AMERICAN UNIVERSITY OF BEIRUT

MOLECULAR MECHANISMS BY WHICH PHOSPHATE MODULATES ANGIOTENSIN II-INDUCED HYPERTENSION

by
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ABSTRACT

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Hypertension or high blood pressure is a critical health burden. Adherence to certain diets lowers the risk of hypertension. Preliminary data have shown a significant decrease in BP in mice receiving high dietary phosphate. High dietary phosphate stimulates the release of FGF23 from bones, and binds to its renal FGFR, causing downregulation in the expression of sodium-phosphate-dependent cotransporters (NaPIIa/c) located at the level of proximal convoluted tubules and encoded by SLC34A1/3 respectively, thus contributing to a decrease in the reuptake of phosphate coupled with sodium ion and increased excretion in urine. Our aim is to investigate the underlying mechanisms by which phosphate modulates hypertension by influencing the activity of these transporters. Initially, hypertension was induced by infusing male mice with Ang II pumps subcutaneously and fed low: 0.15% P, Control: 0.3%, High: 1.5% P. Mice were sacrificed, and kidneys were harvested to determine mRNA expression levels of FGF23 and SLC34A1-3. Urine and plasma phosphorous were analyzed. To assess the effect of phosphate on renal damage, DHE stain for ROS and NGAL were done. Our results showed that in hypertensive mice fed high dietary phosphate there was an alteration in FGF23 and downregulation in SLC34A1-3, and changes in urine phosphate excretion with variation in plasma phosphate. In addition, ROS was found in all groups of hypertensive mice and NGAL was increased in hypertensive mice receiving a high dietary group. Dietary phosphate might be used as a non-pharmacological intervention in the prevention and control of hypertension, however, further investigations into phosphate regulatory mechanisms and its effect on kidney damage should be assessed.

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ABBREVIATIONS

CVD	Cardiovascular Diseases
WHO	World Health Organization
AHA	American Heart Association
DBP	Diastolic Blood Pressure
SBP	Systolic Blood Pressure
ROS	Reactive Oxygen Species
MENA	Middle East and North Africa
CNS	Central Nervous System
ANP	Atrial Natriuretic peptide
SNS	Sympathetic Nervous System
ABP	Atrial Blood Pressure
RAAS	Renin-Angiotensin Aldosterone System
ACE	Angiotensin-Converting Enzyme
JG	Juxtaglomerular Cells
BP	Blood Pressure
AT1	Angiotensin Receptor-1
AT2	Angiotensin Receptor-2
NF-KB	Nuclear Factor Kappa B
JAK/STAT	Janus Kinase and Signal Transducer and Activator of Transcription
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
ERK1/2	Extracellular Signal Regulated Kinase

MAPK	Mitogen Activated Protein Kinase
JNK	Jun-N Terminal Kinase
DASH	Dietary Approches to Stop Hypertension
PTH	Parathyroid Hormone
FGF23	Fibroblast Growth Factor
SLC34	Solute Carrier
NaPIIa	Sodium-Phosphate Co Transporter Type II A
NaPIIc	Sodium-Phosphate Co Transporter Type II C
NaPIIb	Sodium-Phosphate CoTtransporter Type II B
SGK1	Serum-Glucocorticoid Regulated Kinase-1
NHERF	Sodium-Hydrogen Exchanger Regulatory Factor 1
IACUC	Institutional Animal Care and Use Committee
MFI	Mean Flourescence Intensity
PCT	Proximal Convulated Tubule
NCC	Sodium-Chloride Co-transporter
ENAC	Epithelial Sodium Channels
NGAL	Neutrophil-Gelatinase Associated Lipocalin
DHE	Dihydroethidium

CHAPTER 1

INTRODUCTION

1.1. Global Burden of Cardiovascular Diseases

Cardiovascular diseases (CVDs) are the most common cause of death worldwide. According to the World Health Organization (WHO), CVDs killed 17.9 million people globally in 2019, accounting for 32% of all fatalities, with heart attacks and strokes accounting for 85% of total deaths. CVDs will steal the lives of 23.6 million people by 2030. Cardiovascular diseases can be avoided by acknowledging behavioral risk factors such as smoking, bad diet and overweight, lack of exercise, and alcoholism. Low- and middle-income countries account for at least three-quarters of all CVD deaths worldwide. People in low- and middle-income countries frequently lack access to primary healthcare services that allow for the early diagnosis and intervention of people with CVD risk factors.[1] Cardiovascular disease is any condition that affects the blood circulatory system, including the heart and vasculature, which displace and convey blood. This multifactorial condition encompasses a wide range of genetic and acquired diseases.[2] Hypertension is one of the most common causes of CVD and is associated with an increased risk of mortality and morbidity. Lowering blood pressure has been shown to significantly reduce major CV events.[3]

1.2. Hypertension Prevalence and Etiology

According to the American Heart Association (AHA), one in every two adults in the United States has hypertension, accounting for 46 percent of the adult population. By 2019, high blood pressure was a major and leading cause of death for 516,955 US people.[4] Every year, approximately 9.4 million people die due to hypertension-related complications. Arab countries have widespread hypertension (29.5 percent), especially when compared to Sub-Saharan Africa (27.6 percent) and the United States (28 percent). Compared to Egypt and Palestine, Lebanon, a small country, has a high prevalence of hypertension, with one-third of the population affected and 30% prehypertensive. Over the last decade, hypertension in Lebanon has increased threefold. [5]

According to the AHA, hypertension is a "silent killer" since most patients are unaware of the symptoms till they have serious consequences such as a heart attack, stroke, or kidney failure. Patients may, however, experience symptoms such as headache, lightheadedness, and vertigo. Hypertension, also known as high blood pressure, is defined as high arterial blood pressure when blood flows against the arterial wall. Systole and diastole are two phases of the cardiac cycle, with systole measuring pressure during heart contraction and diastole measuring pressure after the heart relaxes. ACC/AHA guidelines 2017 define hypertension as blood pressure readings greater than 130/80 mmHg. High blood pressure can lead to devastating consequences such as renal injury, retinopathy, heart failure, stroke, and impotence. A reduction of 10mmHg in SBP can significantly reduce coronary artery disease by 17%, stroke by 27%, and heart failure by 28%. Non-pharmacological approaches lower blood pressure by 10mmHg and are considered critical interventions for hypertensive adults and hypertension prevention.[4]

Hypertension is classified as essential or primary hypertension, also known as idiopathic, which means that the underlying cause is unknown and varies between populations due to its heterogeneity, with genetics being the most common cause. Secondary hypertension is often associated with underlying diseases such as primary aldosteronism, renal artery stenosis, or pheochromocytoma, and others. [6] Major players in the molecular mechanisms of hypertension include the renin-angiotensin system, sympathetic nervous system, reactive oxidative species (ROS) generation, and irregular G-protein-coupled signaling pathways. [7]

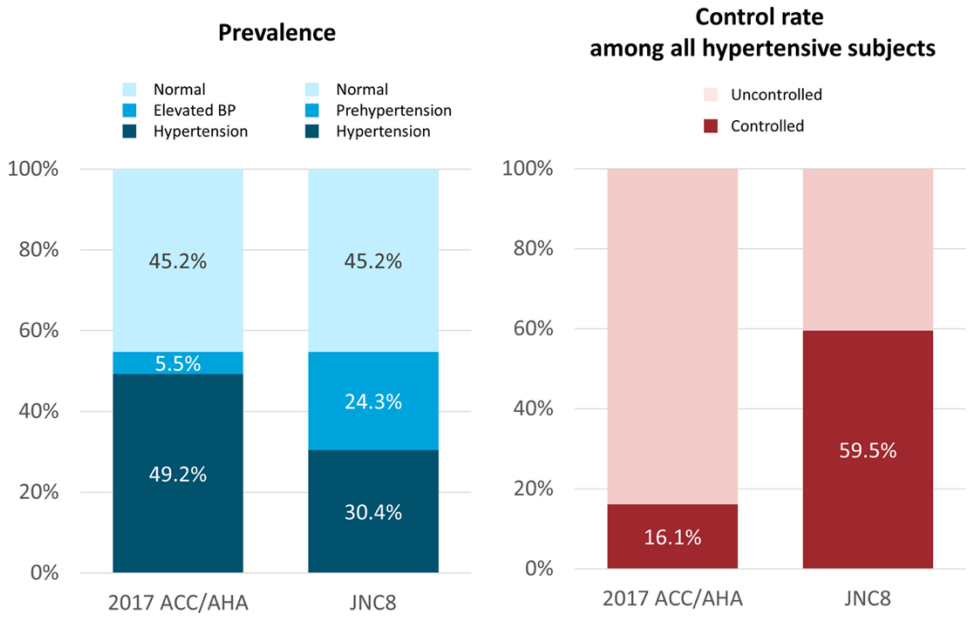

Figure 1. The Impact of the New AHA/ACC 2017 Guideline on the Prevalence and Control Rate of Hypertension among the Korean population. [8]
The prevalence of hypertension was 49.2%, a considerable rise over the previous definitions estimated at 30.4% by JNC. The current definitions of BP aim to enhance public awareness and use non-pharmacological measures as first-line treatment.

1.3. Blood Pressure Homeostasis

Blood pressure is maintained within normal through short and long-term regulations, where the force of cardiac contraction, blood volume, and total peripheral

resistance are key determinants of blood pressure. The central nervous system attempts to control such factors via nerves and the secretion of hormones. [9] Short-term blood pressure regulation via the baroreflex is a key example of homeostasis in which a set point is kept at its optimum value through a physiological control mechanism. For example, blood pressure deviates when standing up and communicates with the central nervous system via baroreflex input from the blood vessel wall. In response, CNS alters the heart and blood vessel wall to compensate by causing tachycardia and returning blood pressure to baseline.[10] Also, the kidneys play an important role in blood pressure regulation by maintaining body fluid volume. As for long-term regulation, hormones circulating like catecholamines, renin-angiotensin system, vasopressin, and atrial natriuretic peptide (ANP) influence the sympathetic nervous system. [10]

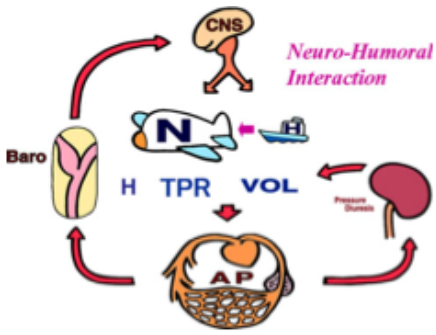

Figure 2. Neuro-Humoral Interaction for Blood Pressure Regulation [9].
Any variations in the ABP will be adjusted by the baroreflexes that sense the CNS to compensate; this is referred to the short-term adjustments in the body. However, the Neuro-humoral is considered a long-term mechanism for maintaining BP where the kidneys regulate fluid volume via their diuretic effect. Simultaneously, hormones released by the kidneys and afferent neurons originating in the kidneys play a role in blood pressure regulation.

1.4. Pathogenesis of Hypertension

1.4.1. Sympathetic Nervous System

The sympathetic nervous system modulates arterial blood pressure (ABP), and changes in sympathetic function have been connected to the genesis of cardiovascular illnesses, including hypertension. [11] The adrenergic overdrive affects elevated blood pressure progression and implements end-organ damage due to hypertension. [12] Sympathetic upregulation is correlated with increased heart rate. It appears to perpetuate cardiac and vascular modifications which may contribute to the development of major hypertension abnormalities such as atrial fibrillation, left ventricular hypertrophy, and thickening of the arteries. Actions are stimulated by adrenergic neurotransmitters mainly noradrenaline, adrenaline, and dopamine. These affect cardio-metabolic systems by hitting components involved in homeostasis control such as the heart, kidneys, veins, and arterioles, resulting in renin release, salt retention. As an outcome, these SNS targets lead to an elevation in blood pressure. [13]

1.4.2. Renin-Angiotensin-Aldosterone System

The renin-angiotensin-aldosterone system (RAAS) regulates BP, fluid volume, and sodium and potassium electrolyte balance. It is one of the most significant hormonal systems in preserving hemodynamic stability. As a result, any disturbance in the elements that constitute RAAS leads to the development of hypertension. [14] Angiotensinogen, a major component, is synthesized by the liver converted to angiotensin I (inactive decapeptide) via renin secretion by the kidneys mainly from juxtaglomerular (JG) cells. Angiotensin-Converting Enzyme (ACE) mediates the conversion of angiotensin I to an active compound by removing the C-terminal dipeptide to generate octapeptide Angiotensin II a biologically active and powerful

vasoconstrictor. ACE is an exopeptidase bound to plasma membranes and found mainly in vascular and microvilli brush border endothelial cells. It is also known as kininase II as it converts various proteins into inactive forms including bradykinins and kallidin. As a result, the effects of ACE cause significant vasoconstriction. Renin is an inactive hormone and through proteolysis active renin is formed and is stored in JG and considered a rate-limiting component in the RAAS system. Renin release is stimulated by a drop in BP via baroreceptors, NaCl concentration at the macula densa that senses any changes in chloride amounts, SNS activation through beta-1 adrenergic receptors, and the autoregulatory actions of Ang II on JG cells to compensate any changes. [15, 16] Angiotensin II acts mainly on two isoforms angiotensin 1 and 2 receptors (AT1-R and AT2-R) where the majority of Ang II's established hypertrophy and fibrosis are via the AT1-R, but it can also connect to the AT2-R, causing the reverse effect as that of the AT1-R. [15] AT2-R activation causes NO release while inhibiting the NF-B and JAK/STAT signaling pathways resulting in cardiovascular protection. [17] Ang II via the AT1 receptor in the adrenal cortex induces the production of aldosterone, an effector molecule in RAAS, thus leading to sodium and water retention, elimination of potassium and magnesium on the renal distal nephron, consequently altering extracellular fluid volume and BP. Ang II also acts on vascular smooth muscle cells and constricts both afferent and efferent arterioles drastically reducing the kidney's blood flow and glomerular filtration. Ang II enhances the production of reactive oxygen species (ROS) through the activation of NADPH oxidase, decreasing the vasodilating effect of NO. [14] Notably, the RAS system generates Ang (1-7) that acts on Mas receptors and antagonizes the effects of Ang II, therefore mediating vasodilation. [17]

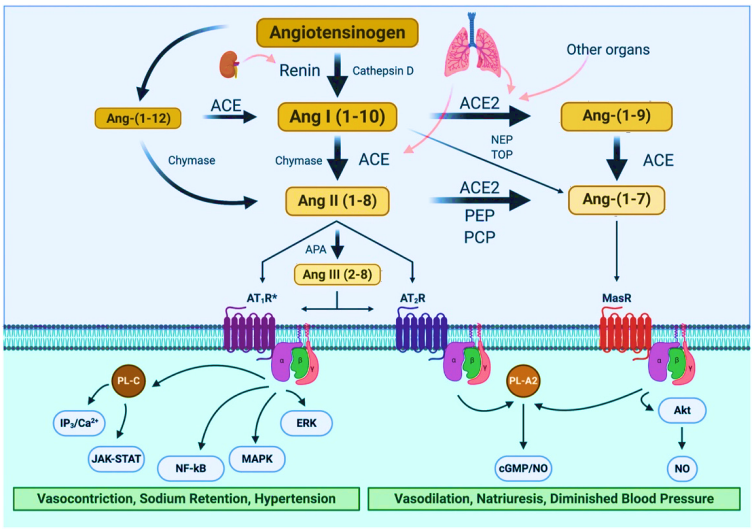

Figure 3.The Classical Renin-Angiotensin System [18].
Angiotensinogen, a key peptide in the RAS is converted to Angiotensin I after the secretion of renin by the kidneys and converted to Angiotensin II by ACE, which is a powerful vasoconstrictor. Angiotensin mediates this effect through binding to AT1R which activates various signaling pathways and elevates BP. As negative feedback, the action of Ang II on AT2R and the effect of Ang(11-7) on MasR will counteract the elevation in BP.

1.4.3. Oxidative Stress-Induced Hypertension

Reactive oxygen species play a crucial function in maintaining the arterial wall and in the pathophysiology of increased blood pressure. Oxidative stress has been connected to one of the fundamental mechanisms responsible for the development of hypertension. Evidence from both hypertensive animals and humans showed that there's increased ROS generation, decreased levels of nitric oxide (NO), and impaired antioxidants and recognized that vascular oxidative stress is an underlying mechanism in hypertension. [18] Substances such as Ang II can aggravate mitochondrial superoxide production. Eventually, ROS participates in multiple consequences such as a rise in systemic BP, endothelial dysfunction, vascular constriction, and sodium retention. ROS activates ERK1/2, p38MAPK, and JNK members of the mitogen-activated protein kinase (MAPK) family thus affecting the cellular proliferation, migration, hypertrophy,

and inflammation within the heart and vessels. In addition, inflammation in kidneys and heart are promoted by activating transcription factors [NF] κB, signal transducer and activator of transcription [STAT] activator protein 1 [AP-1], and hypoxia-inducible factor 1 [HIF-1]. [19] NADPH oxidase is the most well-studied generator of ROS and is highly expressed in vascular smooth muscles. Five subfamilies of NADPH in animals are recognized with Nox1,2,4 mostly expressed in vascular cells. Nox is responsible for producing O2- which can be scavenged by nitric oxide to form peroxy-nitrite (ONOO-) or converted to hydrogen peroxide through metabolism by superoxide dismutase. Hydrogen peroxide can be further reduced by catalase or glutathione peroxidase. [20] Notably, eNOS is a common isoform of NO and important in maintaining NO synthesis in the vessel well, uncoupling of eNOS after the formation of ONOO- will lead to endothelial dysfunction and elevates BP.

Angiotensin II, through its stimulation to NADPH oxidase, contributes to tubulointerstitial damage progression and the advancement of obstructive nephropathy and causing renal hypertrophy. [21]

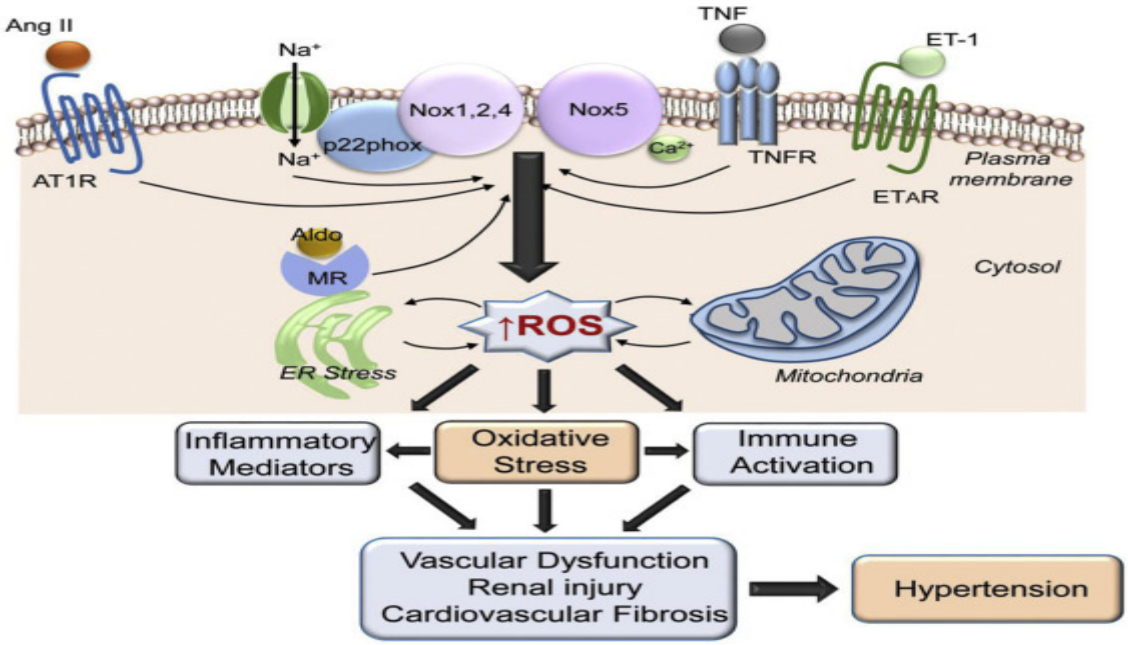

Figure 4. The Role of Oxidative Stress in HTN [20].
The actions of Ang II on AT1R allow the activation of Nox 1,2,4 and Nox 5 (subfamilies of NADPH oxidases) and are considered generators of ROS. ROS exacerbates hypertension and is considered one of the fundamental mechanisms in the formation of HTN via causing vascular and renal damage.

1.5. Protective Mechanisms in the Regulation of Blood Pressure

1.5.1. Kallikrein System

Bradykinin, an autocoid peptide that belongs to the Kallikrein-kinin system, has multiple physiologic actions and is crucial for maintaining blood pressure. The system is known to be present in the kidney, cardiac muscle, and vasculature. The main function of the Kallikrein-kinin system in the kidney may be to prevent salt retention at the collecting duct level. It works by interacting with two transmembranes G-protein coupled B1 and B2 receptors. There is evidence in the literature that bradykinin tends to play a central role in the regulation of hypertension through vasodilation extensively in various regions of the circulation, a decrease in overall arterial stiffness, control of sodium excretion from the kidney by inhibiting sodium chloride and water reabsorption by the distal nephron thus counter regulating the elevation in blood pressure caused by the renin-angiotensin system. Dysfunction in this system contributes to the development of elevated blood pressure and causes end-organ damage. [22, 23]

1.5.2. Role of Vascular Hormones: NOS and e-NOS

As a crucial paracrine modulator of the vasculature, NO plays a vital function and has numerous positive impacts. Reducing NO bioavailability in the vasculature lowers vasodilator capability, which correlates to hypertension. The enzyme NOS, a family of enzymes, mediates NO production from oxygen and arginine. In the vessel wall, eNOS is the most common NOS isoform. NO counteracts the effects of Ang II, ET1, and ROS, NO diffuses as a gas into nearby smooth muscle and interacts with guanylyl cyclase. eNOS is considered the most common isoform of NO in the vessel wall and to maintain NO synthesis, L-arginine, and BH4 (tetrahydrobiopterin) serve as important co-factors for eNOS. Any lack of oxidation in this pathway will lead to eNOS

uncoupling thus resulting in decreased NO synthesis and increased eNOS mediated superoxide generation. [21]

1.6. Importance of Lifestyle in Hypertension

Genetic and environmental factors that contribute to hypertension and end up with damaging organs and death highlight the importance of non-pharmacological measures as cornerstones in preventing and managing elevated blood pressure. Dietary changes, considered a major part of lifestyle interventions, were shown to lower and maintain blood pressure by decreasing body weight and losing fat, reducing alcohol intake, and boosting levels of vitamins. This will result in long-term improvements in blood pressure and lower the risk of heart diseases. People with stage 1 or 2 hypertension who focus on adjusting their lifestyle will significantly decrease their blood pressure. They might be highly desirable as 1st line treatment than lowering blood pressure drugs. [24] The DASH diet, which stands for dietary approaches to stop hypertension, is highly recommended internationally and focuses on eating nutritious food such as fruits and vegetables, low-fat dairy products, whole grains, nuts, poultry, and fish, and at the same time, consuming low portions of red meat, sugar-containing food, and saturated fat and cholesterol where blood pressure is significantly decreased in normal and hypertensive people. Clinical experiments in the US and Europe have demonstrated that lowering salt intake decreases blood pressure. Guidelines highly advocate limiting sodium consumption to 100mmol, equal to 2.3 g of sodium or 5.8 g of sodium chloride. [25] The impact of other vitamins on hypertension, such as calcium, magnesium, and phosphate remains confusing. [26]

1.6.1. Phosphate, an important mineral

Phosphate is a crucial mineral in osteogenesis, an effective molecule in signaling pathways, and a vital component in the cell wall structure, Moreover, it plays a key role in forming the helical structure of DNA and RNA as it is considered a crucial element in high-energy molecules such as AMP, ADP, and ATP. [27] Limited amounts of this mineral will cause physiological events to be impaired such that the apoptosis of chondrocytes is prevented, vascular invasion and bone remodeling are altered leading to rickets and stunted growth. As a result of low amounts of Pi, osteomalacia is seen in the rest of the skeleton due to the inadequate production of hydroxyapatite. In the human body, phosphate is the most prevalent anion as it accounts for 1% of body composition and is considered an intracellular anion with a 100-fold higher percentage compared to plasma. Bones and teeth are major sources of phosphorous accounting for 85% of the total amount and the 15% remaining constitute other tissues with 1% in extracellular fluid. Pi is largely crosslinked with calcium as hydroxyapatite crystals, and the remainder appears as amorphous calcium phosphate. In soft tissues and cell membranes, phosphorous is found as phosphate esters, phosphoproteins, and free ions. One-tenth of phosphorous found in the extracellular space is bound to proteins and one-third bound to sodium, calcium, and magnesium and the rest is present as inorganic phosphate. [28] For adequate bone mineralization, normal serum levels of Pi must be maintained otherwise elevated or low serum amounts end up with soft tissue calcification. [27] Pi is widely present in foods and it is absorbed by the intestines efficiently and minimally controlled through it and it seems that the kidney plays a significant role in Pi homeostasis at the proximal tubule level, where most filtered Pi is reabsorbed. Consequently, the kidneys adjust the Pi's re-absorptive capacity to meet Pi's demands. The whole process is

mediated by the presence of sodium-dependent Pi (Na/Pi) mechanisms in the brush-border membrane. [29]

1.6.2. Clinical Relevance of Phosphate and its Effect on Hypertension

It is necessary to sustain normal serum phosphate values since alterations in phosphate availability would cause a variety of organ and system dysfunctions. Given the complexity of phosphate regulation, additional variables can affect the phosphate ion, including decreased renal clearance, increased catabolism, and intestinal loss. Hypophosphatemia is one of the critically ill patients' most common electrolyte abnormalities. Hypophosphatemia can result in various clinical symptoms, including cardiac failure, diaphragmatic weakness, seizures, coma, rhabdomyolysis, and red blood cell dysfunction. A retrospective cohort study involved 1073 adult patients admitted to the ICU. It was shown that patients with hypophosphatemia required greater mechanical ventilation and longer ventilator time than the normal group [30].

The relation between dietary phosphorous and hypertension is still unclear. Americans have relatively a large consumption of phosphorous food such as canned food and food preservatives. Consequently and based on two large cohorts, which involved 15792 and 6814 candidates each, it was found that phosphate intake from dairy products is linked to a reduction in BP and with a decreased incidence of HTN [31]. Another cross-sectional study involved 4680 from different countries highlighting the importance of dietary phosphate as one of the important strategies in lowering BP and preventing hypertension in adults [32]. In contrast to other studies that have shownblood pressure in rats with both normal and elevated blood pressure during the resting condition knowing that both groups had adequate kidney function. The amount of Pi intake in US greatly

exceeded the daily intake since inorganic Pi is widely used in food industries as a preservative, flavor additive, and color stabilizer. [33] Therefore, dietary phosphorus can impact blood phosphorus levels, so establishing the correlation between dietary phosphorus and the risk of developing hypertension is particularly important. Accordingly, determining whether dietary phosphate has a favorable or harmful effect on the cardiovascular system is a critical public health issue as elevated serum phosphorous concentration may raise the incidence of cardiovascular events. [26]

1.7. Phosphate Homeostasis

The regulation of phosphate is maintained by an interplay between its intestinal absorption, kidney uptake and excretion, and its exchange between extracellular and storage in bones. Furthermore, Pi is regulated through the parathyroid hormone (PTH), calcitriol, and phosphatonins which are groups of peptides, and the most well-known is fibroblast growth factor 23 (FGF23). Dietary phosphate, 1,25-dihydroxy vitamin D (1,25(OH)2D3), and PTH are considered the most physiological regulators for phosphate absorption in the intestine. Whereas, at the subcellular level, there are sodium-dependent phosphate transporters belonging to the solute carrier family SLC34 which involve three forms of co-transporters NaPiIIa (SLC34A1), NaPiIIb (SLC34A2), and NaPiIIc (SLC34A3) and they are the center of attention in the absorption of phosphate. The two transporters SLC34A1 and SLC34A3 are both mainly expressed in the apical brush border membrane at the proximal tubules in the kidney and are essential in renal phosphate uptake, whereas NaPiIIb (SLC34A2) is significantly expressed in the small intestine and is controlled by $1,25(OH)_2D_3$.

The transport of Pi is unidirectional in the proximal tubule cell. It includes absorption across the brush border membrane, translocation across the cell, and exudation at the basolateral membrane. The apical membrane is considered a rate-limiting step for phosphate influx and is accomplished by the presence of sodium-phosphate co-transporters typeII SLC34A1 and SLC34A3 on the cell surface and account for 80% of the filtered phosphate reuptake.[28]

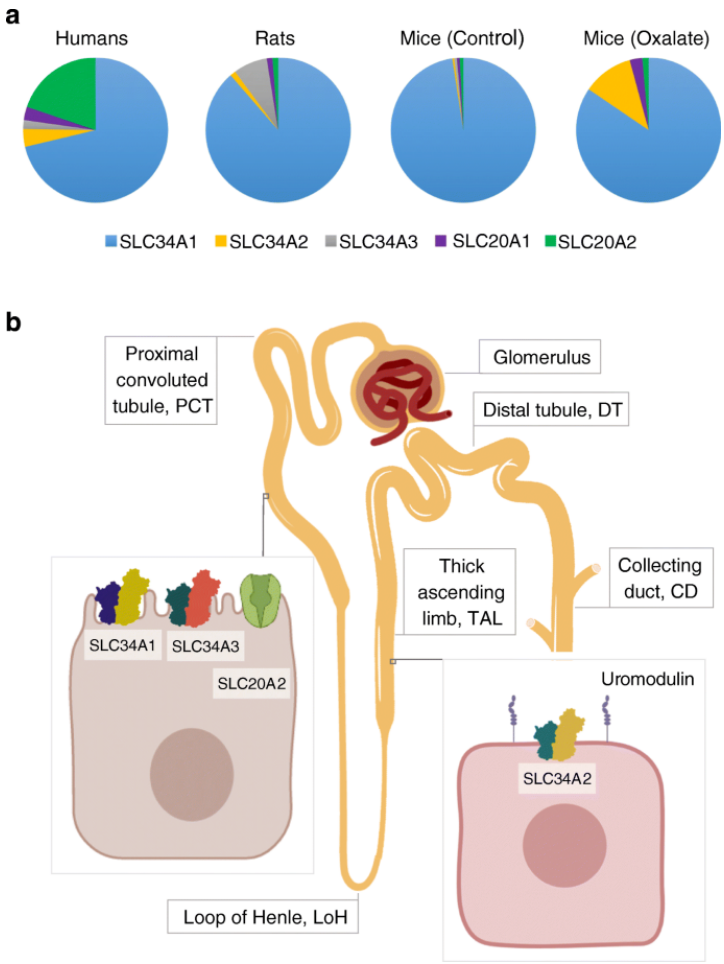

Figure 5. Proximal Renal Regulation of Phosphate [35].
The renal handling of phosphate is mostly governed by the presence of sodium-phosphate co-transporters encoded by the solute carrier family. The most important ones are SLC34A1 an SLC34A3 which are highly expressed at the level of proximal convoluted tubules. However, the expression varies between humans and animals where both highly express SLC34A1-3, however SLC34A3 is more expressed in humans than in mice.

The regulation of phosphate is affected by the amount of phosphorous present in diet or plasma. Thus, limited phosphate consumption or low serum phosphorous concentrations will allow the calcium-sensing receptors in the parathyroid gland to prevent the further release of PTH, and a decrease in FGF23 of PiT1/PiT2 cotransporters found in bones. As a result of decreased PTH and FGF23 levels will sense the kidneys to increase phosphate intake from the glomerular infiltrate, which is maintained by the upregulation of NaPiIIa and NaPiIIc. Moreover, the kidney induces more 1,25(OH)2D that allows the intestine to enhance the uptake of phosphate from diet and this is accomplished by the upregulation of Npt2b found in the intestine. Consequently, the total impact is an increase in serum phosphorous to counteract the limited amount.

In contrast, high dietary phosphate or high plasma concentration conditions increase PTH release and secrete FGF23 from bones that bind to renal FGF receptor and this binding is meadiated by the presence of klotho receptor therefore all contributing to the downregulation of NaPiIIa and NaPiIIc and limiting the reabsorption of phosphate from the glomerular filtrate. Such an effect will allow the kidney to decrease the rise in 1,25(OH)2D so that no longer absorption of phosphate by the intestines since Npt2b is less expressed. [27]

1.8. Hormonal Regulation of Phosphate

1.8.1. Parathyroid Hormone

The main role of PTH is controlling calcium concentrations in bones through enhancing Ca2+ reabsorption in the kidney's distal convoluted tubules (DCT) by increasing the expression of an enzyme known as 25(OH) vitamin D 1-αhydroxylase that is found in the proximal tubules and is responsible for synthesizing the active form

1,25(OH)2 vitamin D. Also, PTH is considered an important regulator of plasma phosphate levels. In vitro and In vivo experiments showed that PTH availability correlates with a decreased expression of sodium phosphate co-transporters. PTH exerts its effect by binding to PTHE1 receptors that are widely spread in the kidney tubules. PTH mediates the activation of the adenylate cAMP system and phospholipase C PKC pathway and MAPK and this can lead to fast lysis and breakdown of sodium-phosphate cotransporters. PTH and vitamin D have antagonistic effects on the kidney and gut, keeping phosphate levels in check while maintaining calcium ion homeostasis. The impact of great levels of PTH under the normal physiologic function of the kidney leads to a reduction in serum Pi. Still, specific illness conditions contribute to the development of rickets and osteomalacia. FGF23 has been found to regulate both PTH and vitamin D production through negative feedback loops, adding to the intricacy of this regulation mechanism. [28]

1.8.2. Fibroblast Growth Factor

In the 1980s, parabiosis experiments were performed in mice with kidney phosphate loss and hypophosphatemia caused by a factor circulating in the bloodstream. It was known as Fibroblast growth factor 23 related to the “Phosphotonin” family. FGF23 is a 32kDa glycoprotein that originated from bones mainly osteoblasts and osteocytes and impacts phosphate reuptake and vitamin D synthesis in the kidney. The hormonal influence of FGF23 on the renal organ is based on the presence of αklotho, a transmembrane or soluble co-receptor. The availability of αklotho mediates the strongest linkage of FGF23 to the FGF receptor on the site of action and it enhances the binding affinity by a factor of 20. FGF23 enhances phosphate excretion at the cellular

level of proximal tubules in the kidneys by blocking phosphate reabsorption from the urine. FGF receptor-1c (FGFR1c) is likely the major FGFR for the FGF23 signaling cascade among the four types of receptors. FGFRs are tyrosine kinases and induce intracellular phosphorylation following ligand-induced dimerization. Studies have shown that mice with FGFR1 knock-out in the proximal renal tubule region are unable to induce 1,25(OH)2D3 downregulation. The influence of FGF23 on proximal kidney tubules is through inhibiting Pi absorption and decreasing the activity of 1α-hydroxylase which is a rate-limiting enzyme in the formation of vitamin D. Prevention of 1α-hydroxylase will lead to the activation of extracellular signal-regulated kinase 1and 2(ERK1/2) and serum/glucocorticoid-regulated kinase-1 (SGK1), resulting in phosphorylation of scaffolding protein Na+/H+ exchange regulatory cofactor (NHERF)-1. Consequently, NHERF-1 internalizes and breaks down NaPiIIa,c, leading to less expression of these co-transporters on the apical membrane, and less phosphate is reabsorbed from the urine. [34].

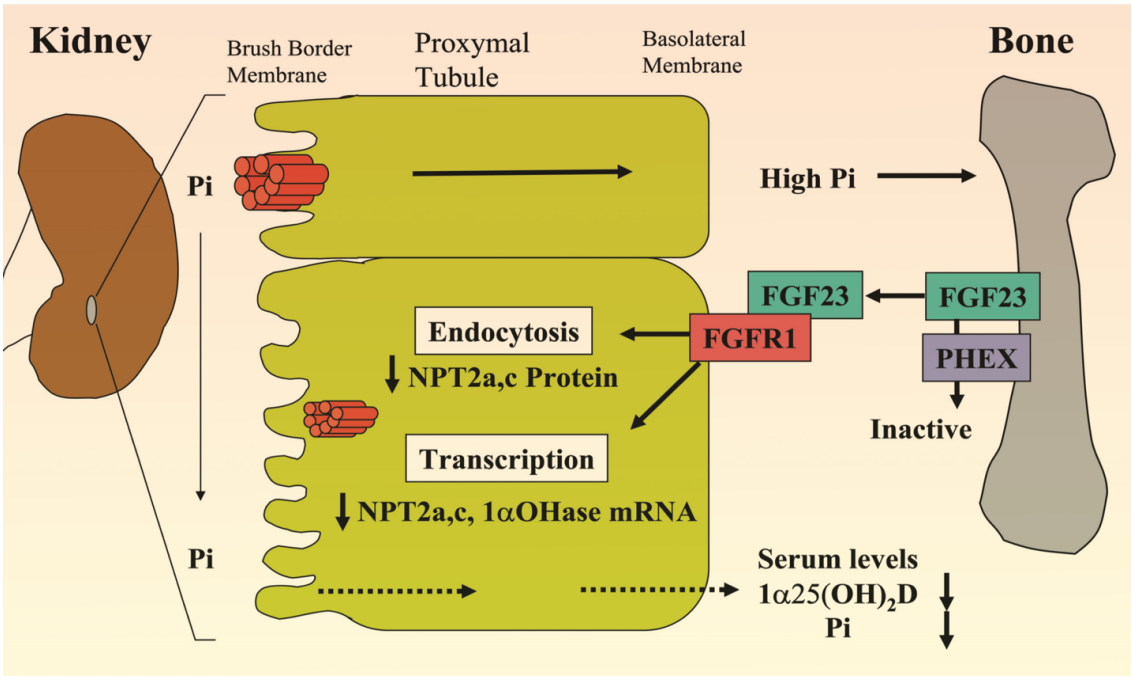

Figure 6. The Role of FGF23 in the Regulation of Phosphate Homeostasis [30]. Renal handling of phosphate constitutes the majority in regulating this mineral where it is responsible for reuptake and excretion. FGF23 is considered a novel hormone in controlling phosphate within normal. High phosphate levels induce the secretion of FGF23 from bones that binds to FGFR1 in the kidney thus causing downregulation of the NaPIIa,c through internalization and degradation of these transporters. Furthermore, via decreasing the synthesis of 1αhydroxylase, an important enzyme contributing to the intestinal absorption of phosphate. The overall action of FGF23 results in a decrease in serum levels of phosphate.

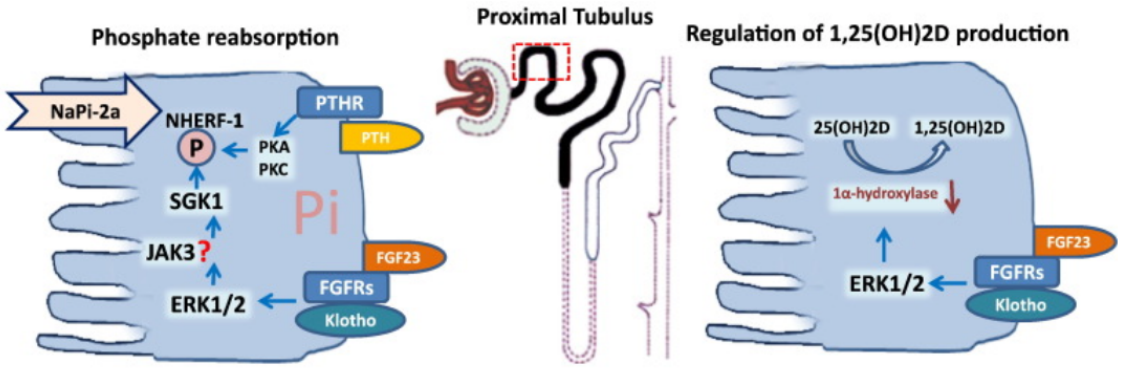

Figure 7.Hormonal Regulation of Phosphate via PTH and FGF23.
This figure illustrates the influence of PTH and FGF23 at the level of PCT under high dietary phosphate conditions where PTH by binding to PTHR activates protein kinases PKA and PKC and FGF23 by binding to FGFR mediated by klotho activates ERK1/2 leading to the stimulation of SGK1, thus both hormones contributing to the phosphorylation of NHERF-1 thus causing internalization and degradation of sodium phosphate co-transporters. In addition, FGF23 controls the intestinal absorption of phosphate since the activation of ERK1/2 limits the transcription of 1αhydroxylase leading to a decrease in 1,25(OH)2D, responsible for intestinal phosphate absorption.

1.9. The Importance of Sodium Phosphate Co-Transporters

1.9.1. Sodium-Phosphate Co-Transporter Type II A Encoded by SLC34A1 Gene

Sodium-phosphate cotransporter type IIa, encoded by the gene SLC34A1 is almost exclusively found in the renal proximal tubules on human chromosome 5. According to animal studies done on rats, SLC34A1 is found in fully differentiated juxtamedullary kidney tubules at birth and its protein expression accelerates through the first two months as the kidney develops. The promoter region of the SLC34A1 contains vitamin D response elements and multiple binding domains for various transcription factors such as c-Jun, C-Fos, AP-1, and others. Furthermore, it contains a phosphate response element linked with the TFE3 binding site. It seems that the PDZ binding motif, which is found at the carboxy-terminal of SLC34A1 is a significant structural characteristic since they are essential for trafficking and placement of the co-transporter at the apical membrane. The presence of the PR motif in the carboxy-terminal is needed

for effective apical expression. Most SLC34A1 is found at the apical membrane where it interacts with a scaffolding protein known as sodium hydrogen exchanger regulatory factor isoform 1 (NHERF1) and contains two PDZ domains. For SLC34A1 to be integrated into the actin cytoskeleton, NHERF1 must bind to ezrin an actin-binding protein, where such combination is required for SLC34A1 to be localized on the membrane. Hence a lack of NHERF1 or ezrin causes abnormal trafficking in SLC34A1. The primary controllers of SLC34A1 expression on the apical membrane are Pi, PTH, FGF23, and dopamine where the low Pi intake enhances the expression of SLC34A1 while high Pi intake, PTH, FGF23, or dopamine will suppress its expression by causing NHERF1 phosphorylation specifically at serine 77 through the simulation of PKA and PKC by PTH and dopamine mainly and MAPK by FGF23 leading to the separation of NHEFR1 from SLC34A1. [27] Since NaPiIIa is responsible for the reuptake of phosphate in the kidney, it couples with sodium for its transportation and it uses the free energy given by the electrochemical gradient for Na+ to facilitate the reabsorption of Pi from primary urine. NaPiIIa is electrogenic and predominantly delivers divalent Pi. It operates with tight sodium: phosphate ratio of 3:1, resulting in one positive charge driving inward each cotransport cycle. [35]

Impairment of NaPiIIa is linked to several interrelated disease conditions, including hypophosphatemic nephrolithiasis with osteoporosis, renal Fanconi's syndrome with chronic kidney disease, and idiopathic infantile hypercalcemia and nephrocalcinosis. Studies have shown that Impairment in the NaPiIIa transporter can induce sodium uptake problems, resulting in a loss of extracellular fluid and kidney phosphate squandering. The latter would result in increased sodium delivery to the distal nephron, and exaggerated sodium and fluid loss from the body. The renin-

angiotensin-aldosterone system (RAAS) appears to be triggered due to the volume loss, leading to secondary hyperaldosteronism. [36] Studies done by Beck et al. showed that knocking out the SLC34A1 gene causes increased urine Pi excretion as well as a 70-80% decrease in brush border membrane NaPIIa dependent transport thus leading to hypophosphatemia. [37].

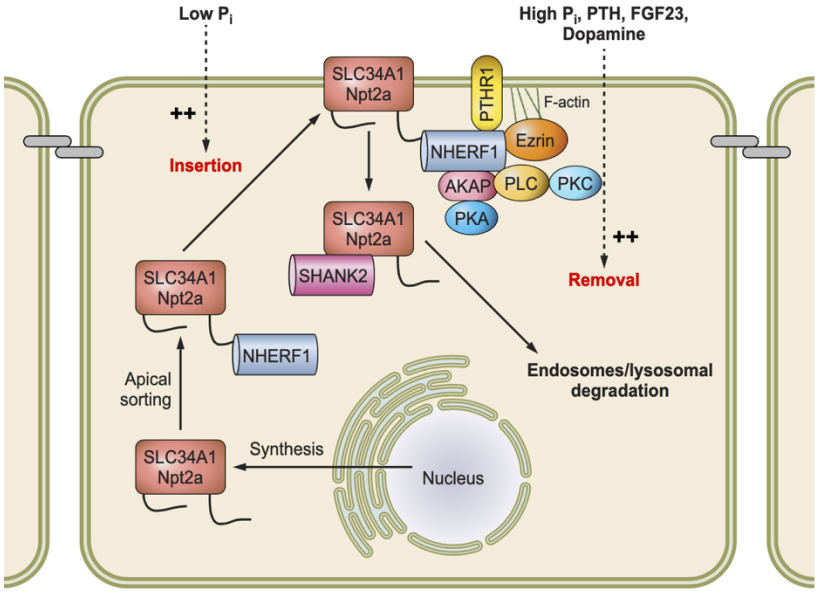

Figure 8. Trafficking of NaPiIIa/SLC34A1 [40]
NaPiIIa is inserted into NHERF1 in conditions of low dietary phosphate where it will be stabilized at the brush border membrane. In high amounts of phosphate, NaPiIIa is internalized and degraded via the activation of multiple pathways and kinases that phosphorylate NHERF1 leading to the dissociation and unstabilized NaPiIIa at the BBM.

1.9.2 Sodium-Phosphate Co-transporter Type II C Encoded by SLC34A3 Gene

Sodium-Phosphate co-transporter type II c encoded by the human gene SLC34A3 is mainly found in humans at chromosome 9. SLC34A3 consists of the same number of exons as SLC34A1 as well as the same binding site for TFE3. The binding sites of the transcription factors in the promoter region for SLC34A3 are different from

those of SLC34A1 like RP58, HOXA5, etc. In contrast to SLC34A1, SLC34A3 is not an electrogenic transport responsible for transporting two ions of Na+. The reason behind this discrepancy is the charged aspartic acid that is exchanged for glycine at position 195 of SLC34A3. Thus, the reabsorption of Pi mediated by transporter type IIc requires coupling two sodium ions. In addition, during birth in mice SLC34A3 is absent unlike SLC34A1, and rises after the weaning period. It seems that SLC34A3 is only expressed in the renal proximal tubules and it engages with PDZ-containing proteins like NHERF1 and NHERF3 where the latter one doesn't appear to participate in a substantial part in SLC34A1 localization on the apical membrane but for SLC34A3 it does appear to be essential for its effective anchoring where NHEFR3 elimination lowers the expression. Pi, PTH, and FGF23 are key regulators of SLC34A3 and the gene suppression by Pi and PTH is likely to be greater than the 1st SLC family. [27] In rats it shown that high Pi load resulted in approximately 4 hours with up to one or two days for SLC34A3 to be intracellularly sequestered and broken down compared to SLC34A1, which takes up to 2 hours. Beck et al showed that NaPiIIc is responsible for 30% of Pi absorption and even though type IIc Na/Pi transporter mRNA levels remained unaltered, type IIc Na/Pi transporter protein levels in NaPiIIa knockout mice were considerably greater than in wild-type mice. Although full overexpression of type IIc Na/Pi transporter protein is insufficient to compensate for the lack of type IIa Na/Pi transporter activity in NaPiIIa knock out, the type IIc Na/Pi transporter does contribute to remaining renal Na/Pi transport. [38] It is found that defective Npt2c gene in rodents was associated with hypercalcemia, hypercalciuria, and elevation in the amounts of 1,25(OH)2 vitamin D3 where Npt2c-/- in humans was the origin of an autosomal recessive disease condition known as HHRH (hereditary hypophosphatemic rickets with

hypercalciuria) and manifested by kidney loss of Pi, low serum Pi concentration, high 1,25(OH)2 vitamin D3 levels, hypercalciuria, rickets, and osteomalacia. Such findings imply that NPT2c plays a crucial role in renal Pi uptake and bone mineralization and is a critical predictor of plasma Pi content in individuals.[39]

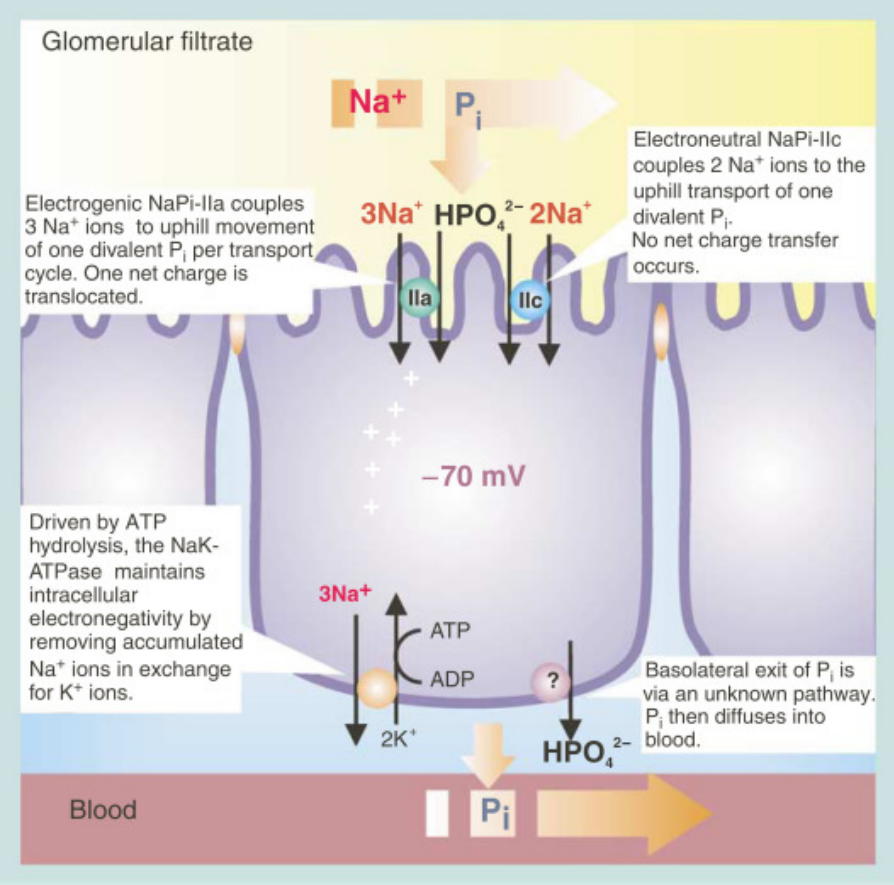

Figure 9. Energetics of Phosphate Re-uptake [43]
Two types of transporters at the luminal membrane of proximal tubules are responsible for the bulk of phosphate reuptake. NaPIIa is responsible for 70% of phosphate reabsorption, which occurs when phosphate combines with three sodium ions at the BBM and is transported into the bloodstream via ATP. In contrast to NaPIIa, which translocates one net charge, NaPIIc is responsible for the remainder of the reuptake, in which two sodium ions bond with phosphate with no net charge transfer.

1.9.3. Sodium- Phosphate Co Transporter Type II B Encoded by SLC34A2 Gene

NaPiII type b encoded by SLC34A2 is responsible for phosphate absorption in the intestines. NaPiIIb may assist in maintaining Pi homeostasis where in mice is highly expressed in the jejunum and ileum, unlike in rats where its expression is limited to the duodenum and jejunum with null expression in the ileum. Such protein was shown to be modulated by a range of stimuli either directly or indirectly including many hormones such as thyroid, estrogens, FGF23, and glucocorticoids. Importantly, conditional deletion of NaPiIIb implies that it is involved in phosphate homeostasis through cooperating intestine and renal phosphate reabsorption. Findings suggest that NaPiIIb is a component of the machinery that controls changes to sustain systemic Pi management through the evidence that was done on NaPiII type c KO mice that showed lower intestinal phosphate absorption but normal serum Pi levels due to the overexpression of kidney Na-Pi cotransporters by the fact that low levels of FGF23 will increase the serum 1,25(OH)2D3 and therefore increasing the expression of NaPiIIa that is responsible for the major reuptake of Pi. According to these studies, Npt2b-dependent phosphate transport accounts for 45 to 50% of overall phosphate transfer in the first hour after a phosphate load.[40]

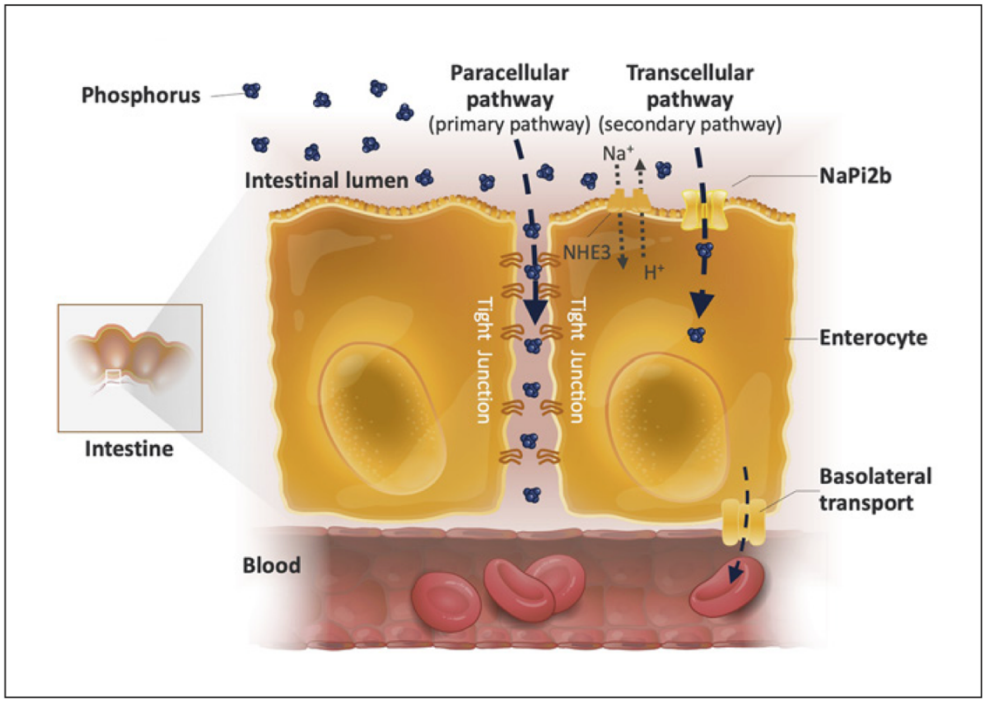

Figure 10. Dietary Phosphate Reabsorption in the Intestine [45].
In the small intestine, dietary Pi absorption occurs via two pathways either transcellular which reflects the active intestinal transport of phosphate and is the dominant one where the maximum amount is attained or it can be accomplished through paracellular route by the presence of tight junctions and passive diffusion which accounts for the vast majority of absorption in humans.

1.10. Gender Influencing Sodium-Phosphate Co-Transporters

It is worth noting that the female hormone estrogen or estradiol strongly participates in triggering bone metabolism in women at menopause, in addition to many health benefits that can arise from the administration of estradiol, such as decreasing the atherosclerotic risk especially pos-menopause. Many hormonal and non-hormonal factors can affect NaPII co-transporters, however, the impact of estradiol remains unclear [41]. Research studies have shown that estrogen increases the production of FGF23 while inhibiting PTH secretion from parathyroid glands in rats. Estrogen appeared to selectively and directly downregulate sodium phosphate co-transporter type IIA by activating estrogen receptor isoforms [42].

CHAPTER 2

OBJECTIVES

Previous studies have been made to assess phosphate's role on cardiovascular diseases, particularly hypertension, and its impact was identified on blood pressure, vascular, and kidneys.

Our preliminary data showed that dietary phosphate of 1.5% (high) can significantly decrease blood pressure in hypertensive mice infused Ang II pump.
To assess this effect, we hypothesized that phosphate modulates elevated blood pressure through sodium-phosphate cotransporters type 2 in the renal proximal tubules responsible for phosphate coupled with sodium ion reuptake and its urinary excretion.
Aim 1: Conduct the mRNA expression of fibroblast growth factor 23 and sodium-phosphate cotransporters type II encoded by the solute carrier family 34 (SLC34) in the proximal renal tubules that express SLC34A1 and SLC34A3 and serve as a final regulatory site for phosphate homeostasis and reuptake mechanisms.
Aim 2: Analyze phosphate in urine and plasma to validate our data.
Aim 3: Determining the effect of phosphate on kidneys by conducting DHE staining to look up ROS and mRNA expression of NGAL, a biomarker for acute kidney injury.

CHAPTER 3

EXPERIMENTAL PROCEDURES

3.1. Animal Model and Approval

C57BL/6 male mice aged 12 weeks were used in this investigation, procured from the Animal Care Facility at the American University of Beirut. The Animal Care Facility Animal Ethics Committee accepted the study's protocols and methods, which were carried out in accordance with the IACUC Guidelines for the Ethical Use of Animals in Research.

Mice were fed Teklad food and water ad libitum for 12 weeks prior to the experiment and for one week during the adaptation period, with baseline blood pressure measurements taken. Mice were kept in cages at 25 degrees Celsius with a 12 hour light/dark cycle.

3.2. Study Design

Male Mice of origin C57BL/6 were given a chow diet during the one-week adaptation phase followed by an additional one week known as " Baseline" during which blood pressure measurements were recorded. After mice were fed a chow diet, a control diet consisting of 0.3% phosphate was given for them to adapt to the new type and shape. Following this interval, mice were randomly assigned to one of two groups: Sham, regarded as the control in our study, or ANG II groups.

To induce hypertension, mini osmotic pumps were subcutaneously implanted delivering 490ng/kg/min of ANG II infusion for 4 weeks. These 4 weeks were divided into two weeks of control diet i.e 0.3% Phosphate and another two weeks during which

the mice were divided into different groups each fed with specific amounts of phosphate diet: low phosphate intake with a percentage of 0.15% phosphate, high phosphate intake 1.5% and part of mice were kept on the control diet 0.3% phosphate.

At the end of the protocol mice of both groups, Sham and ANG were sacrificed using CO2 inhalation in order to undergo further investigations. (Figure 16)

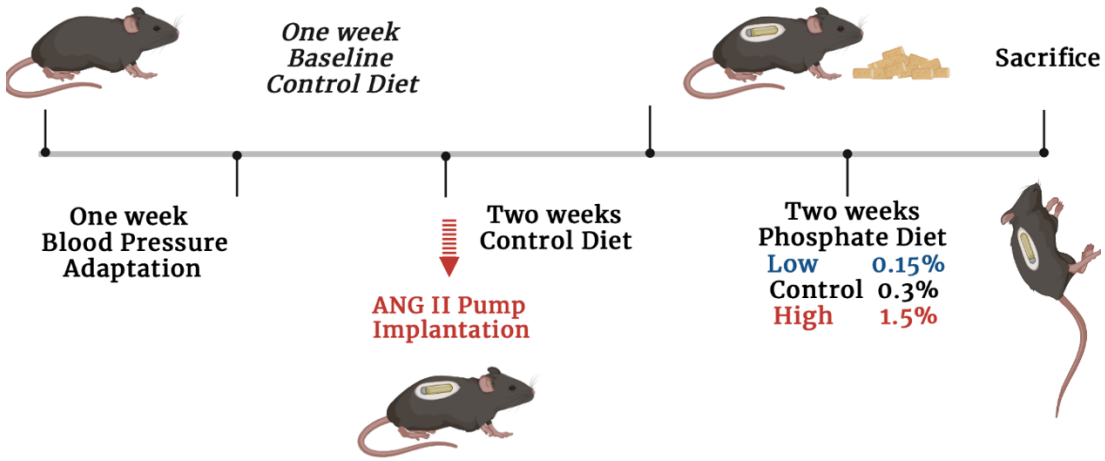

Figure 11. Study Design and Protocol.
Our protocol divided male mice into two groups, Sham and Ang. Ang group were implanted SC with Ang II infusion to induce hypertension while the Sham group are saline infused. After that mice were randomly selected to receive different amounts of phosphate, low, control, and high amounts.

Ingredients	Normal Control Group	0.15% P	0.3% P	1.5% P
Casein	200	200	200	200
L-Methionine	3	3	3	3
Starch	400	367.7	364.7	340.9
Sucrose	232	232	232	232
Oil	70	70	70	70
Cellulose	50	50	50	50
Min Mix	35	35	35	35
Vit Mix	10	10	10	10
Potassium Phosphate	0	0	6.5	59.1
Sodium Chloride	0	0	0	0
Potassium Chloride	0	32.3	28.8	0
Total Weight (g)	1000	1000	1000	1000

Table 1: Diet composition

3.4. Total RNA Extraction

Total RNA was isolated and purified from kidney samples using Trizol™ (Invitrogen, Waltham, MA) reagent using the manufacturer's protocol. First, the kidney tissues were smashed and homogenized using a mortar and pestle (homogenization phase), and then 750 µl of Trizol was added to each sample to induce the lysis of cells. Next, we added 150 µl of chloroform under the hood, vortexed very well, incubated for 5 minutes at room temperature, and centrifuged the samples for 15 minutes at 1200 RCF at 4°C. The upper layer containing RNA was collected. After that, 375 µl of isopropanol was added and samples were placed for around 20mins at -20°C and centrifuged again under the same conditions as mentioned previously. After that, the supernatant was discarded, preserving the pellet. Next, the pellets were washed twice by adding 750 µl of 75% ethanol was used twice (RNA wash). The samples were kept open to air dry for 15-20 minutes. Towards the end, the RNA pellet was resuspended in 30 µl of RNase/DNase free water. The quality (using the 260/280 ratio) and quantity (in ng/l) of the extracted RNA molecules were then determined using a Denovix

spectrophotometric nanodrop machine (DeNovix DS-11 FX Spectrophotometer). Obtained RNA samples were stored at -80°C.

3.5 Complementary DNA (cDNA)

After the extraction of total RNA for the kidneys, the complementary DNA (cDNA) of the RNA molecules were synthesized on ice using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). We took 2 µg RNA molecules. Then, a master mix was made, following the manufacturer's protocol, containing 10x RT Buffer, Multiscribe RT enzyme, dNTPs 100mM, 10x RT random Primers, and RNase/DNase free water. A cDNA reaction tube has a total volume of 20µl of RNA samples and master mix. The reaction tubes were spinned down briefly and loaded into T100 thermal cycler from Bio-Rad using the following protocol: 10 minutes at 25°C,120 minutes at 37°C, and 5 minutes at 85°C. Finally, the cDNA molecules synthesized were diluted by adding 20 µl of RNase/DNase free water, rendering DNA samples of concentration equal to 1 µg/µl. The cDNA molecules were placed at -20°C for long-term storage.

3.6 Quantitative Real Time-Polymerase Chain Reaction (RT-qPCR) Analysis

Gene expression was quantitatively analyzed by real-time RT-PCR. First, a master mix containing: 5 µl of 2x SensiFAST SYBR No-ROX Mix (meridian, BIOSCIENCES, Michigan, USA), 0.5 µl of each of the forward and reverse RNA probes, and 3 µl of RNase/DNase free water was prepared for each well. Next, the mixture was vortexed and dispersed into the wells of a Bio-Rad 384 well PCR plate. Finally, 1 µl of synthesized cDNA molecules were added to each well, rendering a total

volume of 10µ l in each well. A no-template control well was utilized for each gene, where 1 µl of the sample was replaced by RNase/Dnase-free water, to assure the absence of primer dimer formation. After that, the plate was sealed and briefly centrifuged for 1 minute at a high speed (2500 rpm). Using the Bio-Rad CFX384 machine, the following protocol was used: 2 minutes at 95°C, 5 seconds at 95°C (denaturing step), 10 seconds at 60.3°C (annealing step), and 15 seconds at 72°C(extension step). All conditions were performed in duplicates. Fold changes in the expression (Ct) of the genes of interest were calculated using the ΔΔCt method, based on the following rule:2

$$Ct = \left(Ct_{gene\ tested} - Ct_{housekeeping\ gene}\right)_{hypertensive\ mice} - \bar{X}\left(Ct_{gene\ tested} - Ct_{housekeeping\ gene}\right)_{healthy\ mice}$$

All Ct values obtained were normalized using GAPDH as a housekeeping gene. The primers used for amplification are listed in Table 2.

Gene	Sense	Antisense
SLC34A1	ACTAGGATAGGCAGGAGTAAGG	GCAAGGAGGTGTGCAAATTC
SLC34A3	CAAATGGGCGGTAGGCGTG	CCAGGTTTCCGGGCCCTCAC
FGF23	GGTGATAACAGGAGCCATGAC	TGCTTCTGCGACAAGTAGAC
NGAL	TCA CCT CCG TCC TGT TTA GG	CGA AGT CAG CTC CTT GGT TC
GAPDH	GGTGTGAACCATGAGAAGTATGA	GAGTCCTTCCACGATACCAAAG

Table 2. Primers used for qRT-PCR

3.7 Urine and Plasma Phosphorous:

Urine and Plasma samples were defrosted. Urine and plasma phosphate was measured using VITROS 350 analyzer (Ortho Clinical Diagnostics, Johnson and Johnson, Buckinghamshire, UK). Plasma phosphate was measured by direct potentiometry while concentrated urine samples were diluted by a ratio of 1:3.

Initially, before running the samples, VITROS 350 was well-calibrated using a calibrator kit 1, phosphate verifier I, and phosphate verifier II, in addition to two phosphate cartridges for urine and plasma, knowing that these cartridges were kept at room temperature to warm up before using since they were stored at 4°C. The next day, urine and plasma samples were loaded into trays (each tray is loaded with 10 samples), and measurements in mg/dL are detected on the screen.

3.8 Histology

For morphological evaluations, kidney sections were stained using dihydroethidium (DHE) to detect reactive oxygen species (ROS). Red DHE fluorescence was detected using ZEISS Axio Observer and areas of ROS were measured using the Zen lite program. All images were taken at the same setting using a 20x lens and all fixed at gamma=1.

3.9 Statistical Analysis

GraphPad Prism software, version 8 (GraphPad Software Inc.) was used for statistical analyses. All data are expressed as mean ± standard error of the mean. One-way and two-way ANOVA with multiple comparisons was employed.

CHAPTER 4

RESULTS

Our preliminary data have shown that dietary phosphate has a significant impact on blood pressure. Blood pressure results after the administration of a high phosphate diet over 2 weeks to hypertensive mice reduced blood pressure levels similar to that of the normotensive mice. Figure 18 shows that Ang II-infused mice were hypertensive at day 14 (159.978 ±5.091 mmHg), yet upon high phosphate diet feeding, mice's BP was significantly reduced (135.222 ±3.076 mmHg). It was discovered that the SBP on the hypertensive mice fed a high phosphate diet was significantly reduced by 24.8 mmHg. Likewise, its DBP was reduced considerably by 19.32 mmHg. SBP of Ang II mice fed a low phosphate diet (0.15%) was not altered.

The systolic blood pressure of Ang II-infused mice fed the control diet (0.3%P) was reduced from 154.4±4.431 mmHg on day 14 to 148.4±3.823 mmHg on day 28 by 6.1±0.608 mmHg. Similarly, the DBP was reduced from 91.86±4.573 mmHg on day 14 to 83.11 ±4.198 mmHg on day 28 by 8.75±0.375 mmHg.

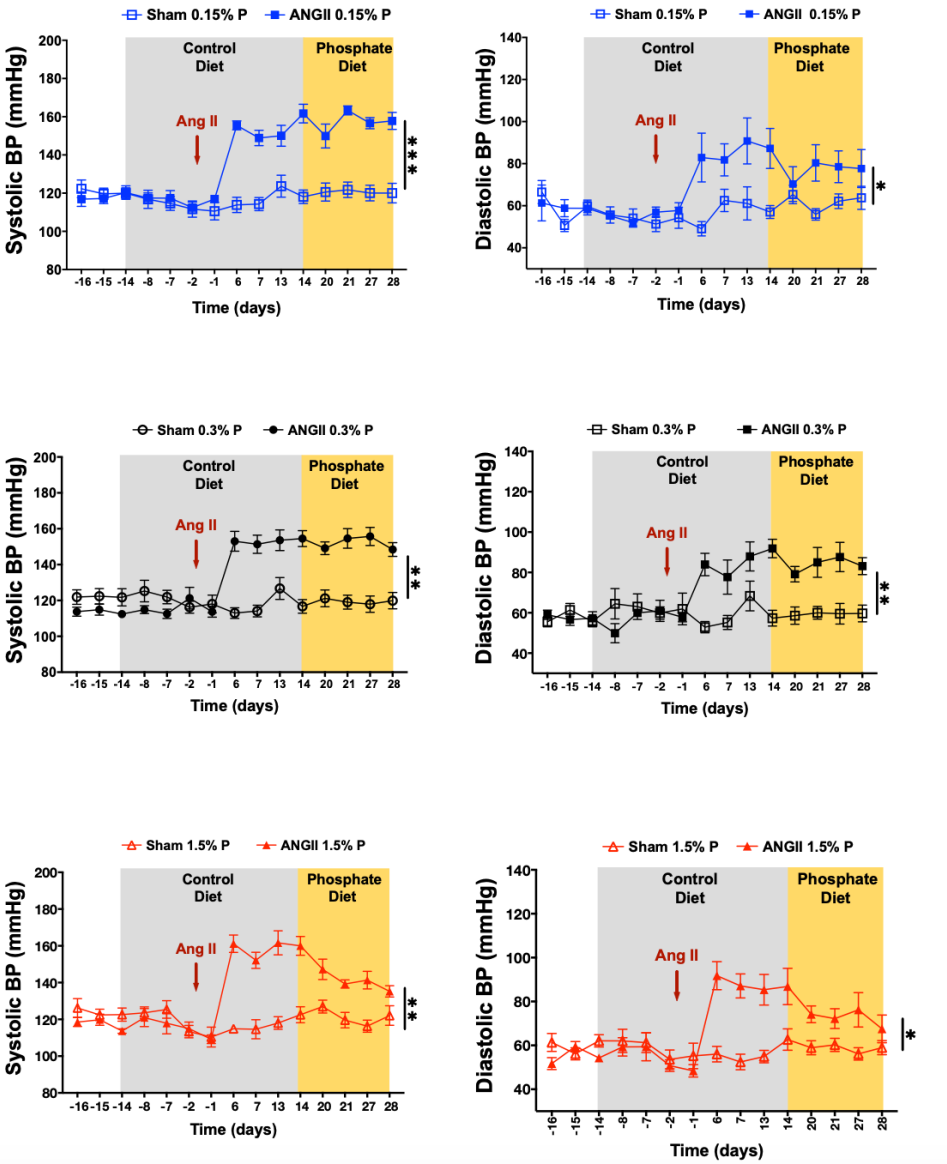

Figure 12. Previous Data on Blood Pressure Measurements.
Tail-Cuff SBP and DBP in response to 4 weeks angiotensin infusion followed by phosphate intake (0.15%, 0.3%. 1.5%). Data were analyzed using Two-Way ANOVA with repeated measurements, n=12-13 per group.

4.1. Reduced Expression of SLC34A1 in Response to High Dietary Phosphate Intake.

The mRNA expression levels for SLC34A1 encoding for NaPIIa were high during the administration of low dietary phosphate (0.15%) in both groups (Sham and

Ang). However, the levels start to decrease gradually with an increase in the amount of phosphate and this is seen in the control groups (0.3%) of both Ang and Sham.
The mRNA expression levels of SLC34A1 in the Ang of control (0.3%P) were downregulated and significance was shown between Sham and Ang both of control (0.3% P) with a P-value=0.0278 (*P-value <0.05).

Also, A reduction in the expression was observed in Ang at 0.3% representing a significance with a P-value=0.0178 (*P-value<0.05) in comparison with Ang receiving a low phosphate diet (0.15%).

In addition, SLC34A1 expression in Ang II receiving high dietary phosphate (1.5%) was shown to be significantly reduced in comparison to Ang II infused mice receiving low dietary phosphate (0.15%) with a P-value= 0.007 (**P-value< 0.01).

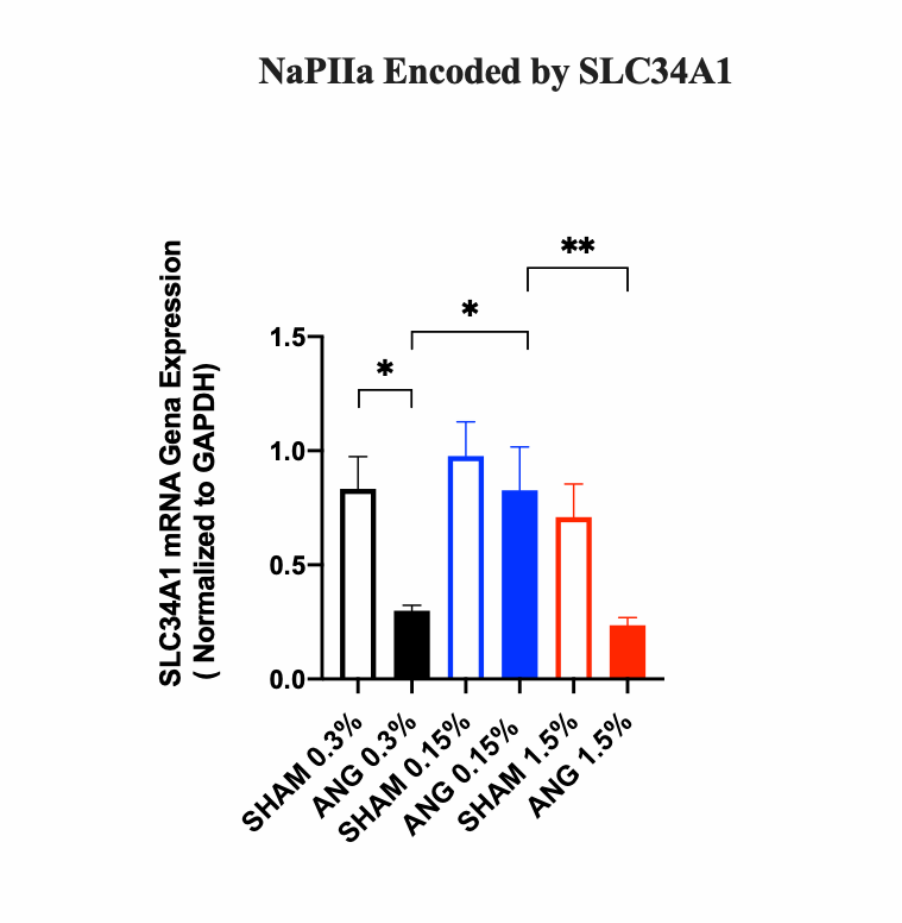

Figure 13. SLC34A1 mRNA Expression Levels.

Real-time PCR was done on kidney tissues to detect the mRNA expression level of SLC34A1 encoding for NaPIIa in Sham and Ang II infused receiving different phosphate amounts (0.15%, 0.3%, 1.5%). GAPDH was used as a housekeeping gene. Data were analyzed using Ordinary one-way ANOVA and Tukey's multiple comparisons test. N=8-12 per group. * P<0.05, **P<0.01.

4.2 Reduced Expression of SLC34A3 in Response to High Dietary Phosphate Intake

The mRNA expression level of SLC34A3 in the Ang II-infused mice receiving a control phosphate diet (0.3%) was vastly reduced when opposed to Sham of the same group (0.3%) with a P-value= 0.0001 (***P < 0.001).

Notably, downregulation in the expression was observed in Ang at 0.3% representing a significance with a P-value=0.0027 (**P-value<0.01) as compared with Ang receiving a low phosphate diet (0.15%).

SLC34A3 expression in Ang II (1.5%) was shown to be markedly lower than Ang (0.15%) with a P-value= 0.0008 (***P-value< 0.001).

Regarding Sham groups, downregulation in SLC34A1 in the sham group receiving high dietary phosphate (1.5%) was shown and appeared to be significant with the sham receiving low dietary phosphate (0.15%). *Pvalue=0.0385 (*Pvalue<0.5).

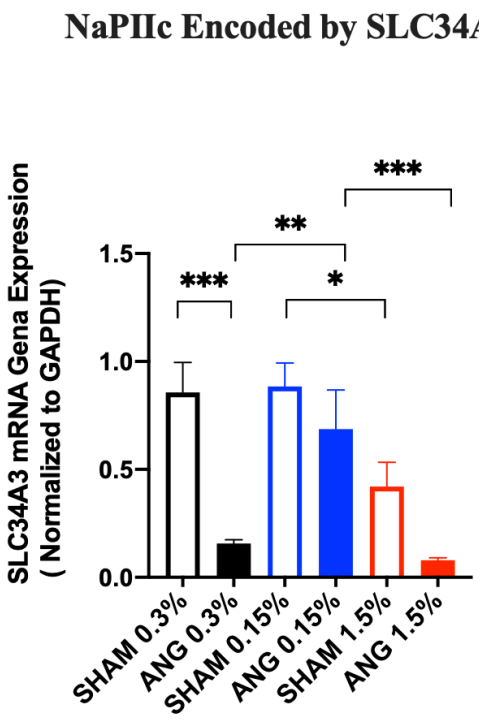

Figure 14. SLC34A3 mRNA Expression Levels.
Real-time PCR was done on kidney tissues to detect the mRNA expression level of SLC34A3 encoding for NaPIIc in Sham and Ang II infused receiving different phosphate amounts (0.15%, 0.3%, 1.5%). GAPDH was used as a housekeeping gene. Data were analyzed using Ordinary one-way ANOVA and Tukey's multiple comparisons test. N=8-12 per group. *P<0.5, **P<0.01, ***P<0.001.

4.3. Upregulation of FGF23 in Response to Dietary Phosphate Intake

FGF23 mRNA expression levels were found in both the sham and Ang groups consuming various percentages of dietary phosphate, as FGF23 is a key mediator of Pi homeostasis.

FGF23 was maximally upregulated in the Ang group receiving control diet of 0.3% and shown to be significant with its respective sham and with Ang receiving low dietary phosphate (0.15%) with a *P value= 0.012 and 0.0327 respectively. (Pvalue<0.5).

No significance was detected between Ang II infused mice receiving high dietary phosphate.

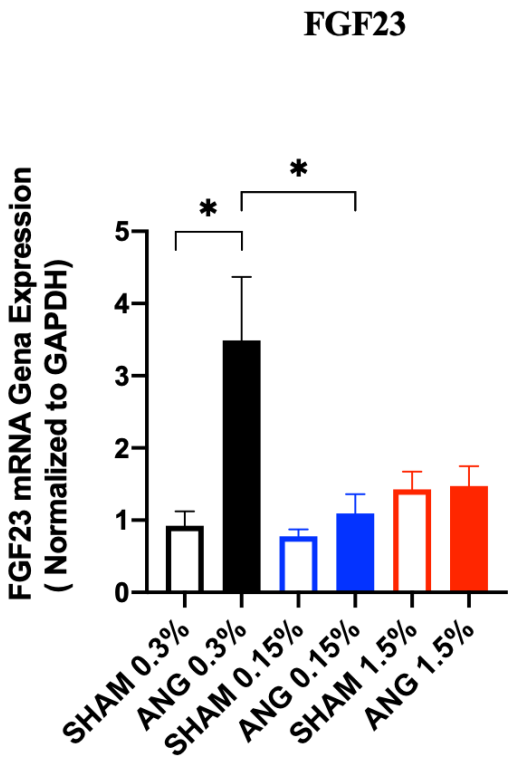

Figure 15. FGF23 mRNA Expression Levels.

Real-time PCR was done on kidney tissues to detect the mRNA expression levels of the FGF233 gene in Sham and Ang II infused receiving different phosphate amounts (0.15%, 0.3%, 1.5%). GAPDH was used as a housekeeping gene. Data were analyzed using Ordinary one-way ANOVA and Tukey's multiple comparisons test. N=7-12 per group *P<0.05.

4.4. Increased Urinary Phosphate Excretion with Increased Amounts of Dietary Phosphate

To validate and correlate our results, the excretion of phosphate was evaluated by measuring its levels in urine using VITROS 350 analyzer.

There's a trend to increase phosphate excretion in urine upon increased phosphate supplementation.

Increased excretion was shown in the sham group receiving high dietary phosphate and appeared to be significant with the sham receiving low dietary phosphate (0.15%).

***P-value=0.0003 <0.001.

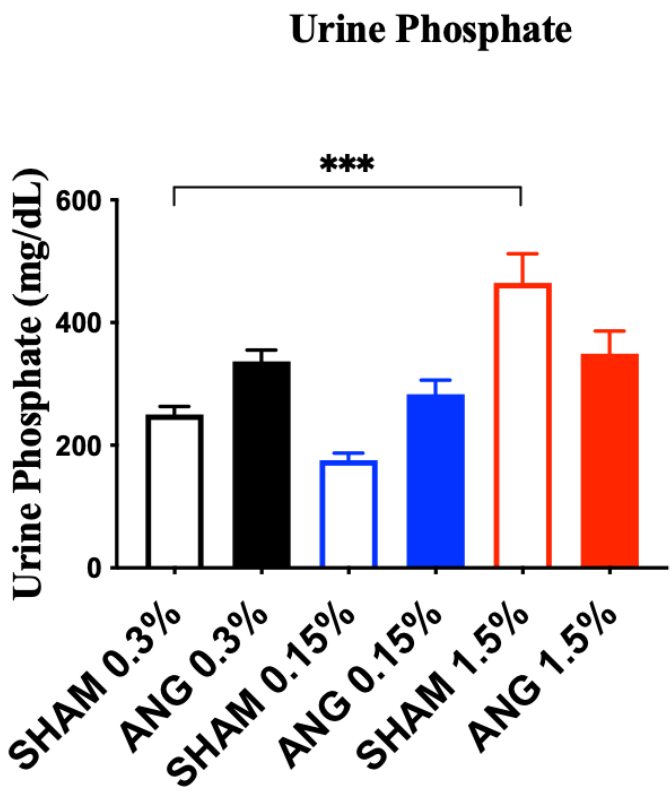

Figure 16. Renal Phosphate Excretion.
The phosphate levels were measured in the urine in both Sham and Ang II infused mice receiving 0.15%, 0.3%, and 1.5% phosphate diet. Data were analyzed using Ordinary one-way ANOVA and Tukey's multiple comparisons test. Data expressed as mean ±SEM with N= 5-13 in each group. ***P<0.001.

4.5. Plasma Phosphate Levels, No Significant Variation

To validate and correlate our results, phosphate levels in serum were detected. It is found that plasma phosphate levels didn't change remarkably between groups. However, a slight decrease in plasma phosphate was detected when comparing Sham 0.15% (9.17 ± 1.37 mg/dL) with Ang 0.3% (8.31± 0.58 mg/dL) and with Ang 1.5% (8.1± 2.2 mg/dL). However, no significance was estimated between them. Similarly, plasma phosphate of Sham 0.3% was higher (9.4mg/dL ± 1.47) compared to Ang 0.3% (8.31± 0.58 mg/dL) and Ang 1.5% (8.1± 2.2 mg/dL) but no marked difference appeared. (Figure 23).

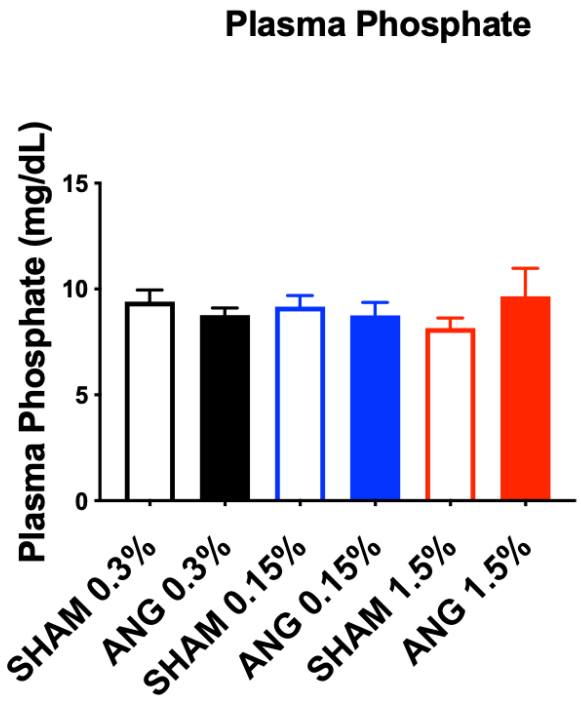

Figure 17. Plasma Phosphate Levels.
The phosphate levels were measured in the serum in both Sham and Ang II infused mice receiving 0.15%, 0.3%, and 1.5% phosphate diet. Data were analyzed using Ordinary one-way ANOVA and Tukey's multiple comparisons test. Data expressed as mean ±SEM with N= 8-12 in each group.

4.6. Exacerbated Kidney Injury in Angiotensin Infused Mice Receiving High Dietary Phosphate

To assess the acute renal injury, mRNA expression levels for Neutrophil Gelatinase-Associated Lipocalin (NGAL), were detected in both Sham and Ang II infused mice receiving dietary phosphate (0.15%, 0.3%, 1.5%).

NGAL expression levels are upregulated in the Ang II-infused mice compared to the Sham groups. Maximal expression was shown in Ang II infused mice receiving high dietary phosphate (1.5%) and significance appeared between Ang 1.5%P with its respective Sham group ****Pvalue<0.0001.

Another significance is detected between Ang (1.5%P) with Ang (0.15%P) and with Ang (0.3%P) with a ***P-value<0.001 and ****Pvalue<0.0001 respectively.

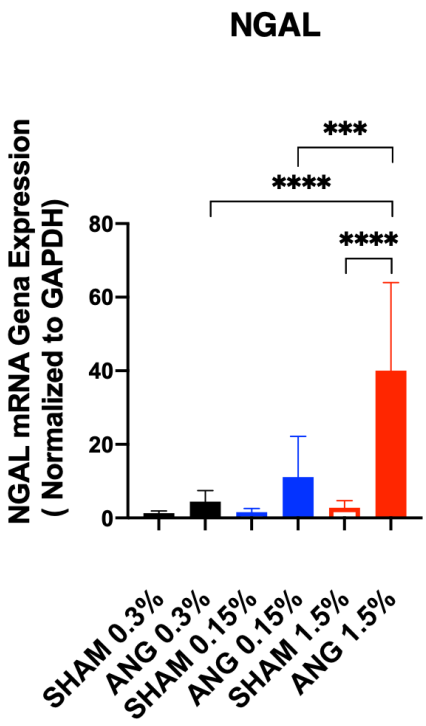

Figure 18. NGAL, AKI Biomarker mRNA Expression.
Real-time PCR was done on kidney tissues to detect the mRNA expression levels of the NGAL gene in Sham and Ang II infused receiving different phosphate amounts (0.15%, 0.3%, 1.5%). GAPDH was used as a housekeeping gene. Data were analyzed using Ordinary one-way ANOVA and Tukey's multiple comparisons test. N=8-12 per group ***P-value<0.001 and ****P<0.0001.

4.7. High ROS in Ang II Infused Groups

To assess the morphology of the kidney in response to angiotensin and dietary phosphate, kidney sections were stained with dihydroethidium (DHE), and confocal representative images were taken. Moreover, fields were analyzed to see the influence of both ang and phosphate on ROS generation.

Visually, representative figures reveal the presence of ROS in both Sham and Ang groups. However, Angiotensin has a notable effect, as ROS are substantially expressed in Ang kidney sections (Figure 25).

Data analysis in the sham group fed a low phosphate diet showed less MFI (1787.52 ± 304.34) compared to the Ang II infused mice receiving 0.15% P, where MFI was highly increased (3247.77 ± 584.54). Significance was shown with a P-value= 0.0037 (**P-value < 0.001).

Similarly, ROS was shown to be more in the Ang II group fed a control diet (0.3% P), (MFI= 2411.13 ± 630.5) in comparison to it sham group which revealed a less generation of ROS (MFI= 1762.73 ± 317.64). A slight significance was indicated between them with a P-value of 0.0195. (*P-value<0.05)

A noticeable difference was detected between Ang (MFI=3053.61 ± 847.77) and sham (MFI= 1706.08 ± 599.1) receiving high dietary phosphate, as Ang, 1.5% revealed a great increase in ROS where the significance was shown with a P-value= 0.0014 (***P-value < 0.001). (Figure 26)

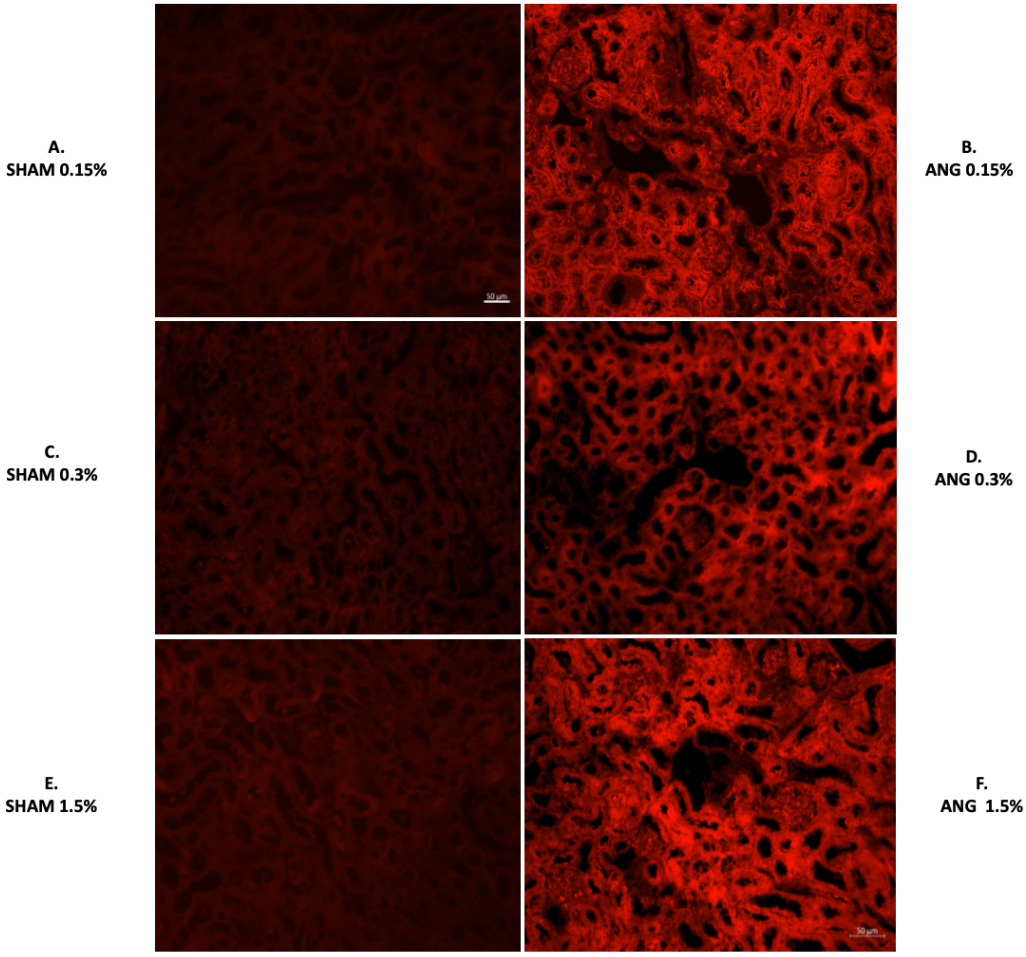

Figure 19. Representative Images of DHE Stained Kidneys.
Evaluation f production of ROS in A versus B (Sham 0.15% vs Ang 0.15%), C versus D (Sham 0.3% vs Ang 0.3%), and E versus F (Sham 1.5% vs. Ang 1.5%). Images were taken using ZEISS AXIO OBSERVER under the same setting. Magnification of 20x, Gamma=1, Scale bar= 50 μm.

DHE

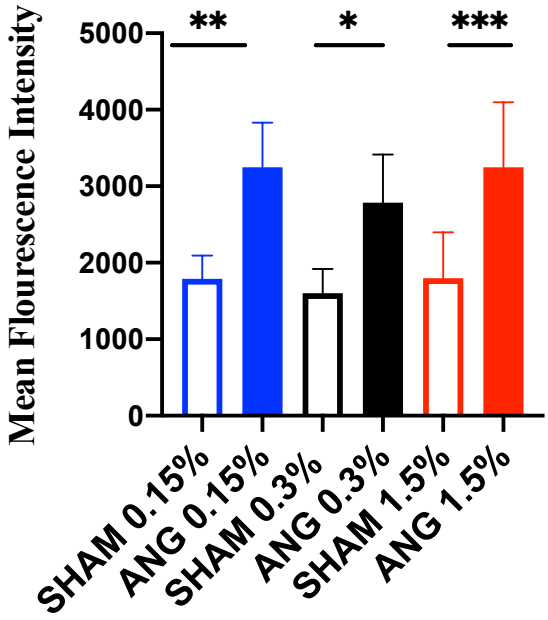

Figure 20. Mean Fluorescence Intensity Reflecting Reactive Oxygen Species. Analysis of kidney reactive oxygen species production in sham and Ang II infused mice receiving different % of phosphate diet. Superoxide production is increased in Ang-infused mice compared to the sham mice in all groups. One-Way ANOVA and multiple comparisons are conducted. Values are means ± S.E. *P-value<0.05, **P-value<0.01, ***P-value < 0.001.

CHAPTER 5

DISCUSSION

To investigate the effect of phosphate on hypertension, C57BL/6 male mice were infused with Ang II pumps subcutaneously and fed different amounts of phosphate from 0.15% to 0.3% reaching a high concentration of 1.5%. Our lab investigated the effect of phosphate on BP, immunity, and vasculature and discovered that the SBP on the hypertensive mice fed a high phosphate diet was significantly reduced by 24.8 mmHg. Likewise, its DBP was reduced considerably by 19.32 mmHg, suggesting that the readings were almost similar to normotensive. In mice that received a control diet of 0.3%, BP was also significantly reduced but did not reach the BP of the normotensive ones. The mechanism by which high dietary phosphate decreases BP is still unclear.

The drop in BP in hypertensive mice following a high-phosphate meal administration is related to decreased expression and/or activity of the sodium-dependent phosphate cotransport SLC34A1-3 in the kidneys due to a high level of serum phosphate. This decrease will negatively impact phosphate and sodium reabsorption through those co-transporters, where both phosphate and sodium couple together, resulting in their elimination in urine and, consequently, lowering blood pressure.

To understand how phosphate modulates hypertension, mRNA expression levels on kidney tissues were evaluated to glance at the SLC34 family mainly SLC34A1 SLC34A3, and FGF23, key modulators in phosphate homeostasis.

Starting with a discussion about SLC34A1 and SLC34A3 encoding for NaPiII type and c responsible for the reabsorption of 70% and 30% of phosphate respectively

where both revealed the highest expression in mice fed a low dietary phosphate (0.15%) in both sham and ang groups. However, as the concentration of dietary phosphate increases to control (0.3%) and high diet (1.5%), downregulation in the expression occurred.

Our findings have shown in both SLC34A1-3 downregulations in Ang receiving the control diet (0.3%P) which was significant compared with it respective sham. It seems that Ang has an impact on downregulating the abundance of these transporters regardless of the diet based on the comparison to its sham. In contrast to our results, Brison et al. investigated that acute ANG II infusions in rats can enhance the redistribution of NaPII thus leading to sodium and water retention. [43] Xu. et al showed that angiotensin II can increase the abundance of NaPIIa at the level of brush border membrane. [44]

A decreased expression was shown in Ang groups with increased amounts of dietary phosphate in both SLC34A1-3 and in addition SLC34A3 demonstrated considerable downregulation between sham 0.15% and sham of 1.5%. These results are in accordance with Wubuli et al, who developed a strong relationship in SLC34A1 overexpression in the renal cortex between high and low phosphate groups, with low phosphate values showing more than 3 times greater expression than high phosphate groups with low SLC34A1 expression. [45] Our study results support the concept of phosphate homeostasis, under which a low dietary or serum phosphate upregulates SLC34A1 to prompt phosphate reabsorption at the level of PCT, while a high dietary or serum phosphate downregulates these levels to prevent further phosphate reuptake and increase excretion of phosphate.

Another possible mechanism for reducing SLC34A1 is angiotensin mediating renal damage. Rubinger et al. discovered a reduction in NaPII proteins in acute renal failure. [46]

A rise in phosphate concentration can decrease the expression of NaPIIa, limiting phosphate absorption and increasing its excretion where it partners with sodium in the urine, resulting in considerably lower blood pressure. Research on rats fed high dietary phosphate has shown a decrease in the expression of NaPIIa levels. [47] Furthermore, a possible reason for the decrease in BP in mice receiving high dietary phosphate (1.5%) can be related to the activation of certain paracrine factors that can exert negative feedback, thus regulating BP. Zhang et al studies on humans showed that high phosphorus intake was observed to cause a drop in serum renin and Ang II, a considerable decrease in serum aldosterone, and a notable increase in the atrial natriuretic peptide (ANP) that inhibits NCC and ENaC in the distal tubules thus promoting urine excretion of the increased salt loading. [48]

It seems that the presence of angiotensin with the presence of increased amounts of dietary phosphate have the potential in supressing more the expression to decrease BP and maintain homeostasis. Studies done by Makhanova showed that the expression of SLC34A3 and many other transporters had been decreased following Ang II infusion. [49] In addition, angiotensin can further decrease SLC34A3 expression regardless of the amount of phosphate present because ang can influence renal damage. Rubinger et al. discovered a reduction in NaPII proteins in acute renal failure. [46]

Regarding FGF23, a key modulator in the phosphate homeostasis is where it senses the amount of serum or dietary phosphate and stimulates SLC34A1-3 at the level of kidneys through the klotho receptor to manage and maintain phosphate balance.

Our results show that FGF23 has the least expression with low dietary phosphate in both groups sham and ang, however, the levels were upregulated in the Ang group receiving control diet of 0.3%. One possible mechanism that aims to achieve homeostasis is explained by the fact that low dietary phosphate can decrease the release of FGF23 from bones, thus low amounts of FGF23 will greatly sense SLC34A1-3 in kidneys to achieve reuptake of phosphate and vice-versa. Wang et al. conducted studies on hens that received high dietary phosphate and showed that the mRNA expression levels of FGF23 increased dramatically in the calvaria, femur, and tibia. Unlike, hens that received a low phosphate diet, FGF23 expression levels were suppressed. [50]

FGF23 reached its maximum in the ang group fed a control diet of 0.3%P, and this group showed significance with Sham 0.3%P and Ang 0.15%P. Our BP results are expected to be highest in the group of ang 1.5% since with high dietary phosphate, FGF23 is expected to be the highest in comparison with other groups as it causes massive suppression in the SLC34A1-3 thus contributing to the decrease in BP. However, it might be reasonable to discuss the maximal increase in Ang 0.3% and highlighting the relation between FGF23 and CKD. Erban et al. stated that FGF23 is highly elevated in the early stages to correct the imbalance of phosphate; however, in late stages, FGF23 is no longer highly elevated to overcome the increase in phosphate, which might explain why in ang 0.3% FGF23 is more upregulated than Ang 1.5% however further investigation on kidney disease should be assessed. [34]

To validate our data, urine excretion of phosphate was measured and showed a trend with increasing amounts of dietary phosphate, the excretion of phosphate in urine is elevated. A significance was detected when between sham of control and sham receiving high dietary phosphate. This takes us back to phosphate homeostasis. The

fact that the body is maintaining the balance where in low serum or low dietary phosphate, the body preserves phosphate by increasing its uptake through NaPII present in kidneys therefore, less phosphate appears in the urine. When the phosphate levels are elevated the body gets rid of excess phosphate through renal excretion thus more phosphate levels appears in the urine. In addition, Ang might have an impact on phosphate excretion. Our results didn't show any considerable variations in urinary phosphate in hypertensive mice but according to Xu et al. investigated the effect of Ang II on rats and found that phosphate excretion was elevated following Ang II implantation. [44]

To further validate our data, plasma phosphate levels were measured however no significance appeared between the groups, and no considerable difference in the level of phosphate between groups was detected. Phosphate is tightly regulated, and serum phosphate is maintained by the cooperation of multiple organs. Not only do the kidneys play a major role, but also the intestine and the expression of NaPIIb play a key role in maintaining serum phosphate. Our results are in accordance with studies done by Dwaib et al. where rats fed phosphate didn't show any significance in their serum phosphorous levels [51].

We conducted studies on the kidney to assess phosphate's effect in Ang II-infused mice.

NGAL, a biomarker for acute kidney injury, has been shown to be increased in the angiotensin groups compared to the sham groups. This is related to the damaging effect of angiotensin on the kidney. Kuwabara et al. studied mice to highlight the influence of the renin-angiotensin system in acute renal injury, where employing

Candesartan, an angiotensin receptor blocker, drastically decreased the levels of NGAL in urine, implying that NGAL is raised by RAS activation. [52]

Considerable significance in the ang 1.5% P group with its sham and with ang 0.3%P, ang 0.15%P suggesting that high dietary phosphate attained a massive kidney injury compared to the other groups. High dietary phosphorus has been shown in investigations to initiate and/or worsen the progress of renal failure, whereas dietary phosphate limitation reverses and/or limits the dysfunction. [53]

Another experiment was done to evaluate ROS generation's effect on kidneys. DHE staining showed significance between the following: sham 0.15% and ang 0.15%, sham 0.3% vs. a 0.3%, sham 1.5% vs ang 1.5% suggesting the fact that angiotensin is the main contributor to the formation of ROS in the kidneys. Li et. Al research on WT mice showed that Ang II infusion significantly increased NADPH oxidase activity and enhanced the formation of ROS.[54]

CHAPTER 6

CONCLUSION

In conclusion, our animal model (WT C57BL/6 male mice) was subjected to a sham and ANG infusion where both groups received different phosphate amounts, low (0.15% P), control (0.3%P), and high (1.5%P). Our previous data showed that blood pressure was significantly reduced exclusively in hypertensive mice receving 1.5%P

One possible mechanism behind the modulation of BP can be related to our results that showed a significance in the SLC34A1-3 and regulated by the expression of FGF23. SLC34A1-3 refer to the gene coding of Sodium Phosphate Co-transporters type IIA-C respectively, whereupon high intake of phosphate, SLC34A1-3 showed decreased expression, thus contributing to decreased phosphate coupled with sodium intake and increased excretion. Excretion of phosphate was shown to be increased in urine and no significant variation in serum phosphate.

In contrast to the protective phosphate levels on blood pressure, the effects on kidneys didn't show any protection. ROS was highly detected in groups of mice that are Ang II infused explaining and confirming that Ang II has deleterious effect on kidneys and NGAL was maximal in the Ang II infused group receiving high dietary phosphate.

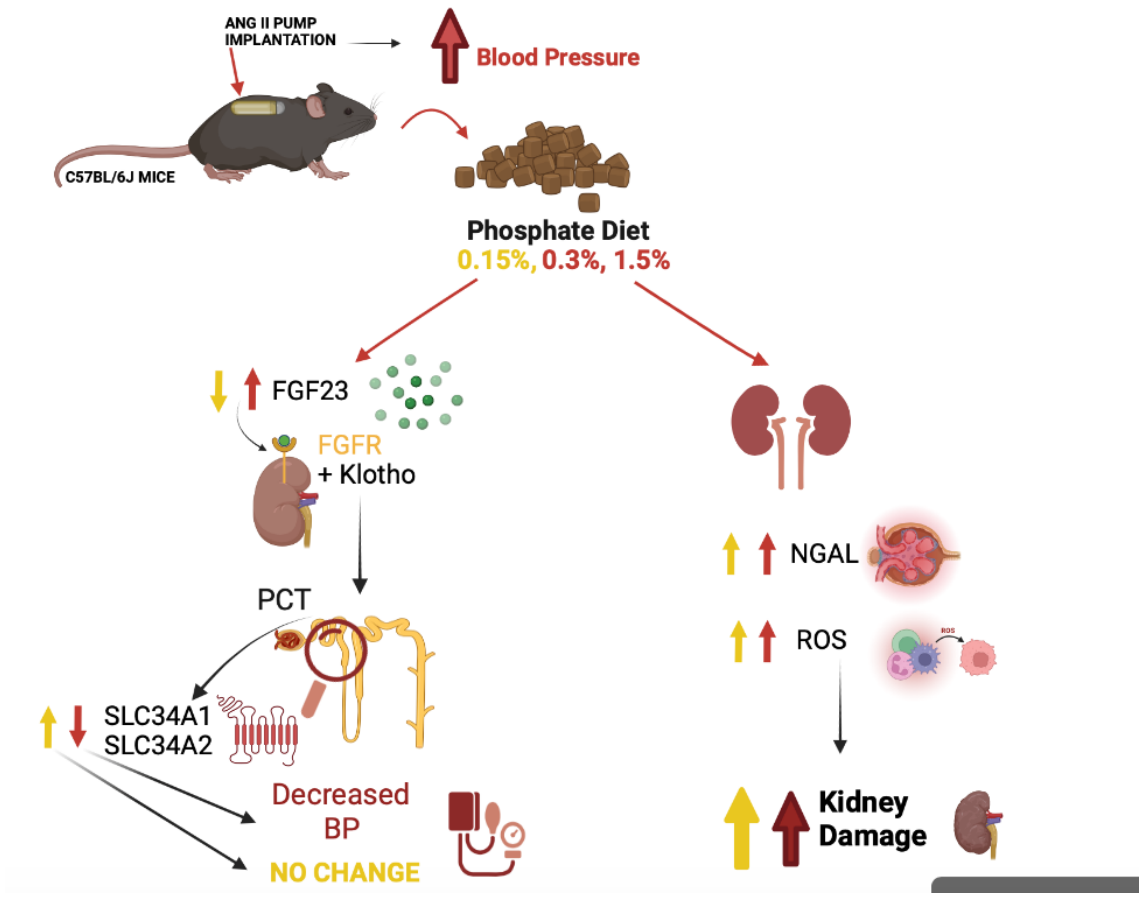

Figure 21. Summary of the Findings.
Our findings reveal that low dietary phosphate levels reduce FGF23 expression, triggering upregulation of SLC34A1-3 expression to compensate for the low levels and promote phosphate reabsorption. In contrast, high dietary phosphate means greater concentration detected by FGF23, which would downregulate the expression levels of SLC34A1-3. All concentrations demonstrated higher levels of NGAL and ROS by the hypothesis that angiotensin mediates the activation of ROS and renal injury, which can be aggravated by increasing phosphate concentrations.

CHAPTER 7

LIMITATIONS AND FUTURE PERSPECTIVES

To better understand and validate the exact mechanism by which phosphate modulates hypertension and significantly lowers BP, we aim in the future to:

1. Analyze sodium in serum and urine.
2. Quantitatively analyze mRNA gene expression of NaPIIB in the intestinal level to validate the data of plasma phosphate.
3. Western Blott for NaPiIIa/s to give an idea about any posttranslational mechanisms.
4. Assess the activity of these transporters, an alternation between mouse models under conditions of high versus low dietary phosphate is reasonable, such as:
    - Using a female mouse model to assess gender differences impact on transporters' expression levels.
    - Knocking out Sodium-Phosphate Co-transporters Type IIa/c to determine the importance of these transporters in phosphate homeostasis.
    - Pharmacological Inhibition of these transporters.

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