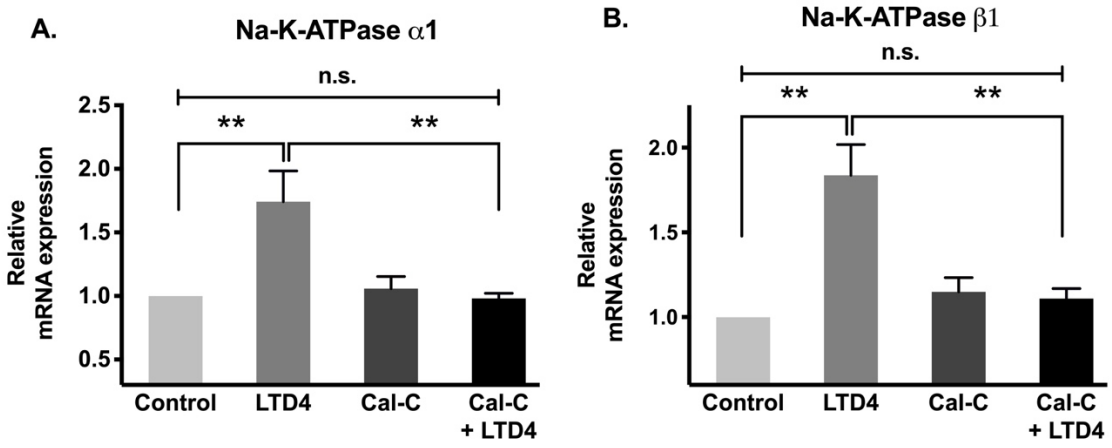


**Figure 24. QRT-PCR analysis of IEC-18 cells.** Values are relative to control and normalized to β-actin. **A.** Na-K-ATPase α1. **B.** Na-K-ATPase β1. Values are represented as mean ± SEM, n=4. **, $P < 0.01$; n.s., not significant.

### 4.4.8. Na-K-ATPase α1 and Na-K-ATPase β1 subunit protein expression during LTD4 treatment

To determine whether changes in Na-K-ATPase activities are due to altered transcription of the Na-K-ATPase α1 and β1 subunits protein, Western blot analysis of plasma membrane (Figure 25) and immunocytochemistry of IEC-18 cells (Figure 26) was performed. Densitometric analysis of relative protein expression revealed that the level of Na-K-ATPase α1 protein expression was significantly increased by LTD4 treatment, whereas it remained at control levels when treated with Cal-C+LTD4 (Figure 25B and 26). Similarly, there was also a significant increase in Na-K-ATPase β1 subunit protein expression in the plasma membrane (Figures 25A & C) when treated with LTD4, and this level reverted to normal when treated with Cal-C+LTD4 (Figures 25A & C). Therefore, the expression of Na-K-ATPase α1 and Na-K-ATPase β1 subunits mRNA expression correlated with its protein expression in the plasma membrane. This shows that LTD4 regulates Na-K-ATPase transcriptionally by increasing mRNA and protein levels of the α1 and β1 subunits.

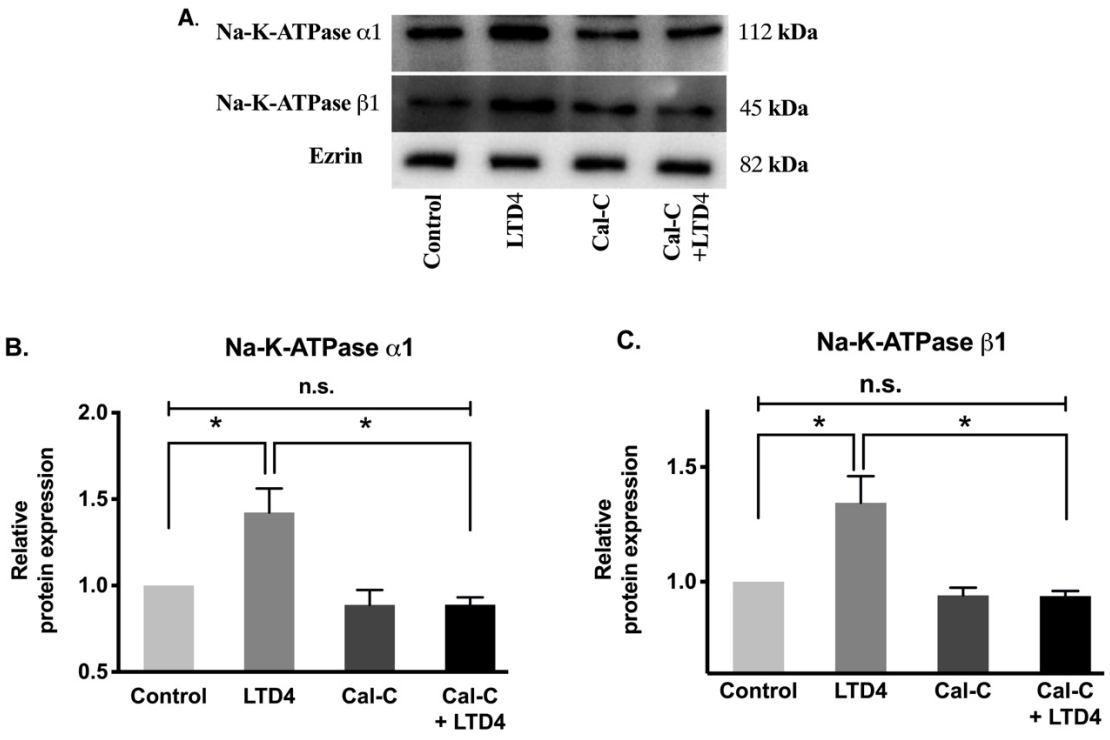


**Figure 25. Western blot analysis of IEC-18 cells when treated with LTD4 and Calphostin-C (PKC inhibitor). A.** Representative western blots of Na-K-ATPase α1, Na-K-ATPase β1 and Ezrin (internal control). Densitometric quantitation of blots, **B.** Na-K-ATPase α1 and **C.** Na-K-ATPase β1. Values are relative to control and normalized to Ezrin. Values are represented as mean ± SEM, n=4. *, $P < 0.05$; n.s., not significant.

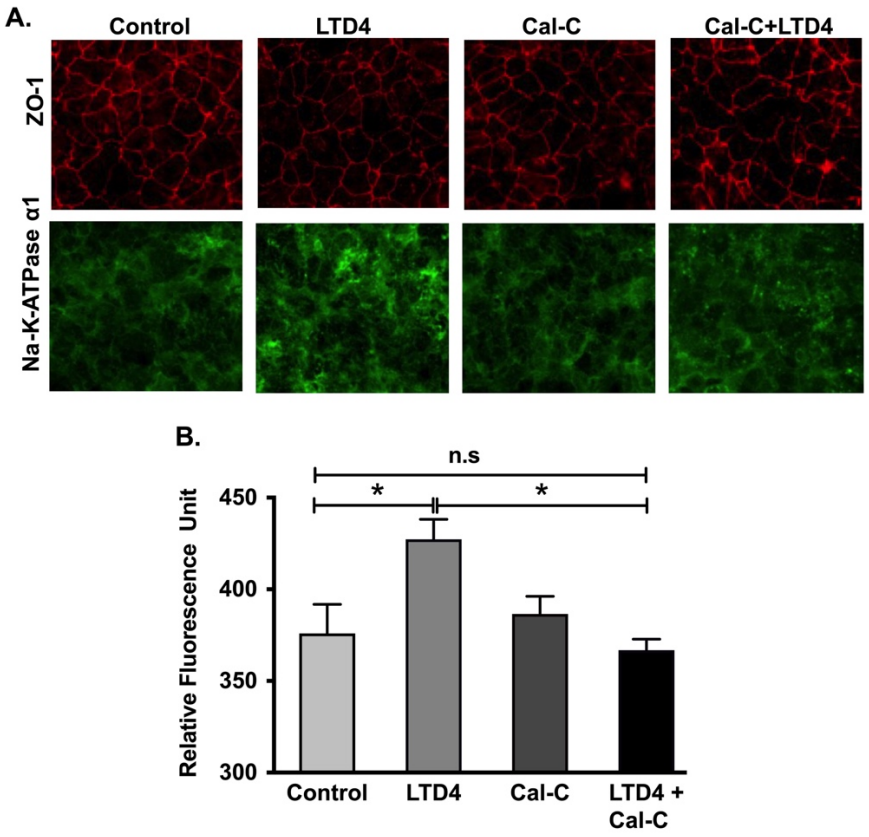


**Figure 26. Immunocytochemistry of IEC-18 cells when treated with LTD4 and Calphostin C (PKC inhibitor). A.** Representative images of Na-K-ATPase α1(green), and ZO-1(Red), (20X). **B. (**Quantification of Na-K-ATPaseα1 (green channel). Values are represented as mean ± SEM, n=6. *, $P < 0.05$; n.s., not significant.

## 4.5. DISCUSSION

In the chronically inflamed intestine, Na-glutamine co-transport, mediated by SN2, is stimulated at the cellular level secondary to the stimulation of Na-K-ATPase. Immune-inflammatory pathway inhibition indicated that these unique changes might mediate through the leukotriene pathway (S. Singh et al., 2018). Indeed, LTD4 was shown to be elevated in the chronically inflamed intestinal mucosa. These observations led to the hypothesis that LTD4 may stimulate Na-K-ATPase thereby facilitating the stimulation of SN2 in crypt cells during chronic intestinal inflammation. These studies for the first time provided the mechanism of stimulation of Na-K-ATPase by LTD4 in crypt cells during chronic intestinal inflammation.

To explore the mechanism described above, Rat intestinal epithelial cells (IEC-18) were used. These cells at 0-day post-confluence phenotypically resemble intestinal crypt cells, and by day 4 post-confluence mature into villus like cells. Comparable to native crypt cells, IEC-18 cells at day 0, have SN2 on the BBM but not SGLT1, NHE3 or any other absorptive process (S. Singh & Sundaram, 2015). Also comparable to crypt cells, IEC-18 cells have Na-K-ATPase, located on the BLM, to provide the favorable Na gradient for SN2. Thus, these cells are an ideal in vitro model of intestinal crypt cells.

Alterations in intestinal epithelial cell absorption and secretion are mediated by immune inflammatory mediators, known to be released in the chronically inflamed intestine (Arthur et al., 2012; Coon et al., 2010; Coon et al., 2005; Saha et al., 2012). The alteration in absorptive mechanisms during intestinal inflammation may be due to many immune inflammatory mediators including prostaglandins, leukotrienes (LTs), chemokines, interleukins, and/or reactive nitrogen and oxygen species (Sartor & Powell, 1991). Indeed, many of these inflammatory mediators have been shown to regulate nutrient and electrolyte transport processes during

chronic intestinal inflammation (Manokas, Fromkes, & Sundaram, 2000; Sundaram & West, 1997; Sundaram, Wisel, & Coon, 2005; Sundaram, Wisel, & Fromkes, 1998b; Sundaram, Wisel, Rajendren, & West, 1997).

Of the arachidonic acid metabolites known to be increased in the chronically inflamed intestinal mucosa, there is a wealth of evidence that LTs (LTB4, LTC4, LTD4, and LTE4) are involved in the alteration of intestinal electrolyte transport in IBD patients (Peskar et al., 1986). Other studies have shown that the LOX pathway is involved in the stimulation of Na-glutamine transporter (SN2) and Na-K-ATPase activity in crypt cells in the inflamed intestine of rabbits (S. Singh et al., 2018). Hence, it was reasonable to postulate that LTD4 mediates the stimulation of Na-K-ATPase in crypt cells and identify the specific mechanisms by which LTD4 stimulates Na-K-ATPase.

This study demonstrated that 1 μM LTD4 for 24 hours is both non-toxic and optimal for stimulation of Na-K-ATPase in IEC-18 cells. This stimulation of Na-K-ATPase is through LTD4 receptor activation. At the level of the intracellular signaling, receptors CysLT1 and CystLT2 mediate their action through various G-proteins that subsequently alter intracellular $Ca^{2+}$ or cAMP (Bouchelouche, Ahnfelt-Ronne, & Thomsen, 1990; Chan et al., 1994; Gronroos, Thodeti, & Sjolander, 1998; Han, Park, Lee, Lee, & Park, 1999; Thodeti & Sjolander, 2002). This study demonstrated that LTD4 increased intracellular $Ca^{2+}$ when exposed to LTD4; thus, when Ca was chelated, the reversal in the stimulation of Na-K-ATPase was observed; therefore, indicating that stimulation of Na-K-ATPase is mediated through increased $[Ca^{2+}]_i$.

LTD4 mediated increase of $[Ca^{2+}]_i$, may in-turn activate $Ca^{2+}$dependent kinases and downstream signaling (Bouchelouche et al., 1990; Chan et al., 1994; Gronroos et al., 1998; Han et al., 1999; Thodeti & Sjolander, 2002). Based on the external stimulus, PKC has been shown to

be involved in the reduction or activation of Na-K-ATPase activity, depending upon species and tissues studied (Borghini, Geering, Gjinovci, Wollheim, & Pralong, 1994; Feschenko & Sweadner, 1995). Moreover, the stimulation of various isoforms of PKC can have a differential effect on Na-K-ATPase. Conventional PKC α and β are associated with decreased activity of Na-K-ATPase by endocytosis (Chibalin, Pedemonte, et al., 1998; Feraille & Doucet, 2001), whereas other isoforms of PKC (ε and δ) have been shown to stimulate Na-K-ATPase activity through the ERK1/2 pathway (Galuska et al., 2011; Zhong et al., 2005). In the present study, the PKC pathway was activated by LTD4. When calphostin-c inhibited PKC, LTD4-mediated stimulation of Na-K-ATPase was attenuated, thus indicating that $Ca^{2+}$ activated PKC is responsible for stimulation of Na-K-ATPase. Future studies will need to identify the exact PKC isoform responsible for the LTD4-mediated stimulation of Na-K-ATPase.

Upon activation of PKC, its modulation of Na-K-ATPase might be due to 1) direct phosphorylation of Na-K-ATPase α and γ subunits, or 2) alteration in transcription factors associated with various subunits of Na-K-ATPase (Li & Langhans, 2015). In the present study, LTD4 increased the transcription of the message for the α1 and β1 subunits of Na-K-ATPase and subsequently the protein levels of these subunits in the plasma membrane. Transcription factors such specific protein (Sp); Sp1, Sp2, and Sp3 are ubiquitously expressed whereas Sp4 is mainly confined to neurons and testis (Li & Langhans, 2015). Among these, Sp1 and Sp3 found to increase the transcription of the α1 and β1subunits by binding to the promoter region of these subunits (Johar et al., 2012; Kobayashi et al., 1997). Similarly, phosphorylation of transcription factor CREB induces the increased α1 mRNA level without altering β1subuint mRNA level (Dagenais et al., 2001). Also, the binding of ZEB1 (AREB6) to the promoter region of the α1 subunit increases the protein level of the α1 subunit in skeletal muscle (Galuska et al., 2011).

Future studies will decipher the potential transcription factor via which LTD4 may stimulate Na-K-ATPase in crypt cells.

## 4.6. CONCLUSION

In conclusion, as shown in Figure 27, LTD4-mediated stimulation of Na-K-ATPase in intestinal crypt cells is due to activation of Ca-mediated PKC, which in-turn activates different transcription factors regulating expression of α1 and β1 subunits of Na-K-ATPase. Thus, in the chronically inflamed intestine, LTD4 mediated stimulation of crypt cell Na-K-ATPase which facilitates the stimulation of the lone nutrient absorptive pathway of these cells, namely SN2, is via PKC and subsequently enhanced transcription of α1 and β1 subunits of Na-K-ATPase.

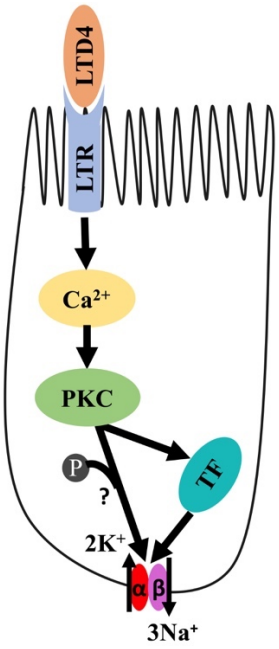


**Figure 27. Proposed mechanism of LTD4–mediated regulation of Na-K-ATPase in intestinal epithelial cells.** LTD4-Leukotriene-D4, LTR-Leukotriene receptor, PKC-Protein Kinase C, TF-Transcription Factor.

## 4.7. SUPPLEMENTARY FIGURES

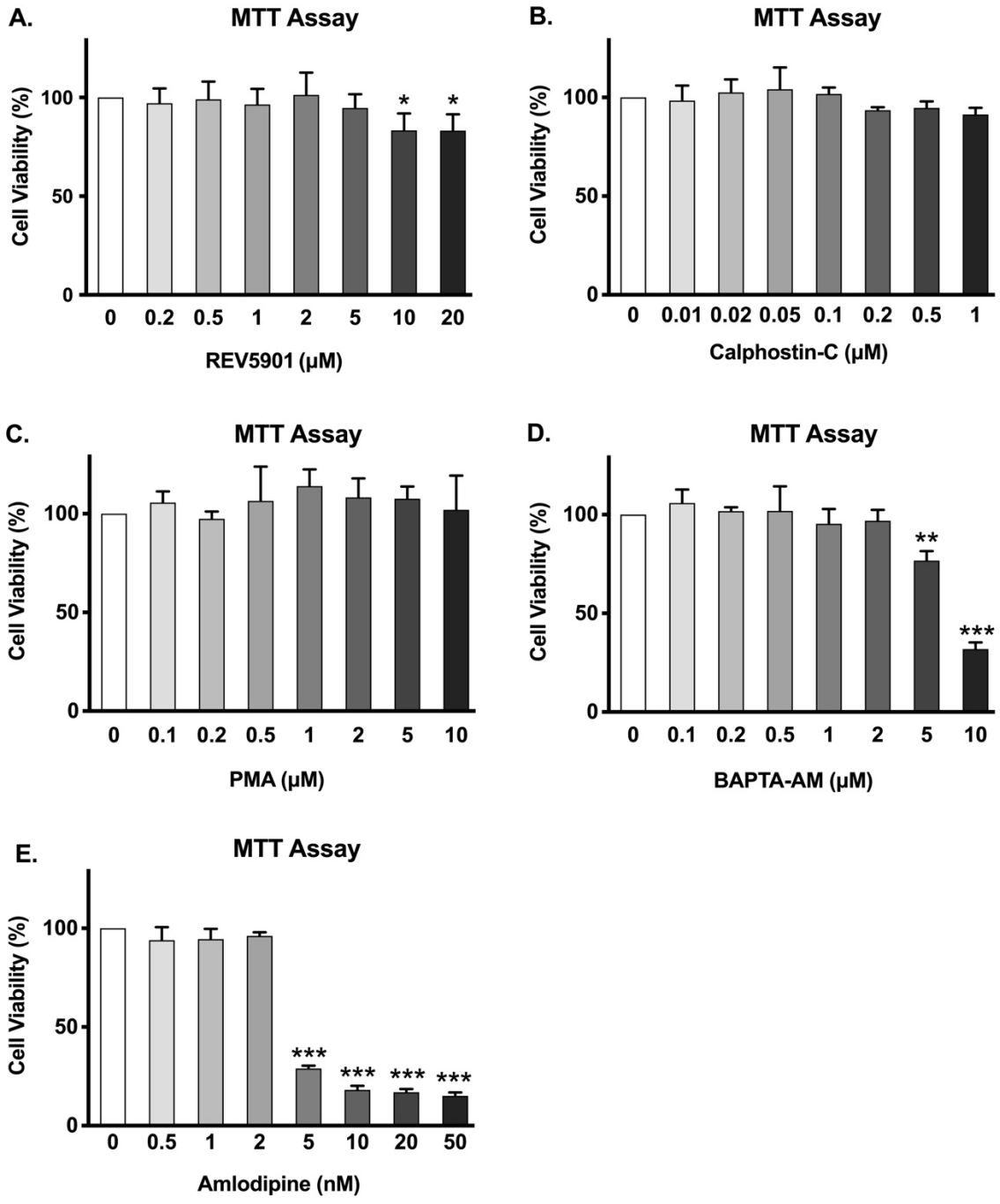


**Figure 28. Effect of different concentration of reagents (drugs) exposure for 24 hours on cell viability. A.** REV5901. **B.** calphostin-C. **C.** PMA (Phorbol 12-myristate 13-acetate). **D.** BAPTA-AM. **E.** Amlodipine. Measurement of cell Viability by MTT assay. Values are relative to 0 μM and are represented as means ± SEM, n=6. *, $P < 0.05$ vs 0 μM, **, $P < 0.01$ vs 0 μM, ***, $P < 0.001$ vs 0 μM.

## ACKNOWLEDGEMENT

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## CONFLICTS OF INTEREST

The authors have declared that no conflicts of interest exist.

# CHAPTER 5: MECHANISM OF NA-K-ATPASE INHIBITION BY PGE2 IN INTESTINAL EPITHELIAL CELLS

Unpublished Manuscript

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## 5.1. ABSTRACT

In the mammalian small intestine, villus cells absorb nutrients, electrolytes, and water. The primary means of absorption of nutrients by villus cells is via Na-dependent nutrient co-transporters located in brush border membrane (BBM) of villus cells. These secondary active co-transport processes require a favorable transcellular Na gradient that is provided by Na-K-ATPase, an integral basolateral membrane (BLM). Thus, inhibition of villus cell Na-K-ATPase during chronic intestinal inflammation results in the malabsorption of essential nutrients (e.g., amino acids, glucose) via Na-nutrient co-transport pathways. Glutamine is the preferred metabolite of intestinal epithelial cells. Its villus cell absorption is via Na-glutamine co-transport, B0AT1. During chronic intestinal inflammation, B0AT1 is inhibited in villus cells, at least partially secondary to an inhibition of Na-K-ATPase in these cells. Further, specific immune inflammatory mediators (e.g., PGE2) are elevated in the inflamed intestinal mucosa. But how PGE2 may mediate the inhibition of Na-K-ATPase in the chronically inflamed intestine is not known. Therefore, this study aims to determine the effect of PGE2 on Na-K-ATPase in intestinal epithelial cells and define its mechanism of action. An in vitro model of rat intestinal epithelial cells (IEC-18 cells line), was used, which resemble villus cells 4-days post-confluence. In IEC-18 cells, PGE2 (0.1 μm) significantly reduced Na-K-ATPase activity, as measured by $^{86}Rb^{+}$ uptake and $P_i$ release in the plasma membrane, without affecting cell viability. Since PGE2 mediates its action via cAMP-dependent protein kinase A (PKA), cAMP levels were measured and shown to be increased by PGE2. 8-Bromo-cAMP (cAMP analog) acted similarly to PGE2, while Rp-cAMP (PKA inhibitor) reversed the PGE2-mediated reduction of Na-K-ATPase activity. PGE2 caused a significant decrease in mRNA and plasma membrane protein expression of the Na-K-ATPase α1 and β1 subunits, while Rp-cAMP prevented this inhibition by PGE2.

These studies demonstrated that the PGE2-mediated reduction of Na-K-ATPase is through the cAMP-activated PKA pathway in intestinal epithelial cells. The mechanism of inhibition is secondary to a transcriptional reduction in Na-K-ATPase α1 and β1 subunits. Thus, in an *in vitro* model of intestinal inflammation, PGE2 inhibits Na-K-ATPase activity through PKA by reducing the gene expression of its subunits.

## 5.2. INTRODUCTION

Na-K-ATPase is an integral plasma membrane protein specifically present in the basolateral membrane (BLM) of epithelial cells that transports 3 $Na^+$ out of and 2 $K^+$ into the cell by utilizing one ATP per each transport cycle. Consequently, pumping $Na^+$ out of the cell, Na-K-ATPase generates a favorable transcellular $Na^+$ gradient required for nutrient absorption in mammalian intestinal cells. Thus, Na-K-ATPase drives transporters, specifically Na-dependent transporters present on the brush border membrane (BBM) in absorptive intestinal villus cells.

Three distinct subunits, alpha (α), beta (β) and gamma (γ) subunits constitute a fully functional Na-K-ATPase enzyme. Of these subunits, α and β subunits are crucial for the proper functioning of the Na-K-ATPase, whereas the γ subunit is not essential for its function (Blanco et al., 1999; Geering, 1991; Kaplan, 2002). The catalytic α subunit that does the pumping function can be found in four different isoforms (α1, α2, α3, and α4) and is reported to be expressed in a tissue-specific manner (Blanco et al., 1999; Kaplan, 2002; Mercer, 1993). However, the regulatory β subunit facilitates maturation of the α subunit by forming an α/β heterodimer and transporting this enzyme to the plasma membrane (Geering, 1991). The activity of Na-K-ATPase can be regulated by various mechanisms: 1) trafficking of pump from cytoplasm to the plasma membrane (Blanco et al., 1998), 2) transcriptional regulation of subunits (Musch et al., 1982; Serhan & Kreydiyyeh, 2011), and 3) phosphorylation and dephosphorylation of subunits (Bertorello & Katz, 1993; Ewart & Klip, 1995; McDonough & Farley, 1993).

Inflammatory bowel disease (IBD) is characterized by the malabsorption of nutrients and electrolytes, resulting in severe weight loss and malnutrition. Previous studies have shown that Na-nutrient co-transport pathways in the BBM, namely Na-glucose (SGLT1), Na-alanine (NAcT), Na-glutamine (B0AT1), Na-bile acid cotransporter (ASBT) and Na-adenosine (DMT1),

which all depend on the BLM Na-K-ATPase for their optimal activities, are inhibited in villus cells in a rabbit model of IBD (Ejderhamn et al., 1989; Rachmilewitz et al., 1984; Sundaram & West, 1997; Sundaram, Wisel, & Fromkes, 1998b). In these cases, it has been reported that there is decreased Na-K-ATPase activity in villus cells, which implies that Na-K-ATPase might be at least in part responsible for the improper functioning of those transporters, thus leading to the malabsorption.

It has been well established that during IBD, various immune inflammatory mediators are produced endogenously in the intestine (Stadnyk, 2002). These immune inflammatory mediators work solely or synergistically with one another to affect electrolyte and nutrient transport pathways (Castro, 1991; Hinterleitner & Powell, 1991; Sartor, 1991). Furthermore, immune inflammatory mediators such as arachidonic acid metabolites (AAMs) are prominent in the mucosa of patients with IBD. Specifically, prostaglandin E2 (PGE2) and leukotriene D4 (LTD4) have been associated with the pathogenesis of IBD and several other diseases including rheumatoid arthritis, asthma, psoriasis, and multiple sclerosis. PGE2 has been shown to affect numerous biological activities like water and electrolyte transport in the gut, have vasoactive effects, and induce smooth muscle contraction (Rachmilewitz et al., 1984). Moreover, the cyclooxygenase pathway is also responsible for the reduction of B0AT1 and Na-K-ATPase in villus cells of IBD-induced rabbits (Arthur et al., 2018). However, the molecular mechanisms responsible for PGE2-mediated reduction of Na-K-ATPase activity have not been determined. Therefore, this work aims to address the mechanism(s) of PGE2-mediated reduction of Na-K-ATPase in intestinal epithelial cells.

## 5.3. MATERIALS AND METHODS

### 5.3.1. Reagents

All reagents were purchased from Cayman chemicals (Ann Arbor, Michigan, USA): Prostaglandin E2 (PGE2, Cat# 14010), AH6809 (Cat# 14050), Rp-cAMP (Cat# 16985), and 8-bromo-Cyclic AMP (Cat# 14431) were used in the experiments. All reagents were dissolved in DMSO to make the stock solutions. Final working concentrations of solutions contained less than 0.5% DMSO. Toxicity of all the drugs was assessed, and the safe dose was used for further experiments (Supplementary Figures- Figure 39).

### 5.3.2. Cell culture

The cells used in experiments were rat intestinal epithelial cells (IEC-18, American Type Culture Collection), between passages 5 and 20. IEC-18 cells are a non-transformed, polarized rat epithelial cell line that maintains its integrity and biochemical properties that are similar to *in vivo* mammalian intestinal epithelial cells. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% (vol/vol) fetal bovine serum, 100 U/l human insulin, 0.25 mM B-hydroxybutyric acid, and 100 units/ml penicillin and streptomycin. These cells were cultured in a humidified atmosphere of 10% $CO_2$ at 37°C. Cells were fed with fresh DMEM every other day. When the cells reached 100% confluence, it was considered as 0 day and cells were grown until 4 days post confluence. All of the experiments were conducted on 4 days post confluence when they exhibit villus-like features of small intestine.

### 5.3.3. Cell viability assays

For assessing the cell viability, the MTT (3-(4,5-dimethylthaizol-2-yl)-2,5-diphenlytetrazolium bromide) assay was performed. Vybrant MTT cell proliferation kit (cat#V-13154) was purchased from Thermofisher Scientific. To perform MTT assay, cells were seeded

with an equal number of cells in 96 well plate and cultured, then treated with test chemicals for desired time. The medium was then removed from the wells and replaced with 100 µl of fresh medium. After that, 10 µl of 12 mM MTT solution (prepared in PBS) was added and incubated at 37°C for 2 hours. The whole medium was aspirated and replaced with 100 µl of SDS-HCL solution to each well and mixed thoroughly. The cells were incubated in SDS-HCL solution overnight (~12 hrs) at 37°C. Then the solution was again mixed, and absorbance was read on the spectrophotometer at 570nm. Trypan blue exclusion assay was also performed to measure the cell viability.

### 5.3.4. Crude plasma membrane preparation

Crude plasma membrane was prepared from IEC-18 cells following the method of Havrankova *et al*.(Havrankova et al., 1978). Cells were well mixed with 2.5-fold 0.001 M $NaHCO_3$ and homogenized 3 times (10 seconds each) with homogenizer (IKA, Cat# 823707, T25 S1). These homogenized cells were centrifuged at 600 x g for 30 min. The resultant supernatant was centrifuged for 30 min at 20,000 x g. The membrane was washed twice with 0.001 M $NaHCO_3$. The final pellet was resuspended in 0.04 M Tris-HCl buffer (pH 7.4) containing 0.1% BSA. All procedures were carried out at 4°C.

### 5.3.5. Na-K-ATPase activity assay

Na-K-ATPase activity was measured as *Pi* liberated in plasma membrane fractions from IEC-18 cells according to protocol of Forbush (Forbush, 1983). Enzyme-specific activity was expressed as nanomoles of *Pi* released per milligram protein per minute.

### 5.3.6. RNA isolation and qRT-PCR

RNA was isolated from different experimental groups by using RNeasy mini kit obtained from Qiagen. Real-time quantitative PCR (qRT-PCR) was performed using isolated total RNA

by a two-step method. First, total RNA was used to synthesize cDNA using SuperScript III kit (Invitrogen, Life Technologies). Second, newly synthesized cDNA was used as template to perform real-time PCR using TaqMan Universal PCR master mix from Applied Biosystems (Foster city, CA) according to manufacturer's protocol. Rat specific Na-K-ATPase α1- and β1-specific primers and probe were used for the qRT-PCR studies. Rat specific β-actin primer and probe was used as a housekeeping gene to normalize the expression of samples.

### 5.3.7. $^{86}Rb^{+}$ uptake for Na-K-ATPase activity

For uptake studies, IEC-18 cells were grown on transwell inserts (Corning Transwell # 29442, insert size 24 mm, pore size 0.4 μ) in 24 well plates. IEC-18 cells were plated with equal number of cells. Uptake studies for Na-K-ATPase were done using radioactive Rubidium ($^{86}Rb^{+}$, PerkinElmer). Cells were incubated for 1 hr in serum free DMEM (SFM). The cells were subsequently washed with SFM and incubated for 10 min at 37°C in SFM containing 20 μM monensin on both sides of the membrane. Then cells were washed with SFM. Na-K-ATPase uptake studies were then performed by incubating cells for 15 min with reaction mixture [SFM and $^{86}Rb^{+}$ (~1 μCi/well)] on the basolateral side of the membrane in presence and absence of ouabain (1 mM). The reaction was stopped by addition of ice-cold $MgCl_2$, subsequently washed three times with $MgCl_2$, and the cells were lysed with 800 μl of 1N NaOH and incubation for 30 min at 70°C, which was then mixed with 5 ml of Ecoscint A (National diagnostics). The vials were kept in darkness overnight and radioactivity retained by the cells was determined in a Beckman Coulter 6500 scintillation counter.

### 5.3.8. cAMP measurement

Calcium measurement was performed using the cAMP Direct immunoassay kit (Abcam, cat# ab65355). Cells were seeded with an equal number of cells in 35 $cm^2$ dish and cultured until

4-day post confluent, then treated with test chemicals for desired time. The medium was aspirated followed by addition of 282 µl of 0.1 M HCl. The suspension was homogenized by pipetting up and down several times. Then the suspension was centrifuged for 10 minutes at 14,000 rpm. The resultant supernatant collected was used for further experiments. Next the sample was diluted and mixed with acetylating reagents. After that, the samples were loaded in 96 well plates and incubated with cAMP antibody for an hour; cAMP-HRP was added to each well and incubated for another hour and washed 3 times with wash buffer. Subsequently, HRP developer was added and incubated for 1 hr. The reaction was stopped by 1M HCl and the color developed was read at $OD_{450nm}$ using a plate reader (Spectramax i3x, Molecular Devices).

**5.3.9. Western blot analysis**

Western blot analysis was performed on plasma membrane fractions prepared from different samples as described above. Equal amounts of protein (10 µg) were denatured in sample buffer (10 mM Tris-HCl pH 7, 12% glycerol, 2% SDS, 0.01% bromophenol blue, and freshly added 1 mM Dithiothreitol) and separated by electrophoresis on a 8% polyacrylamide gel. Proteins on the gel were transferred to a polyvinylidene fluoride membrane which was blocked with 5% milk or BSA in TBS (20 mM Tris pH 7.5, 150 mM NaCl) with 0.1% Tween-20 and then incubated with primary antibody against Na-K-ATPase α1 (Millipore, cat# 05-369) or Na-K-ATPase β1 (Abcam, cat# ab2873) overnight at 4°C. Membranes were washed three times each with TBS and TBST, followed by incubation with secondary antibody for 1 hr. Membranes were washed again three times each with TBS and TBST. ECL western blotting detection reagent (GE healthcare Bio-Sciences, Piscataway, NJ) was used to detect the immobilized protein. The chemiluminescence was detected using FluorChem instrument (Alpha Innotech, San

Leandro, CA) and analyzed with its software. Ezrin (Abcam, cat#ab4069) antibody was used to normalize the expression levels of proteins in plasma membrane fractions.

**5.3.10. Immunocytochemistry (ICC) staining**

IEC-18 cells were grown on coverslip to 4-day post confluence. Cells were treated with PGE2 or Rp-cAMP and as mentioned before. Following the treatment for 24 hours, cells were fixed with 100% methanol (chilled at -20°C) at room temperature for 5 min. After cells were permeabilized with PBST (PBS + 0.5% Tween 20) for 10 min, cells were blocked with 3% BSA in PBST for 30 min. Then the cells were incubated for an hour at room temperature with primary antibodies ZO-1 (Anti-Rabbit; Invitrogen cat# 40-2200), and Na-K-ATPase (Anti-mouse; Millipore cat# 05-369). Alexa Fluor secondary antibodies (Invitrogen) were added, and the cells were incubated at room temperature for an hour. Cells were mounted with DAPI-containing mounting medium (Abcam) and sealed with nail polish to prevent cells from drying. An EVOS microscope (Invitrogen) was used to capture images and ImageJ software was used for analysis.

**5.3.11. Protein determination**

Total protein was measured by Lowry method using the DC Protein Assay Kit (Bio-Rad, Hercules, CA). Different concentrations of BSA were used as standards. Samples were diluted to 200 µl with water. The diluted sample was mixed with 250 µl of DC Protein Assay reagent A (cat# 500-0113) and incubated for 2 minutes. Subsequently, the sample was incubated for another 15 minutes after the addition of 2 ml DC Protein Assay reagent B (cat# 500-0114). Finally, the sample was read at $OD_{750nm}$ using a spectrophotometer.

**5.3.12. Statistical analysis**

All groups presented have at least n=4 per group, repeated with a different passage. The values are presented as mean ± SEM, and p-values of $<0.05$ were taken to indicate statistical

significance. All of the data were analyzed using t-test, one-way or two-way analysis of variance (ANOVA) using Graphpad Prism software (San Diego, CA).

## 5.4. RESULTS

### 5.4.1. Effect of PGE2 on Na-K-ATPase in IEC-18 cells

IEC-18 cells were treated with various concentrations of PGE2 for 24 hours (two treatments 12 hours apart), and the activity of Na-K-ATPase was measured by $^{86}Rb^{+}$ uptake. The lowest dose of PGE2 at which Na-K-ATPase activity was significantly diminished was at 0.1 µm of PGE2 (792±53.2 picomole/mg protein/min) compared to control (1656±95.6) (Figure 29A). The reduction of Na-K-ATPase activity in the plasma membrane by 0.1 µm of PGE2 was further corroborated by the Na-K-ATPase inorganic phosphate ($P_i$) release assay (Control-18.9±1.64, PGE2, 1 µm-9.36±1.22 nanomole/mg protein/min; Figure 29B).

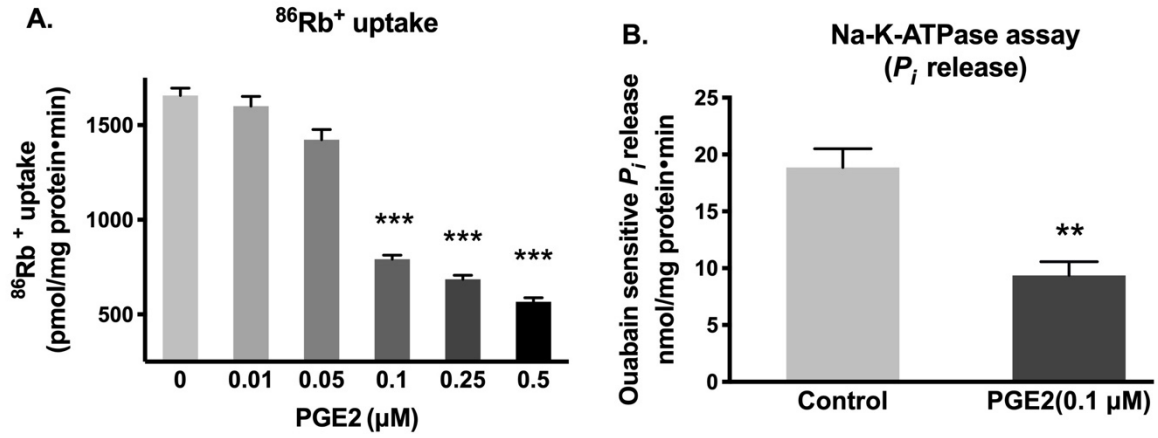


**Figure 29. Effect of PGE2 exposure for 24 hours on Na-K-ATPase activity in IEC-18 cells.** A. Measurement of Na-K-ATPase activity by $^{86}Rb^{+}$ uptake (n=6). B. Measurement of Na-K-ATPase activity by $P_i$ release in plasma membrane preparations (n=4). Values are represented as means ± SEM. **, P < 0.01 and ***, P<0.001 vs 0 µM or control.

### 5.4.2. Effect of PGE2 on cell viability

To ensure that a given dose of PGE2 did not affect the viability of IEC-18 cells, MTT and trypan blue assays were performed. Cell viability was not significantly altered with 0.25 µm or less concentration of PGE2 (Figure 30A). However, there was decreased cell viability in IEC-18 cells with 1 µm or higher concentration of PGE2. Therefore, the concentration we used for our

experiments (0.1 μm) did not cause any cell death, which was also validated with the trypan blue exclusion assay (Figure 30B).

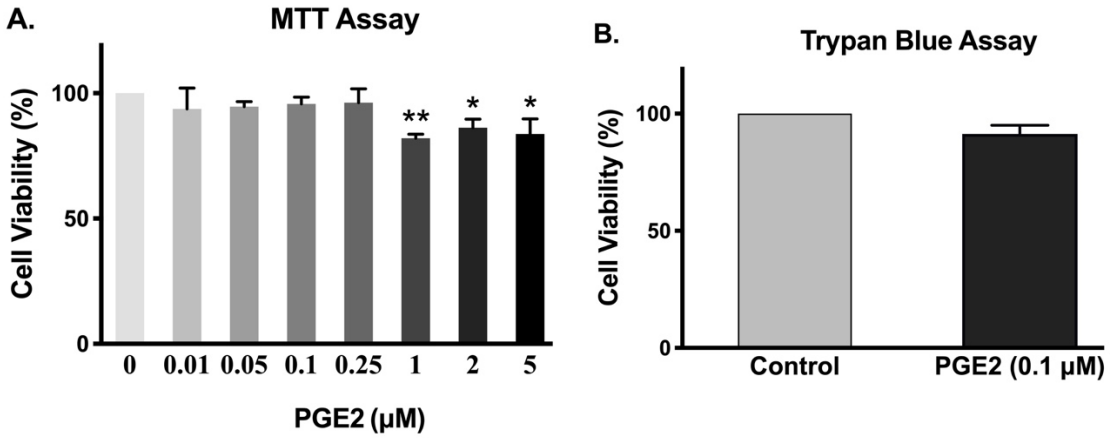


**Figure 30. Effect of PGE2 exposure for 24 hours on cell viability.** A. Measurement of cell viability by MTT assay**.** B. Measurement of cell viability by trypan blue assay. Values are represented as means ± SEM, n=6. *, $P < 0.05$, **, $P < 0.01$ vs 0 μM.

### 5.4.3. Prostaglandin receptor antagonist blocked PGE2 effect on Na-K-ATPase

The PGE2 receptor antagonist AH6809 was used to determine if the PGE2 effect is specifically mediated through its receptor in IEC-18 cells. AH6809 is an EP and DP receptor antagonist which blocks EP1, EP2, EP3, EP4 and DP1 receptors present in cells (Abramovitz et al., 2000). Previous studies from our laboratory have shown that EP2 and EP4 receptors are present in IEC-18 cells (data not shown). In this study, IEC-18 cells were pretreated with 5 μM of AH6809 for an hour followed by PGE2 treatment for 24 hours (two treatments 12 hours apart). As shown in Figure 31, PGE2 significantly inhibited Na-K-ATPase, however, pretreatment with AH6089 followed by PGE2 treatment prevented the inhibition produced by PGE2 (Control-1690±111.1, PGE2-792±81.1, AH6089-1645±115, AH6089+PGE2-1528±85.4 picomole/mg protein/min). This indicates that the inhibitory effect of PGE2 is mediated through its receptors EP2 and/or EP4 in IEC-18 cells.

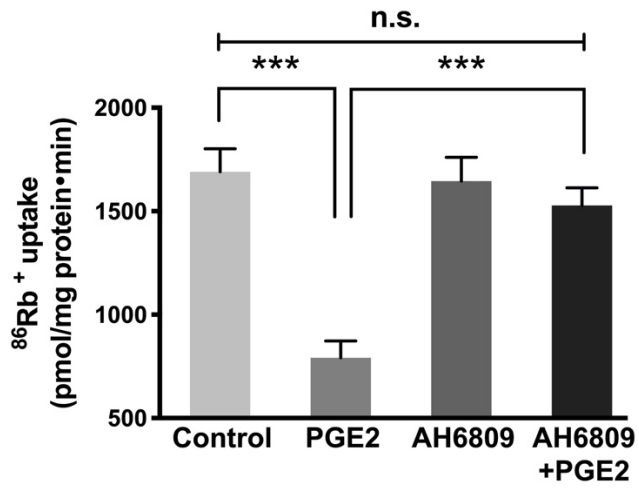


**Figure 31. Effect of PGE2 receptor inhibitor AH6809 (5 µM) on Na-K-ATPase activity in IEC-18 cells.** Measurement of Na-K-ATPase activity by $^{86}Rb^{+}$ uptake. Values are represented as means ± SEM, n=6. ***, $P < 0.001$; n.s., not significant.

### 5.4.4. Effect of PGE2 on intracellular cAMP

In many systems, PGE2 is known to mediate its action via cAMP. This second messenger is involved in various physiological and pathophysiological processes (Rahman, Buck, & Levin, 2013). Thus, measurement of cAMP was conducted. PGE2 treatment increased the cAMP levels in IEC-18 cells as shown in Figure 32. cAMP increased 2.4-fold (Control-11.4±2.3, PGE2-27.09±3.1 picomole/mg protein) within 2 minutes and peaked at 10 minutes (4.3-fold, Control-10.1±0.5, PGE2-43.2±3.3 picomole/mg protein) with PGE2. Levels reduced gradually at later time points (30 minutes, 3.3-fold; Control-11.4±2.9, PGE2-37.7±2.4, 60 minutes, 1.9-fold; Control-12.9±2.2, PGE2-25±2.8, 180 minutes, 1.8-fold; Control-12.5±4,6, PGE2-22.3±0.6 picomole/mg protein) but were still significantly higher than control. Thus, these data indicate that PGE2 increased cAMP levels in IEC-18.

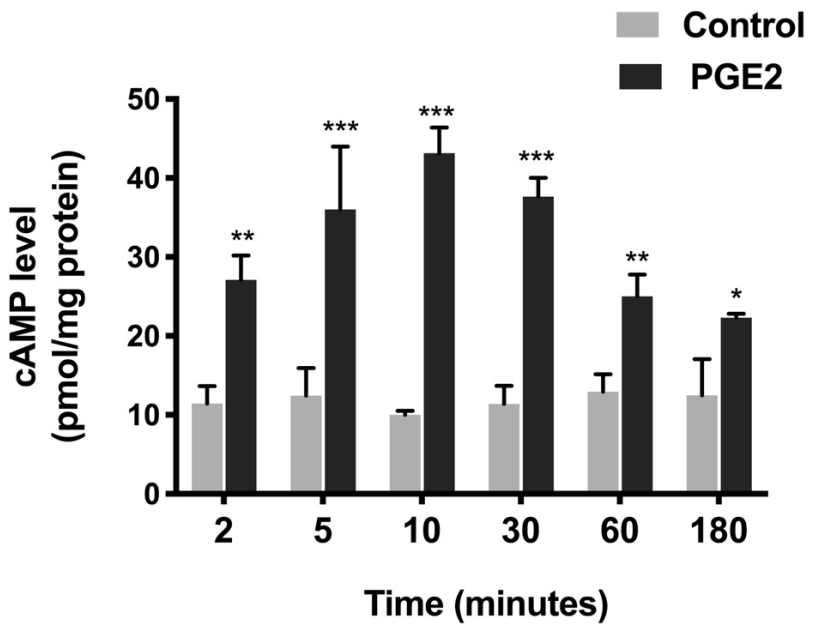


**Figure 32. Level of cAMP at different time points during PGE2 treatment.** Measurement of cAMP by cAMP Elisa kit. Values are represented as means ± SEM, n=8. *, P< 0.05, **, P< 0.01 and ***, P<0.001 vs Control. Control did not change significantly at different time points.

### 5.4.5. Effect of an analogue of cAMP on Na-K-ATPase activity in IEC-18 cells

To know whether increased cAMP is responsible for the reduction of Na-K-ATPase, we used an analog of cAMP: 8-Bromo-cAMP. IEC-18 cells were treated with 0.1 μM 8-Bromo-cAMP for 24 hours (two treatments 12 hours apart) followed by $^{86}Rb^{+}$ uptake. The Na-K-ATPase activity was found to be diminished with 8-bromo-cAMP treatment comparable to PGE2 (Figure 33, Control-1665±108.4, PGE2-780±79.6, 8-Bromo-cAMP-779.8±60.5 picomole/mg protein/min).

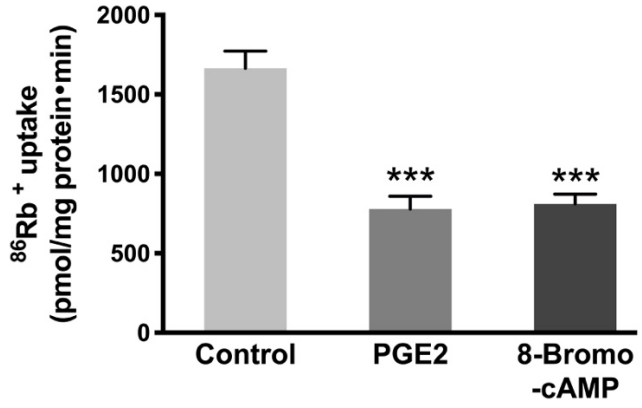


**Figure 33. Effect of cAMP analogue 8-Bromo-cAMP (0.1 µm) on Na-K-ATPase activity in IEC-18 cells.** Measurement of Na-K-ATPase activity by $^{86}Rb^{+}$ uptake. Values are represented as means ± SEM, n=6. ***, P < 0.001 vs Control.

### 5.4.6. Effect of PKA pathway inhibition on Na-K-ATPase activity in IEC-18 cells

It has been demonstrated that cAMP mediates its effects via protein kinase A (PKA) and subsequent phosphorylation of many proteins (Walsh & Van Patten, 1994). To see whether PKA plays an active role in the regulation of Na-K-ATPase by PGE2, cells were pretreated with 10 µM of the PKA inhibitor Rp-cAMP for an hour. After pretreatment, cells were treated with PGE2 for 24 hours (two treatments 12 hours apart) and cellular uptakes for $^{86}Rb^{+}$ were performed. Pretreatment with Rp-cAMP prevented the PGE2-mediated reduction of Na-K-ATPase (Control-1592±100.9, PGE2-746±75.3, Rp-cAMP-1596±67.3, Rp-cAMP+PGE2-1455± 113.5 picomole/mg protein/min; Figure 34).

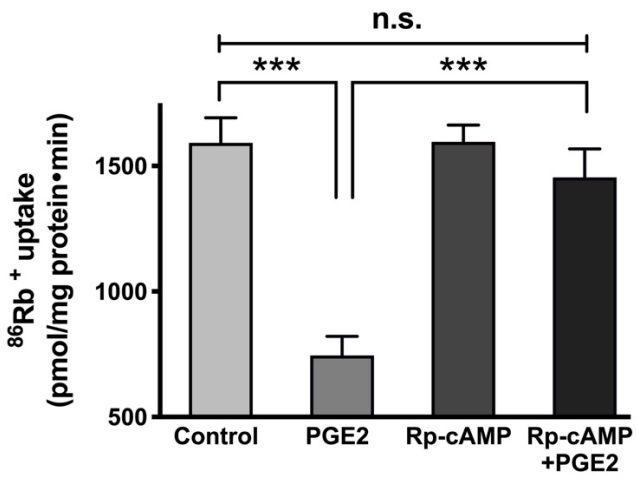


**Figure 34. Effect of PKA inhibitor Rp-cAMP (10 μM) on Na-K-ATPase activity in IEC-18 cells.** Measurement of Na-K-ATPase activity by $^{86}Rb^{+}$ uptake. Values are represented as means ± SEM, n=6. ***, P < 0.001; n.s., not significant.

### 5.4.7. Na-K-ATPase α1 and Na-K-ATPase β1 subunit mRNA abundance after PGE2 treatment

Gene and protein expression levels of Na-K-ATPase subunits, specifically α and β, are correlated with its functional activity. While the α subunit is accountable for Na-K-ATPase pumping activity, the β subunit does not contribute any pumping activity directly but instead supports α subunit by proper folding and transporting α subunit from the cytoplasm to the plasma membrane. Therefore, to determine whether changes in Na-K-ATPase activity mediated by PGE2 are transcriptionally regulated, we performed qRT-PCR analysis. Na-K-ATPase α1 subunit mRNA was significantly decreased when exposed to PGE2, while Rp-cAMP pretreatment prevented the reduction of Na-K-ATPase α1 subunit (Figure 35A, control-1.00X, PGE2-0.65X±0.04, Rp-cAMP-0.88X±0.13, PGE2+Rp-cAMP-0.93X±0.09). Similarly, there was a decrease in Na-K-ATPase β1subunit level when treated with PGE2, but the reduction was partially abolished when pretreated with Rp-cAMP (Figure 35B, control-1.00X, PGE2-0.55X±0.06, Rp-cAMP-1.01X±0.16, PGE2+Rp-cAMP-0.85X±0.05). These data indicate that

Na-K-ATPase α1 and β1 subunits were transcriptionally downregulated when treated with PGE2. Additionally, the PGE2-mediated decrease in Na-K-ATPase α1 and β1 subunit mRNA abundance was prevented with Rp-cAMP+ PGE2, indicating that the PKA pathway is responsible for PGE2-mediated regulation of Na-K-ATPase.

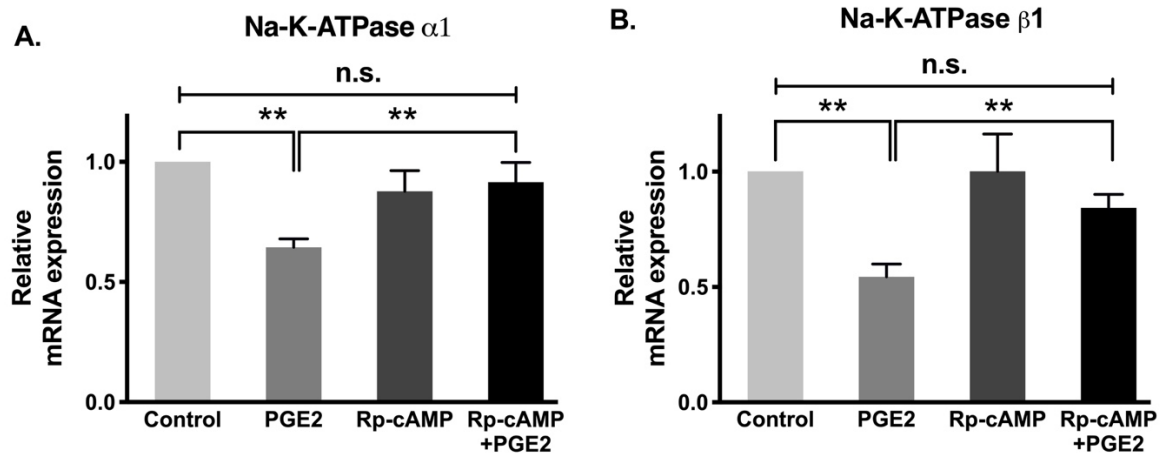


**Figure 35. qRT-PCR analysis of IEC-18 cells.** Values are relative to control and normalized to β-actin. **A.** Na-K-ATPase α1. **B.** Na-K-ATPase β1. Values are represented as mean ± SEM, n=4. *, $P < 0.05$, **, $P < 0.01$; n.s., not significant.

### 5.4.8. Na-K-ATPase α1 and Na-K-ATPase β1 subunit protein expression after PGE2 treatment

Protein levels of Na-K-ATPase α1 and Na-K-ATPase β1 subunits were determined by performing immunocytochemistry of cell monolayers and Western blots analysis on plasma membrane fractions for all experimental conditions. Immunocytochemistry (Figure 36) and Western blot analysis (Figure 37) showed that relative levels of Na-K-ATPase α1 protein expression were significantly lowered after PGE2 treatment, whereas this reduction was blocked when treated with Rp-cAMP+PGE2 (Figure 37A and B, control-1.00, PGE2-0.56±0.26, Rp-cAMP-1.02±0.19, PGE2+Rp-cAMP-0.93±0.21). Similarly, there was also a significant decrease in Na-K-ATPase β1 subunit protein expression in plasma membrane fractions when treated with

PGE2, and this was reversed when treated with Rp-cAMP+ PGE2 (Figure 37A and C, control-1.00, PGE2-0.73±0.1, Rp-cAMP-1.06±0.1, PGE2+Rp-cAMP-1.04±0.08). Therefore, the expression of Na-K-ATPase α1 and β1 subunits mRNA correlated with its protein expression in plasma membrane fractions. These data indicate that PGE2 regulates Na-K-ATPase transcriptionally through the cAMP-activated PKA pathway.

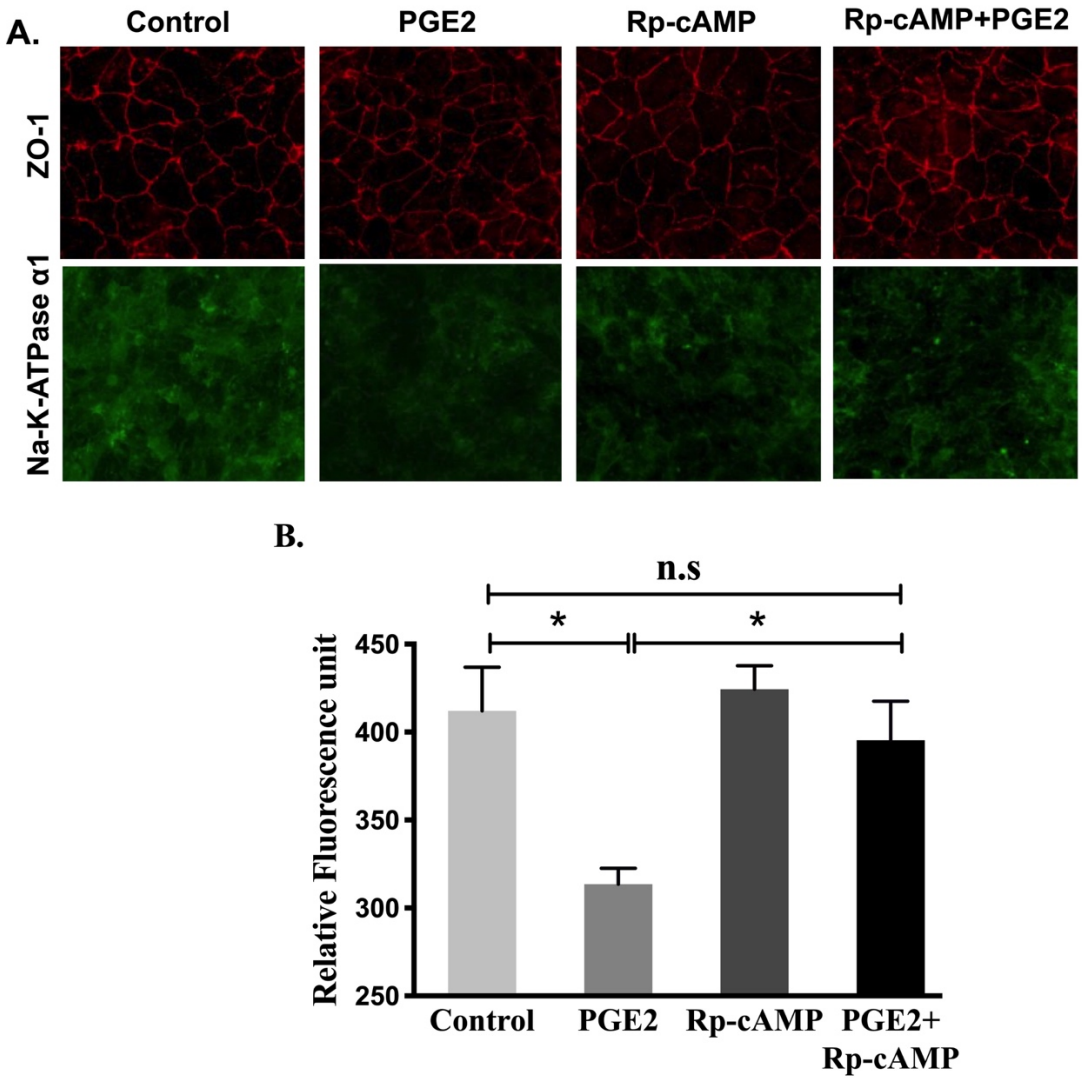


**Figure 36. Immunocytochemistry of IEC-18 cells treated with PGE2 and Rp-cAMP (PKA inhibitor). A.** Representative images of Na-K-ATPase α1(green), and ZO-1(red), (20X). **B.** Quantification of Na-K-ATPase α1 fluorescence. Values are represented as mean ± SEM, n=6. **, $P < 0.01$ vs Control.

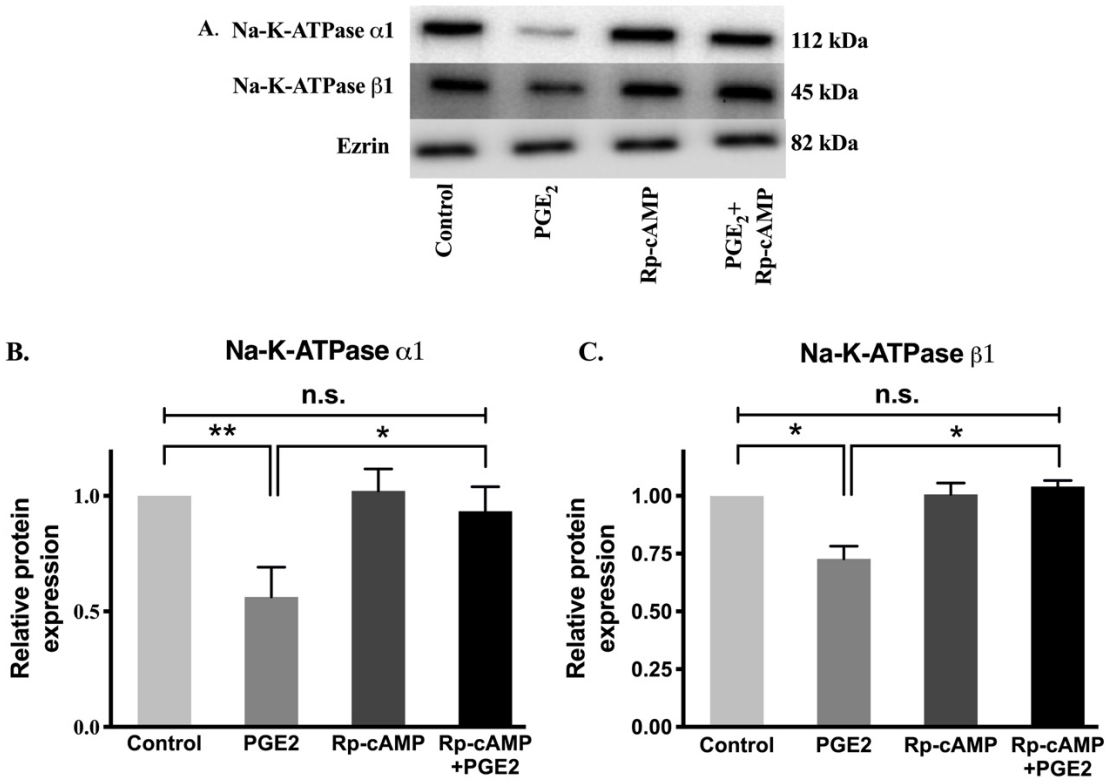


**Figure 37. Western blot analysis of IEC-18 cells treated with PGE2 and Rp-cAMP (PKA inhibitor). A.** Representative western blots of Na-K-ATPase α1, Na-K-ATPase β1 and Ezrin (internal control). Densitometric quantitation of blots, **B.** Na-K-ATPase α1 and **C.** Na-K-ATPase β1. Values are relative to control and normalized to Ezrin. Values are represented as mean ± SEM, n=4. *, $P < 0.05$, **, $P < 0.01$; n.s., not significant.

## 5.5. DISCUSSION

Prostaglandins play an essential role in maintaining normal physiological processes such as intestinal secretion, motility, mucosal protection (Mohajer & Ma, 2000). However, they have also been seen to be increased in the mucosa during chronic enteritis (Ligumsky et al., 1981; Sharon, Ligumsky, Rachmilewitz, & Zor, 1978). PGE2 has been extensively studied in the various process such as apoptosis (Huang et al., 2009), and inflammatory processes (Mohajer & Ma, 2000). Regarding intestinal absorption, PGE2 has been shown to decrease active sodium and chloride absorption and increase chloride secretion in *in vitro* studies (Ligumsky et al., 1981; Sharon et al., 1978). PGE2 has also been involved in the regulation of intestinal epithelial transporters including Na-glucose (SGLT1) (Hyun, Arai, Onaga, & Kato, 1997; Talukder et al., 2014), Na/H exchanger (Hodeify & Kreydiyyeh, 2007; Moeser, Nighot, Ryan, Wooten, & Blikslager, 2006), Na-K-ATPase (Markossian & Kreydiyyeh, 2005) and $Cl^-/HCO3^-$ (Fujii et al., 2016). Moreover, our laboratory has also shown that the cyclooxygenase pathway, which produces prostaglandins, has been involved in the reduction of Na-glutamine uptake (B0AT1) and Na-K-ATPase activity in villus cells in the inflamed intestine of rabbits (Arthur et al., 2012). Given this background, it is evident that PGE2 plays a crucial role in transporters involved in intestinal epithelial cell absorptive pathways, perhaps including Na-K-ATPase during chronic enteritis. However, the mechanism of regulation of Na-K-ATPase by PGE2 in intestinal epithelial cells during chronic intestinal inflammation is not known.

In the present study, we demonstrated the mechanisms of regulation of Na-K-ATPase by the inflammatory mediator PGE2 which in turn regulates several secondary active Na-dependent nutrient and electrolyte transporters. This work revealed that PGE2 reduces Na-K-ATPase activity through the PKA-mediated pathway in rat intestinal epithelial cells (IEC-18 cells), which

physiologically behave like absorptive villus cells when grown four days post-confluent (Saha et al., 2015).

PGE2 is a well-known regulator of Na-K-ATPase activity in numerous tissue types. A PGE2-mediated inhibitory effect has been seen in various organs including heart (Skayian & Kreydiyyeh, 2006), liver (Kreydiyyeh, Riman, Serhan, & Kassardjian, 2007; Seven, Turkozkan, & Cimen, 2005), and kidney (Cimen, Turkozkan, Seven, Unlu, & Karasu, 2004). Similar to other findings, our data also demonstrates that 0.1 μM of PGE2 for 24 hours is optimal for reduction of Na-K-ATPase in IEC-18 cells. Our qRT-PCR data showed that PGE2 transcriptionally reduces Na-K-ATPase α1 and β1, which led to a decrease in plasma membrane protein expression of both subunits as demonstrated by western blot analysis.

Previously, our laboratory has shown that IEC-18 cells have PGE2 receptors (EP1, EP2, EP3, and EP4). The specific inhibitor AH6809 is an EP and DP receptor antagonist and inhibits these receptors with equal affinity (Abramovitz et al., 2000). When IEC-18 cells were pretreated with AH6809, the reduction of Na-K-ATPase due to PGE2 treatment was prevented. These data demonstrate that the decrease of Na-K-ATPase activity is mediated through PGE2 receptors. These receptors mediate their action through secondary messengers such as $Ca2^{+}$ or cAMP (Funk et al., 1993; Woodward, Pepperl, Burkey, & Regan, 1995). We observed that PGE2 increases the intracellular cAMP levels. Therefore, to demonstrate the role of cAMP in the reduction of Na-K-ATPase, we treated cells with a cAMP analog (8-Bromo-cAMP). There was a similar effect produced by 8-Bromo-cAMP comparable to PGE2.

PGE2 has been shown to activate signaling pathways through PKA or PKC, downstream of the second messenger cAMP or $Ca2^{+}$ in various cell types (Sachs, Villarreal, Cunha, Parada, & Ferreira, 2009; Sawasdikosol, Pyarajan, Alzabin, Matejovic, & Burakoff, 2007). PKA has

been shown to have differential regulation (activation, inhibition, or no effect) of Na-K-ATPase based on the tissue types. The discrepancy in the regulation of Na-K-ATPase by PKA might be due to phosphorylation of different isoforms of Na-K-ATPase subunits (Blanco et al., 1998). Oliveira *et al.* reported that short-term treatment of PGE2 has a PKA-mediated inhibitory effect on Na-K-ATPase hippocampal tissue likely due to phosphorylation of the Ser943 residue of the α subunit (Oliveira et al., 2009). On the other hand, cAMP-mediated PKA has also been shown to regulate Na-K-ATPase transcriptionally. Dagenais *et al.* reported that activation of PKA by a cAMP analog subsequently phosphorylates transcription factor CREB and eventually increased the synthesis of mRNA of the α1 subunit but not the β1subunit after 8 hours treatment in isolated rat alveolar epithelial cells (Dagenais et al., 2001). However, there was no significant change in α1 mRNA level after 24 hours of treatment. Additionally, three prostaglandin response elements (PGRE) have been identified in the β1subunit, which in response to PGE1 (4 hours treatment) increased the transcription of the β1subunit by activating transcription factors CRE and CREB (Matlhagela et al., 2005; Matlhagela & Taub, 2006a, 2006b). Contrary to this finding, in our study, we found that a cAMP-mediated PKA is responsible for inhibition of Na-K-ATPase. The discrepancy in the results may be due to the different tissues of origin that we used, the concentration of the PGE2, and/or the exposure time. Another possible reason for this discrepancy might be the observed effect might be an early response.

However, in a study by Mony *et.al,* in the hypoxic condition in cancer, transcription factors hypoxia inducing factor (HIF-1α) and Smad3 bind to the β1 subunit and likely repress the expression of beta-subunit (Mony et al., 2013). Therefore, the prolonged treatment with PGE2 might result in transcription repression of β1 subunit by transcription factors like (HIF-1α) and Smad3.

## 5.6. CONCLUSION

The current study indicates that the mechanism by which PGE2 reduces Na-K-ATPase activity is via the cAMP-mediated PKA pathway, as shown in Figure 38. The mechanism involves binding of PGE2 to prostaglandin receptors, leading to subsequent activation of the PKA pathway through increased intracellular cAMP which eventually leads to a decrease in Na-K-ATPase activity. The reduction in activity is most likely due to transcriptional repression of genes encoding various subunits of Na-K-ATPase.

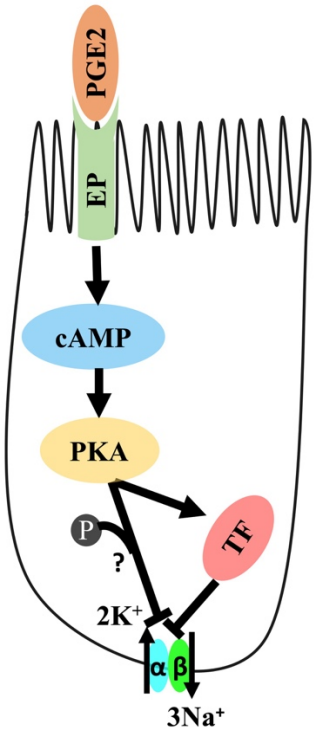


**Figure 38. Proposed mechanism of PGE2-mediated regulation of Na-K-ATPase in intestinal epithelial cells.** PGE2-Prostaglandin-E2, EP-Prostaglandin receptor, PKA-Protein Kinase A, TF-Transcription Factor.

## 5.7. SUPPLEMENTARY FIGURES

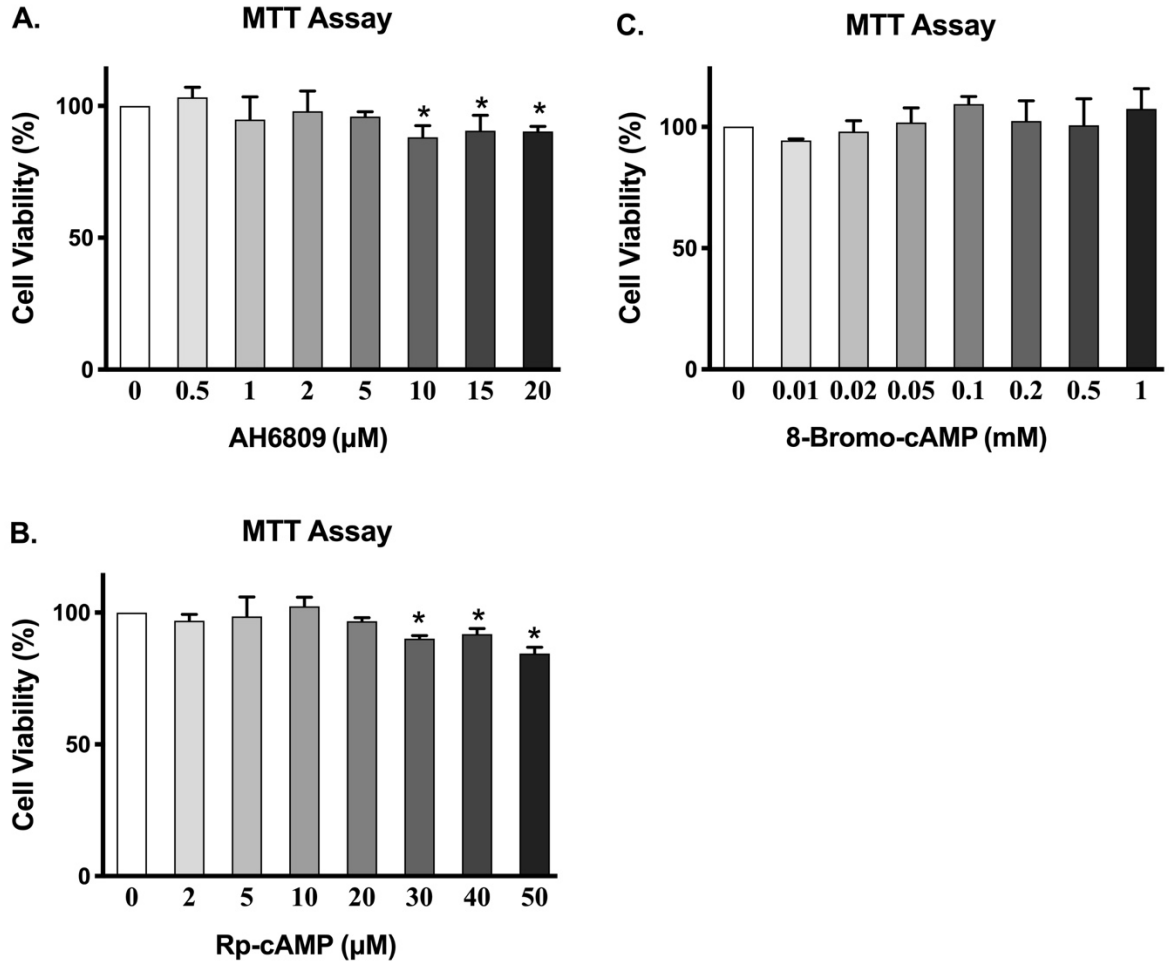


**Figure 39. Effect of different concentration of reagents (drugs) exposure for 24 hours on cell viability.** A. AH6809. B. Rp-cAMP. C. 8-Bromo-cAMP. Measurement of cell Viability by MTT assay. Values are relative to 0 μM and are represented as means ± SEM, n=6. *, $P < 0.05$ vs 0 μM.

## ACKNOWLEDGEMENT

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## CONFLICTS OF INTEREST

The authors have declared that no conflicts of interest exist.

# CHAPTER 6: DISCUSSION AND FUTURE DIRECTIONS

## 6.1. DISCUSSION

The incidence of IBD has been seen to be increasing in the Western world since the 1950s (Lakatos, 2006). Various factors contribute to the pathogenesis of IBD including genetic heterogeneity, intestinal microbiome, and several environmental factors, thus generating complexity in the prognosis of the disease (K. L. Wallace, Zheng, Kanazawa, & Shih, 2014). Individuals suffering from IBD, and specifically with Crohn's disease, are likely to have nutritional deficiency because of chronic diarrhea leading to malabsorption of electrolytes and nutrients (Massironi et al., 2013). Additionally, increased energy expenditure and decreased nutrient uptake during IBD are known to further worsen the disease condition. Among various factors that cause diarrhea in IBD, the effects of inflammation on muscular tone and intestinal transport remain the prominent ones (Wenzl, 2012). The most common treatment to overcome chronic inflammation and nutrient loss during diarrhea to date are enteral nutrition or steroid therapy. However, steroids are the first line of treatment in adults with enteral nutrition as supportive therapy, whereas enteral nutrition remains the preferred treatment in children (Massironi et al., 2013). Though steroids improve or put the disease in remission, they have been reported to have numerous side effects causing additional discomfort to the patients. Therefore, it becomes crucial to identify the immune components that might interfere with the absorption of specific nutrients in the intestine so that drugs can be developed against them to replace steroids for IBD treatment.

A comprehensive understanding of nutrient and electrolyte transport during IBD is of utmost relevance to developing better treatments of IBD. Over the past two decades, there have been extensive studies on nutrient transporters in a rabbit model of chronic intestinal

inflammation resembling IBD (Coon et al., 2010; Saha et al., 2012; Sundaram, Wisel, & Fromkes, 1998a; Sundaram et al., 1997). A series of studies done in this *in vivo* rabbit model of IBD have reported that various nutrient transporters, namely Na-glucose (SGLT1), Na-amino acid [-alanine (ATB0), -glutamine (B0AT1)], Na-bile acid (ASBT), and Na-adenosine cotransport are significantly reduced in villus cells (Coon et al., 2010; Saha et al., 2012; Sundaram et al., 1998a; Sundaram et al., 1997). All these studies indicated that along with decreased nutrient absorption, there was also a decrease in sodium absorption, since all the above indicated cotransporters of different nutrients are sodium-dependent. Additionally, in this animal model of IBD, Na-K-ATPase activity was also significantly decreased in villus cells, indicating decreased transcellular sodium transport, which may be partially responsible for the decreased nutrient at the brush border membrane. On the contrary, it has also been reported that in crypt cells there is increased glutamine absorption mediated by the Na-glutamine cotransporter (SN2) along with increased Na-K-ATPase activity during chronic enteritis in the rabbit model (Saha et al., 2012). The discrepancy in glutamine absorption between villus and crypt cells during inflammation of the intestine might be due to villus atrophy and crypt hypertrophy, and the crypt cells trying to compensate for the loss of glutamine absorption by the villus cells (Saha et al., 2012). However, this increased glutamine absorption in crypts did not compensate for the loss of villus cell glutamine absorption, resulting in a net decrease in glutamine absorption across the villus-crypt axis.

A net decrease in Na and water absorption in the intestine rather than increased anion secretion resulting in watery stool, has also been reported by other studies in IBD (Hawker, McKay, & Turnberg, 1980; Sandle, 2005). The net reduction of Na absorption is likely due to decreased Na-K-ATPase activity (and expression) observed in IBD samples and IBD models

(Tables 3, 4, and 5). The outcome of decreased Na-K-ATPase activity may result in an elevation of intracellular Na level, thus reducing the Na and Na-dependent nutrient absorption capacity of the enterocytes.

Given this background, it becomes important to understand the intracellular signaling and molecular mechanisms responsible for the alteration of sodium absorptive processes in the intestine during IBD. However, *in vivo* systems do not facilitate effective manipulation and dissection of specific cellular processes and protein targets due to their physiological complexity. Therefore, there was a need to develop an *in vitro* model, which could behave like either crypt or villus cells under defined culture conditions. To date, the majority of investigations related to absorptive and inflammatory mechanisms were done in colon cancer cell lines (Caco-2 or T-84), which do not represent the small intestinal enterocytes well (Table 3). Additionally, the complexity generated by the effects of oncogenesis on these cell lines makes them less ideal for studying effects mediated by immune-inflammatory mediators. However, a rat small intestine-derived normal intestinal epithelial cell line called IEC-18 has been successfully used as an *in vitro* model to study enterocytes at the level of either immature crypt-like cells and mature villus-like cells.

Based on this background, the objective of the current study was to dissect the specific molecular mechanisms responsible for the modulation of sodium-dependent nutrient transporters and Na-K-ATPase by specific immune inflammatory mediators in villus and crypt cells. To accomplish this objective, this study was divided into different sub-projects. The first study was to characterize the in *vitro* model (IEC-18) so it could be used as a cell model comparable to both crypt and villus enterocytes. Once establishing IEC-18 as an appropriate *in vitro* system to study

both crypt and villus cells, we then studied the effect of specific immune inflammatory mediators on Na-K-ATPase activity. Our findings are summarized below.

### 6.1.1. Maturation of IEC-18 cells

During the maturation of intestinal absorptive cells from crypt to villus, significant morphological and physiological changes occur. Morphological changes include changes in membrane composition and surface area, so that physiologically mature cells acquire more transporters and therefore increased capacity to absorb nutrients. A study of the crypt-villus axis of piglets has shown that there is upregulation of proteins associated with glycolysis/gluconeogenesis, fatty acid metabolism, amino acid metabolism, and citrate cycle as cells mature from crypt to villus (Yang et al., 2016). Additionally, it has been reported that ALP, aminopeptidase, sucrose, lactase and Na-K-ATPase activities increased along the crypt-villus axis in jejunum and ileum of neonatal pigs (Fan et al., 2001). Similar to these results, our study has shown that there is an increase in ALP and SGLT1 activities as IEC-18 cells mature from crypt-like to villus-like in culture.

### 6.1.2. Regulation of Na-K-ATPase during intestinal cell maturation

There are contradictory reports in the literature addressing the levels of Na-K-ATPase activity during the maturation of intestinal epithelial cells (Blostein et al., 1983; Brugnara & de Franceschi, 1993; Furukawa et al., 1981). The mechanism involved in the reduction of activity was found to be due to the shedding of Na-K-ATPase into exosomes or due to degradation by energy-dependent mechanisms (Mairbaurl et al., 2000). Moreover, studies from our lab have demonstrated that the activity of Na-K-ATPase is elevated in villus cells compared to the crypt cells (Saha et al., 2012). In these studies, Na-K-ATPase activity varied depending upon the physiological nutritional demand of the intestinal epithelial cells during the maturation process.

Supporting this notion, the present study has shown that there is an increase in Na-K-ATPase activity as IEC-18 cells mature from crypt-like to villus-like.

While intestinal epithelial cells mature from crypt to villus, Na-K-ATPase activity also increases to support the increasing absorptive capacity of the enterocytes. The results of this study showed that the increased Na-K-ATPase activity in villus-like cells compared to crypt-like cells is likely due to the change in the affinity of the transporter rather than an elevated expression of Na-K-ATPase. Moreover, the differences in affinity are due to changes in phosphorylation of the α1 subunit of Na-K-ATPase, specifically at serine and tyrosine residues.

### 6.1.3. Regulation of Na-K-ATPase during induced inflammatory condition (LTD4 treatment) in crypt-like cells

Various inflammatory mediators such as prostaglandins, leukotrienes, chemokines, interleukins, and/or reactive nitrogen and oxygen species are known to modulate intestinal nutrient and electrolyte transporters (Sartor & Powell, 1991). The mechanisms of modulation by these factors are unique to the transporters during chronic intestinal inflammation (Manokas et al., 2000; Sundaram & West, 1997; Sundaram et al., 2005; Sundaram et al., 1998b; Sundaram et al., 1997). Previously, our laboratory has shown that the LOX pathway, which produces leukotrienes, is involved in the stimulation of Na-glutamine transporter (SN2) and Na-K-ATPase activity in crypt cells in the inflamed intestine of rabbits (S. Singh et al., 2018). In agreement with this study, the present study also demonstrates that there is increased Na-K-ATPase activity during LTD4 exposure to crypt-like cells. Subsequently, the LTD4-mediated stimulation of Na-K-ATPase in intestinal epithelial cells is due to Ca-mediated activation of the PKC (Figure 40), which in turn may activate different transcription factors upregulating the expression of the Na-K-ATPase α1 and β1 subunits.

### 6.1.4. Regulation of Na-K-ATPase during induced inflammatory condition (PGE2 treatment) in villus-like cells

PGE2-mediated inhibitory effect on transporter has been seen in various organs including the heart (Skayian & Kreydiyyeh, 2006), liver (Kreydiyyeh et al., 2007; Seven et al., 2005), and kidney (Cimen et al., 2004). Additionally, our laboratory has also shown that the cyclooxygenase pathway, which produces various prostaglandins, has been involved in the reduction of Na-glutamine (B0AT1) and Na-K-ATPase activity in villus cells in the inflamed intestine of rabbits (Arthur et al., 2018). Similar to these findings, the present study also demonstrates that PGE2 reduces the activity of Na-K-ATPase in villus-like cells *in vitro*. Moreover, the mechanism of inhibition of Na-K-ATPase in intestinal epithelial cells is due to cAMP-mediated activation of the PKA pathway (Figure 40), which in turn may be modulating the transcription factors downregulating the expression of the Na-K-ATPase α1 and β1 subunits.

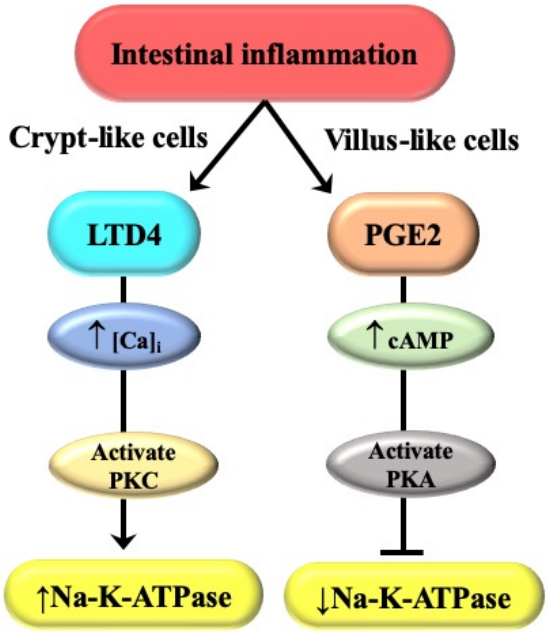


**Figure 40. Schematic representation of mechanisms of alteration of Na-K-ATPase activity by inflammatory mediators (LTD4 and PGE2) during intestinal inflammation.**

## 6.2. FUTURE DIRECTIONS

### 6.2.1. Regulation of Na-K-ATPase during maturation of intestinal cells

The present study provides the potential regulation of Na-K-ATPase during the maturation of intestinal cells. While the study is novel, the limitation of this study is that the model is *in vitro,* and further studies need to be done *ex vivo* or *in vivo* to support the current findings. For example, we can sequentially isolate cells from the immature crypt base to the mature villus tips in rabbit or rat intestine and determine if comparable changes occur in vivo as it pertains to Na-K-ATPase. In addition to investigating the molecular mechanisms involved in the altered activity of Na-K-ATPase during the maturation process, further studies need to be done to decipher external cellular factors that bring about these alterations. As was demonstrated, PGE2 and LTD4 have very different effects on Na-K-ATPase in crypt-like and villus-like states of IEC-18 cells. Thus, such external mediators may regulate Na-K-ATPase during maturation.

#### 6.2.1.1. Locating the phosphorylation residues involved in modulation of Na-K-ATPase during maturation

Our data revealed that there is alteration of phosphorylation levels of serine and tyrosine residues in the Na-K-ATPase α1 subunit during the maturation of intestinal epithelial cells from crypt to villus. There are several phosphorylation targets in Na-K-ATPase α1, which are known to modulate the activity of the enzyme depending on the tissue type and experimental conditions (Beguin et al., 1994; Chibalin et al., 1992). Additionally, we observed in this study that Na-K-ATPase α1-$Ser^{23}$ phosphorylation increases as cells mature, along with increases in Na-K-ATPase activity. Besides $Ser^{23}$, there are phosphorylation sites at $Tyr^{5}$ and $Ser^{943}$ in the α1 subunit that are also known to modulate the activity of Na-K-ATPase (Beguin et al., 1994; Efendiev et al., 2000). Thus, further studies with point mutations of potential serine and tyrosine

phosphorylation sites will help to decipher and understand the mechanisms of regulation of Na-K-ATPase during maturation.

#### 6.2.1.2. Finding the role of FXYD subunit

There are 12 different members of the FXYD protein family that are known to be expressed differentially in cells and tissues (J. Y. Cheung et al., 2010). A specific domain at the N terminus of FXYD is known to interact with Na-K-ATPase thereby regulating the activity of Na-K-ATPase, whereas the C terminus consists of phosphorylation sites that could be potential targets of activation by kinases (Dey et al., 2013). In the present study, we did not explore the role of FXYD in the regulation of Na-K-ATPase. Therefore, future studies should be conducted to explore the potential role of FXYD in the modulation of Na-K-ATPase activity during the maturation of intestinal epithelial cells.

### 6.2.2. Regulation of Na-K-ATPase by inflammatory mediators (LTD4 and PGE2 treatment)

In addition to investigating the mechanism of how inflammatory mediators LTD4 and PGE2 works in crypt and villus, we also treated crypt cells with PGE2 and villus cells with LTD4 (Figure 41). We observed that PGE2 and LTD4 did not have any effect on Na-K-ATPase activities in crypt and villus cells, respectively. However, the question remains on the fact that the epithelial cells lose their sensitivity to LTD4 treatment and acquire PGE2 sensitivity. To explore this interesting phenomenon, further studies need to be conducted to investigate the LTD4 and PGE2 receptor development/transition during the maturation of intestinal epithelial cells. Changes subsequent to agonist-receptor interaction, specifically at the G protein level is another possibility which needs to be explored as well.

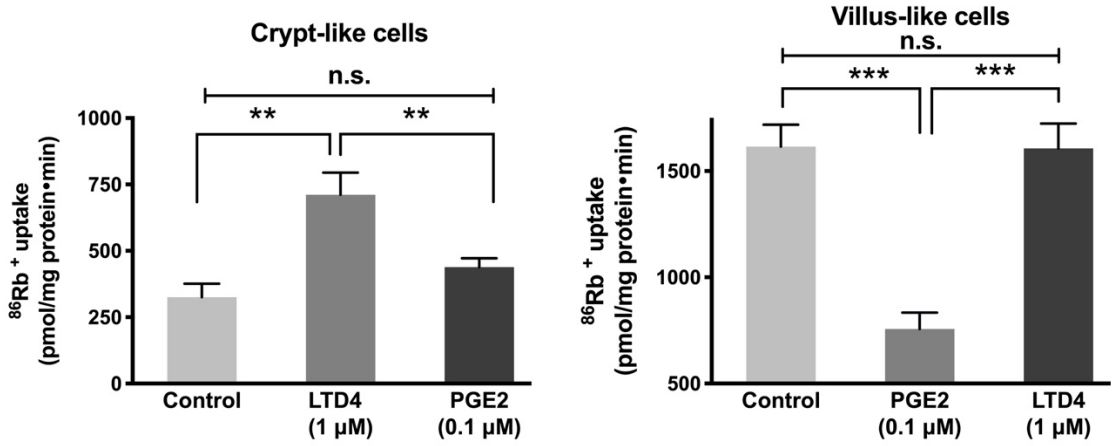


**Figure 41. Effect of LTD4 and PGE2 exposure for 24 hours on Na-K-ATPase activity in IEC-18 cells.** A. Crypt-like cells (0-day confluence). B. Villus-like cells (4-day confluence). Measurement of Na-K-ATPase activity by $^{86}Rb^{+}$ uptake. Values are represented as means ± SEM, n=6. **, P < 0.01 Vs Control, ***, P < 0.001 Vs Control.

#### 6.2.2.1. Exploring the role of different PKC isoforms during LTD4-mediated stimulation of Na-K-ATPase

Based on the external stimulus, PKC has been involved in reduction or activation of Na-K-ATPase activity depending upon species and tissues (Borghini et al., 1994; Feschenko & Sweadner, 1995). Moreover, the stimulation of various isoforms of PKC can have a differential effect on Na-K-ATPase. Conventional PKC α and β are associated with decrease in activity of Na-K-ATPase by endocytosis (Chibalin, Pedemonte, et al., 1998; Feraille & Doucet, 2001), whereas other isoforms of PKC, ε and δ have been shown to stimulate Na-K-ATPase activity through the ERK1/2 pathway (Galuska et al., 2011; Zhong et al., 2005). In the present study, we used calphostin-c, a non-isoform specific PKC inhibitor. Therefore, using specific isoform inhibitors will further dissect the mechanism of LTD4-mediated stimulation in intestinal epithelial cells.

#### 6.2.2.2. Exploring the role of transcriptional factors during LTD4-mediated stimulation of Na-K-ATPase

We observed the transcriptional activation of the Na-K-ATPase α1 and β1 subunits during LTD4 treatment in crypt-like cells. Transcription factors such as Sp1 and Sp3 are known to increase the transcription of the α1 and β1 subunits by binding to the promoter region of these subunit genes (Johar et al., 2012; Kobayashi et al., 1997). Similarly, phosphorylation of transcription factor CREB is known to induce the increased α1 mRNA level (Dagenais et al., 2001). Also, the binding of ZEB1 (AREB6) to the promoter region of the α1 subunit gene increases the protein level of the α1 subunit in skeletal muscle (Galuska et al., 2011). Therefore, future studies observing the role of these transcription factors might be helpful in understanding the transcriptional modulation of LTD4-mediated stimulation of Na-K-ATPase in intestinal epithelial cells.

#### 6.2.2.3. Exploring the role of transcriptional factors during PGE2-mediated reduction of Na-K-ATPase

In the present study, we observed the transcriptional repression of the Na-K-ATPase α1 and β1 subunits during PGE2 treatment in villus-like cells. It has been reported that TGF-β2 reduces the mRNA level of the β1 subunit through transcription factors HIF and Smad3 (Mony et al., 2013). Therefore, these transcription factors might play a potential role in the PGE2-mediated reduction of Na-K-ATPase activity and hence need to be studied. However, there is no report suggesting a potential transcription repressor for the α1 subunit; therefore, further studies need to be done to explore the involvement of potential transcriptional repressors of the α1 subunit.

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# APPENDIX A: INSTITUTIONAL REVIEW BOARD APPROVAL

MARSHALL UNIVERSITY.
www.marshall.edu

Office of Research Integrity

April 15, 2019

Niraj Nepal
1416 7th Ave, Apt #11
Huntington, WV 25701

Dear Niraj:

This letter is in response to the submitted dissertation abstract entitled *"Regulation of Na-K-ATPase by Arachidonic Acid Metabolites in Intestinal Epithelial Cells."* After assessing the abstract it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Code of Federal Regulations (45CFR46) has set forth the criteria utilized in making this determination. Since the study does not involve human subjects as defined in DHHS regulation 45 CFR §46.102(f) it is not considered human subject research. If there are any changes to the abstract you provided then you would need to resubmit that information to the Office of Research Integrity for review and determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

Bruce F. Day, ThD, CIP
Director

## APPENDIX B: ABBREVIATIONS

| | |
|---|---|
| AA | Arachidonic acid |
| AAMs | Arachidonic acid metabolites |
| ADP | Adenosine Diphosphate |
| ALP | Alkaline phosphatase activity |
| ANOVA | Analysis of Variance |
| ASBT | Apical sodium bile acid transporter |
| ATF | Activating transcription factor |
| ATK | Arachidonyl Trifluoromethyl Ketone |
| ATP | Adenosine Triphosphate |
| B0AT1 | System B0 amino acid transporter 1 |
| BBM | Brush border membrane |
| BLM | Basolateral membrane |
| BSA | Bovine serum albumin |
| C-terminal | COOH-terminal |
| $Ca^{2+}$ | Calcium ion |
| $CaCl_2$ | Calcium chloride |
| Cal-c | Calphostin-C |
| cAMP | Cyclic adenosine monophosphate |
| CD | Crohn's Disease |
| cDNA | Complementary Deoxyribonucleic acid |
| CFTR | Cystic fibrosis transmembrane conductance regulator |
| cGMP | Cyclic guanosine monophosphate |

| | |
|---|---|
| $Cl^-$ | Chloride ion |
| COX | Cyclooxygenase |
| CRE | cAMP response element |
| CREB | cAMP response element binding protein |
| CTF | Cardiotonic factor |
| CysLT | Cysteinyl leukotriene receptors |
| DMEM | Dulbecco's modified eagle media |
| DMSO | Dimethyl sulfoxide |
| DMT1 | Divalent metal transporter 1 |
| ECL | Enhanced chemiluminescence |
| EGFR | Epidermal growth factor receptor |
| ERK | Extracellular signal-regulated kinases |
| GI | Gastrointestinal |
| GPCR | G-protein-coupled receptors |
| GPI | Glycosylphosphatidylinositol |
| GRE | Glucocorticoid response element |
| $H^+$ | Hydrogen ion |
| HCl | Hydrochloric acid |
| $HCO_3^-$ | Bicarbonate ion |
| HEPES | 4-(2-hydroxyethyl-1-piperazineethanesulfonic acid) |
| HETEs | Hydroxy eicosatetraenoic acids |
| HIF | Hypoxic inducing factors |
| HPETEs | Hydroperoxyeicosateraenoic acids |

| | |
|---|---|
| HRE | Hypoxia response element |
| HRP | Horse radish peroxide |
| IBD | Inflammatory Bowel Disease |
| ICC | Immunocytochemistry |
| IEC | Intestinal epithelial cells |
| IFN-γ | Interferon gamma |
| IHC | Immunohistochemistry |
| IL | Interleukin |
| IL-1b | Interleukin-1b |
| IP | Immunoprecipitation |
| IP3R | Inositol triphosphate receptor |
| *Isc* | Short-circuit current |
| $K^+$ | Potassium ion |
| KCl | Potassium chloride |
| kDa | Kilo Dalton |
| $K_m$ | Michaelis-Menten constant |
| LOX | Lipoxygenase |
| LT | Leukotriene |
| LTB4 | Leukotriene B4 |
| LTC4 | Leukotriene C4 |
| LTD4 | Leukotriene D4 |
| LTE4 | Leukotriene E4 |
| LTR | Leukotriene receptor |

| | |
|---|---|
| LXA4 | Lipoxins A4 |
| LXB4 | Lipoxins B4 |
| M | Molar |
| MAPK | Microtubule associated protein kinase |
| $MgCl_2$ | Magnesium chloride |
| $MgSO_4$ | Magnesium sulphate |
| mM | Millimolar |
| MMC | Migrating motor complex |
| MRE | Mineralocorticoid response element |
| mRNA | Messenger Ribonucleic acid |
| MTT | 3-(4,5-dimethylthaizol-2-yl)-2,5-diphenlytetrazolium bromide |
| N-terminal | $NH_2$-terminal |
| N/A | Not available |
| Na-K-ATPase | Sodium Potassium Adenosine Triphosphate |
| $Na^+$ | Sodium ion |
| NaCl | Sodium chloride |
| NAcT | Sodium alanine co-transporter |
| $NaHCO_3$ | Sodium bicarbonate |
| NaOH | Sodium hydroxide |
| NB | Northern blotting |
| NHE | Na-Hydrogen exchanger |
| NSAID | Nonsteroidal inflammatory drugs |
| OD | Optical Density |

| | |
|---|---|
| OMG | O-Methyl Glucose |
| PBS | Phosphate buffer saline |
| PG | Prostaglandin |
| PGD2 | Prostaglandin D2 |
| PGE2 | Prostaglandin E2 |
| PGF2 | Prostaglandin F2 |
| PGG2 | Prostaglandin G2 |
| PGH2 | Prostaglandin H2 |
| PGI2 | Prostacyclin I2 |
| PGRE | Prostaglandin response element |
| P*i* | Inorganic phosphate |
| PI3K | Phosphoinositide 3-kinase |
| PKA | Protein kinase A |
| PKC | Protein kinase C |
| PKG | Protein kinase G |
| PLA2 | Phospholipase $A_2$ |
| PLC | Phospholipase C |
| PMA | Phorbol 12-myristate 13-acetate |
| $PO_4^{2-}$ | Phosphate ion |
| PRE | Progesterone element |
| PRX | Piroxicam |
| PTK | Protein tyrosine kinase |
| PTP | Protein tyrosine phosphatase |

| | |
|---|---|
| qRT-PCR | Quantitive real time -Polymerase chain reaction |
| $RB^+$ | Rubidium ion |
| RbCl | Rubidium chloride |
| ROS | Reactive oxygen species |
| Rp-cAMP | Rp-Cyclic adenosine monophosphate |
| SBD | Smad binding domain |
| SDS | Sodium dodecyl sulphate |
| SEM | Standard error of mean |
| Ser | Serine |
| SFM | Serum free medium |
| SGLT1 | Sodium-Glucose Transporter 1 |
| SN2 | System N transporter 2 |
| $SO_4^{2-}$ | Sulphate ion |
| Sp | Specificity protein |
| Src | Sarcoma |
| STAT | Signal transducer and activator of transcription |
| TBS | Tris buffer saline |
| TBST | Tris buffer saline tween20 |
| TF | Transcription Factor |
| TJ | Tight junction |
| TNF-a | Tumor necrosis factor-a |
| TRE | Thyroid response elements |
| TXA2 | Thromboxane A2 |

| | |
|---|---|
| TXB2 | Thromboxane B2 |
| Tyr | Tyrosine |
| UC | Ulcerative colitis |
| *Vmax* | Maximum rate of reaction |
| WB | Western blot |
| WB-PM | Western blot in plasma membrane |
| WB-WC | Western blot in Whole cell |
| ZEB | Zinc finger E-Box Binding Homeobox 1 |
| ZO | Zonula occludens |
| µM | Micromolar |
| ↑ | Up-regulation in in activity and expression |
| ↓ | Down regulation in activity and expression |
| ⇔ | No change |

## APPENDIX C: CURRICULUM VITAE

**NIRAJ NEPAL**

1416 7th Ave, #11
Huntington, 25701-WV
Email: nepal@marshall.edu
TEL: 304-541-8043

# EDUCATION

**Phd. Biomedical Science**, Marshall University, Huntington WV (May 2019).
**Master of Science**, West Virginia State University (May 2012).
Major: Biotechnology
**B-Tech: Biotechnology**, Kathmandu University, Nepal (July 2007).

# HONORS AND AWARDS

| | |
|---|---|
| Kathmandu University Merit Scholar | Kathmandu University, 2003-2007 |
| Dean's Honor | Spring 2004- Fall2007 |
| Research Assistant from Guss R Douglass, Land Grant Institute | Jan 2010-Aug-2010. |
| Research Assistant from NSF grant for Thesis research | Sept 2010- 2012. |
| Graduate Research assistant | July2013-May 2019 |

**Research Skills**

**Techniques:**
Cell culture and viability analysis; Cell proliferation assay; Immunofluorescence; Quantitative PCR and data analysis, Immunohistochemistry; Soft agar assay; Invasion assay; Transfection; Virus preparation; Transduction; Protein purification and quantification; Extraction of DNA/RNA/Protein from animal tissues; Agarose gel electrophoresis; Western blot; Bacterial culture and analysis; Fluorescent Insitu hybridization; Invitro Fertilization; Elisa, Cloning and sequencing, Microarray and data analysis; Ion Exchange Chromatography; Thin-layer Chromatography; Fractional distillation; UV-Vis Spectroscopy; Statistical analysis and graphing. Radioactive uptake assay (Sodium, Glutamine, Rubidium), Smooth muscles contractions on organ bath.

**Animal Models:**
Sprague-Dawley Rats (Proper handling and Tissue Collection); Sepsis Model in Sprague- Dawley Rats; Obese and lean Zucker rats (Proper Handling and Tissue Collection)

**Cell Lines:**
I have handle and worked on following cell lines:
A7R5 (Smooth muscle), RAW264.7 (Mouse leukemic monocyte macrophage), H9C2 (Rat Cardiac myoblast), C2C12 (Mouse myoblast), L6 (Rat Skeletal muscle), HEP (Hepatocytes), HEK (Human embryonic kidney), CH157 (meningioma Cell line) and HUVEC (Human vascular endothelial cell), IEC-18 (Rat Intestinal epithelial Cells).

## COMPUTER SKILLS

- MS software MS word, MS Excel, Power Point
- Programming Language C and C++
- Bioinformatics tools (Blast, QTL)
- Statistics Software (Graph pad prism)
- Data analysis (R programming)
- Cystoscope and protein networking
- Basic Hardware and Software knowledge
- Involved in maintaining GLP (Good Laboratory Practice) through Quartzy (Online Inventory) and Sparklix (Online Notebook for Lab)

## PUBLICATION:

Liu, H., Blough, E. R., Arvapalli, R., Wang, Y., Reiser, P. J., Paturi, S., *Nepal, N* ., Wu, M. (2012). Regulation of contractile proteins and protein translational signaling in disused muscle. *Cell Physiol Biochem, 30*(5), 1202-1214. doi:10.1159/000343310

Manne, N. D., Arvapalli, R., *Nepal, N.*, Shokuhfar, T., Rice, K. M., Asano, S., & Blough, E. R. (2015). Cerium oxide nanoparticles attenuate acute kidney injury induced by intra-abdominal infection in Sprague-Dawley rats. *J Nanobiotechnology, 13*, 75. doi:10.1186/s12951-015-0135-z

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*Nepal, N.*, Arthur, S., & Sundaram, U. (2019). Unique Regulation of Na-K-ATPase during Growth and Maturation of Intestinal Epithelial Cells. *Cells, 8*(6). doi:10.3390/cells8060593

Palaniappan, B., Arthur, S., Sundaram, V. L., Butts, M., Sundaram, S., Mani, K., *Nepal, N.*, Sundaram, U. (2019). Inhibition of intestinal villus cell Na/K-ATPase mediates altered glucose and NaCl absorption in obesity-associated diabetes and hypertension. *FASEB J*, fj201802673R. doi:10.1096/fj.201802673R

Selvaraj, V., *Nepal, N.*, Rogers, S., Manne, N. D., Arvapalli, R., Rice, K. M., . . . Blough, E. R. (2015a). Cerium oxide nanoparticles inhibit lipopolysaccharide induced MAP kinase/NF-kB mediated severe sepsis. *Data Brief, 4*, 105-115. doi:10.1016/j.dib.2015.04.023

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Selvaraj, V., *Nepal, N.*, Rogers, S., Manne, N. D., Arvapalli, R., Rice, K. M., . . . Blough, E. R. (2015). Inhibition of MAP kinase/NF-kB mediated signaling and attenuation of lipopolysaccharide induced severe sepsis by cerium oxide nanoparticles. *Biomaterials, 59*, 160-171. doi:10.1016/j.biomaterials.2015.04.025

## Poster Presentation:

- Nandini Manne, Ravikumar Arvapalli, **Niraj Nepal,** Geeta Nandyala, Kevin Rice, Asano Shinichi, Eric Blough. ***Therapeutic efficacy of cerium oxide nanoparticles in treatment of sepsis induced renal failure (LB608).*** The FASEB Journal, 2014

- N Manne, R Arvapalli, **N Nepal**, K Rice, E Blough - ***Cerium Oxide Nanoparticles Confer Protection against Severe Sepsis Induced Hepatic Inflammation and Injury in Sprague Dawley Rats.*** The FASEB Journal, 2015

- Shinichi Asano, Arvapalli Ravikumar, Nandini Manne, Geeta Nandyala, Bing Ma, Mani Maheshwari, **Niraj Nepal**, Vellaisamy Selvaraj, Kevin Rice, Eric Blough. ***Cerium Oxide Nanoparticles (CeO2 NP) Treatment Ameliorates Sepsis Induced Diaphragm Dysfunction.*** The FASEB Journal, 2015

- Ata Abbas, Humaira Qureshi, Nii KK Koney, **Niraj Nepal**, Sergei Nechaev, Archana Dhasarathy, Philippe T Georgel, ***Potential Role for Omega-3 Fatty Acid in the Inhibition of Breast Cancer Metastasis Mediated by Modulation of Human Leukocyte Antigen-g (hla-g) Expression***. Tissue Antigens. 2015 /8/1

- Soudamani Singh, **Niraj Nepal**, Molly R Butts, Travis Parkulo, Subha Arthur, Uma Sundaram***. Mammalian Intestinal Crypt to Villus Maturation Mediated Differentiation Na-Glutamine Co-Transporters SN2 to B0AT1***. Gastroenterology 2016/ 04/01.

- Nandini Manne, Ravikumar Arvapalli, **Niraj Nepal,** Geeta Nandyala, Kevin Rice, Asano Shinichi, Eric Blough. **Therapeutic efficacy of cerium oxide nanoparticles in treatment of sepsis induced renal failure (LB608).** Federation of American Societies for Experimental Biology, 2014/04/01.

- **Niraj Nepal**, Uma Sundaram. **Regulation of Na/K-Atpase During Differentiation of Intestinal Crypt to Villus Cells.** Gastroenterology 2017/ 04/30.

- **Niraj Nepal**, Subha Arthur, Uma Sundaram. **Mechanism of inhibition of Villus cell Na-K-ATPase by PGE2 in the chronically inflamed intestine.** Gastroenterology 2019/05/18. 156(6), S-688.

- **Niraj Nepal**, Subha Arthur, Uma Sundaram. **Altered Phosphorylation of the alpha-1 subunit regulates Na-K-ATPase activity in the maturation of intestinal epithelial cells.** Gastroenterology 2018/06/03. 154(6), S-269