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THE ROLE OF DIETARY PHOSPHATE IN HYPERTENSION, VASCULAR FUNCTION AND INFLAMMATION.

by
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ABSTRACT
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Endothelial dysfunction is a hallmark of hypertension and other vascular diseases. Endothelial nitric oxide synthase (eNOS) is essential for endothelial function and homeostasis, and eNOS dysregulation is a key common pathophysiologic step in several cardiovascular disease states. In this study, we examined the vascular function in response to dietary phosphate, which has been reported to regulate hypertension. C57bL/6 male mice were subcutaneously implanted with either saline or Ang II Infusion mini-osmotic pumps (0.25 uL/hour for 28 days) before the diet protocol and underwent the 2-week control diet of 0.3% and 2-week phosphate diet (0.15%, 0.3%, 1.5%P). After that, the mice were sacrificed, and flow cytometry for the analysis of immune cells was performed on the aorta of control and Ang II-infused mice receiving the corresponding P-rich diet (0.15%,0.3%,1.5%P).Vascular reactivity studies by Wire myography for thoracic aortas were performed. To further assess vascular function, eNOS protein and superoxide are to be measured in aorta samples. Our data showed that high phosphate diet (1.5% P) reduced blood pressure following hypertensive stimuli. However, high phosphate diet exhibited a deleterious effect on the endothelial function and vascular inflammation especially in the hypertensive Ang II-infused mice. Thus, the role of phosphate in hypertension requires additional studies to dissect the mechanisms involved in mediating its effect on the vasculature.

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ABBREVIATIONS

ACE	Angiotensin-converting enzyme
Ach	Acetylcholine
ADH	Antidiuretic hormone
ADP	Adenosine diphosphate
AGT	Angiotensinogen
AHA	American Heart Association
Akt	Ak Strain transforming/protein kinase B
Ang I	Angiotensin I
Ang II	Angiotensin II
ARIC	Atherosclerosis Risk in Communities
AT-1R	Angiotensin II type 1 receptor
AT-2R	Angiotensin II type 2 receptor
ATP	Adenosine triphosphate
B1R	Bradykinin receptor type 1
B2R	Bradykinin receptor type 2
BH4	Tetrahydrobiopterin
BP	Blood pressure
CAD	Coronary artery disease
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
ECM	Extracellular matrix

eNOS	Endothelial nitric oxide synthase
FACS	Fluorescence-activated cell sorting
FGF23	Fibroblast growth factor 23
GPCR	G-protein coupled receptor
IACUC	Institutional Animal Care and Use Committee
JG	Juxtaglomerular cells
KKS	Kallikrein kinin system
LVH	Left ventricular hypertrophy
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
P	Phosphate
PAD	Peripheral artery disease
PE	Phenylephrine
PG	Prostaglandin
Pi	Inorganic phosphate
PKC	Protein Kinase C
PSS	Physiological salt solution
RAAS	Renin Angiotensin Aldosterone System
ROS	Reactive oxygen species
SBP	Systolic blood pressure
SLC	Solute carrier family
SMC	Smooth muscle cells
SNP	Sodium nitroprusside
SNS	Sympathetic nervous system

TCM	Central T memory cells
TEM	Effector T memory cells
TIA	Transient ischemic attack
WHO	World Health Organization
XPR1	Phosphate export protein

CHAPTER 1

INTRODUCTION

According to the WHO, cardiovascular diseases (CVD) are the primary cause of death worldwide, estimating that by the year 2030, about 23.6 million people will die due to CVD (1). In the Middle East, the increase in CVD-associated mortalities is the highest with CVD risk factors (2). The CVD risk factors include non-modifiable and modifiable factors. The non-modifiable risk factors cannot be changed, such as age, gender, and family history. The modifiable risk factors include physical inactivity, smoking, diabetes, obesity, unhealthy diet, dyslipidemia, and hypertension.

CVD include coronary artery disease (CAD), characterized by myocardial infarction, anginas, and heart failure due to decreased blood and oxygen supply to the coronary artery and increased demand (3). CVD also include Peripheral artery disease (PAD), which occurs due to the constriction of the peripheral, and Aortic atherosclerosis which is characterized by the appearance of abdominal aortic aneurysm (3).

According to a study done in 2015 to determine the prevalence of modifiable CVDs risk factors among the Lebanese population in the capital Beirut and five districts: Mount Lebanon, North, Beqaa, Nabatiyeh, and the South on 1000 Lebanese participants above the age of 45 years old, showed a high prevalence of modifiable CVD risk factor with hypertension being the most remarkable risk factor (29.8%) (2).

Hypertension is a critical risk factor leading to major cardiovascular events and increased morbidity and mortality(4). Hypertension targets multiple major organs, leading to pathological changes in their morphology and function, causing end-organ damage (5). According to the guidelines of the European Society of Cardiology and the

European Society of Hypertension, these major organs include the heart, brain, retina, kidney, and vasculature. Modifications in these organs eventually lead to left ventricular hypertrophy, myocardial infarction, stroke, and chronic kidney disease (6). It is important to treat hypertension early before it leads to irreversible changes in these organs and tissues.

Hypertension is a major health crisis that leads to premature mortality worldwide by causing brain problems such as stroke or TIA, cardiovascular diseases, kidney diseases, and much more (7). According to the 'WHO', it has been noted that about 1.13 billion of the population suffer from hypertension, with one in every four men and one in every five women estimated (4). These statistics raise many concerns regarding hypertension and the importance of managing it before it is too late.

Blood pressure is defined as the blood's force against our vessels and arteries circulating in our body. The blood pressure relies on the heart to pump the blood, the vessels that are a container for that blood, and the volume of the blood itself. An indicative sign of hypertension is measuring the ratio of systolic blood pressure to diastolic blood pressure. While Systolic blood pressure is known as the heart's highest blood pressure when it contracts and pumps the blood into the arteries, diastolic blood pressure is the lowest blood pressure value and corresponds to the period during which the ventricles relax to receive the blood. Hypertension is defined by a systolic blood pressure of $\geq$ 130 mmHg and diastolic blood pressure of $\geq$ 85 mmHg (8).

There are two types of hypertension classified according to the leading cause: primary hypertension and secondary hypertension (9) (10). Primary hypertension, also known as "Essential Hypertension," occurs without a known cause, and it is the most common type of hypertension among people. Unlike primary hypertension, secondary

hypertension occurs due to an identifiable cause which might be due to evident medical conditions and diseases such as kidney failure, hyperthyroidism, renovascular problems, or the intake of medications that eventually lead to high blood pressure.

According to a Lebanese cross-sectional study performed on adults from 5 different districts between 2014 and 2015, about 399 out of 1362 adults suffered from hypertension, and only 166 knew that they had high blood pressure (11). This sheds light on how hypertension is seen as a "silent killer," which was also stated by the WHO and the AHA journals. This study indicated a high prevalence of hypertension in Lebanon especially starting in males of 18-29 years old. All these indicate the need to control and treat hypertension among the Lebanese population.

1.1. The Regulation of Blood Pressure

Blood pressure is tightly regulated by different control mechanisms involving short-term regulation and long-term regulation. The autonomic nervous system plays a crucial role in the regulation of blood pressure. Baroreceptors, involved in short-term regulation, are stretch receptors located in the blood vessels and the heart that sense any changes in the arterial blood pressure (12). When the blood pressure increases, these stretch receptors are activated, and fire action potentials to increase the SA node's vagal tone and inhibit the sympathetic tone, thus causing a decrease in the heart rate to restore the blood pressure. The opposite occurs when low blood pressure is sensed (12) (13). The regulation of arterial pressure starts within seconds by the intervention of the baroreceptors and other different components of the nervous system pressure controls. The intermediate pressure control takes over, followed by the long-term pressure control through the intervention of "Renal Blood Fluid Mechanisms" to stabilize the

arterial blood pressure. This long-term regulation has many interactions with the RAAS, the nervous system, and other mechanisms (14).

There are different systems implicated in the regulation of blood pressure such as the Renin Angiotensin Aldosterone System and the Kallikrein Kinin System (14) (15) (24).

1.1.1. Renin Angiotensin Aldosterone System (RAAS)

The Renin-angiotensin system is involved in the regulation blood pressure and volume (14) (15). This system participates significantly in the pathophysiology of hypertension and eventually leads to renal damage and failure. The over-activation of the RAAS causes the glomerular capillary pressure to rise leading to glomerular hypertrophy. Both the RAAS and especially Ang II lead to oxidative stress and inflammation, thus causing kidney disease (15).

The RAAS pathway starts with the component "Angiotensinogen" (AGT), as seen in figure 1, a globular protein that serves as the precursor of the active molecule involved in the RAS pathway. It is synthesized, and primarily secreted by the liver. AGT is cleaved by "Renin" which is stored in vesicles in the juxtaglomerular cells of the kidney (14). Renin, a highly specific aspartyl protease, is first released as pro-renin into the blood and then activated in response to low tubular sodium levels which are sensed by the Macula Densa. Once in the active form, it cleaves AGT into a decapeptide "Angiotensin I" (Ang I). Ang I is further cleaved by an "angiotensin-converting enzyme" (ACE), found in the lungs and blood vessels, into the octapeptide Ang II. Ang II, the most active vasoconstrictor in the RAS pathway, stimulates the release of aldosterone, which is a mineralocorticoid hormone secreted by the adrenal cortex to

increase the blood pressure by causing fluid and salt retention, and in addition to that, it will activate the thirst centers in the brain.

Both Ang II and aldosterone lead to the activation the enzyme NADPH oxidase, leading to the production of oxidative stress and decreased nitric oxide levels (16).

A non-ACE conversion pathway leads to the production of Ang II from Ang I through a secretory serine protease known as “Chymase”. Ang can be further cleaved into Angiotensin 1-7, which plays an opposite role to Ang II (14) (17).

This system can also be classified as a classical endocrine system. When the levels of Ang II are increased, it cycles back and switches off the production of renin, thus decreasing plasma renin levels and performing a negative feedback loop. Hence, it is important to regulate the levels of renin to have a well-regulated control over the RAAS system through the production of Ang II.

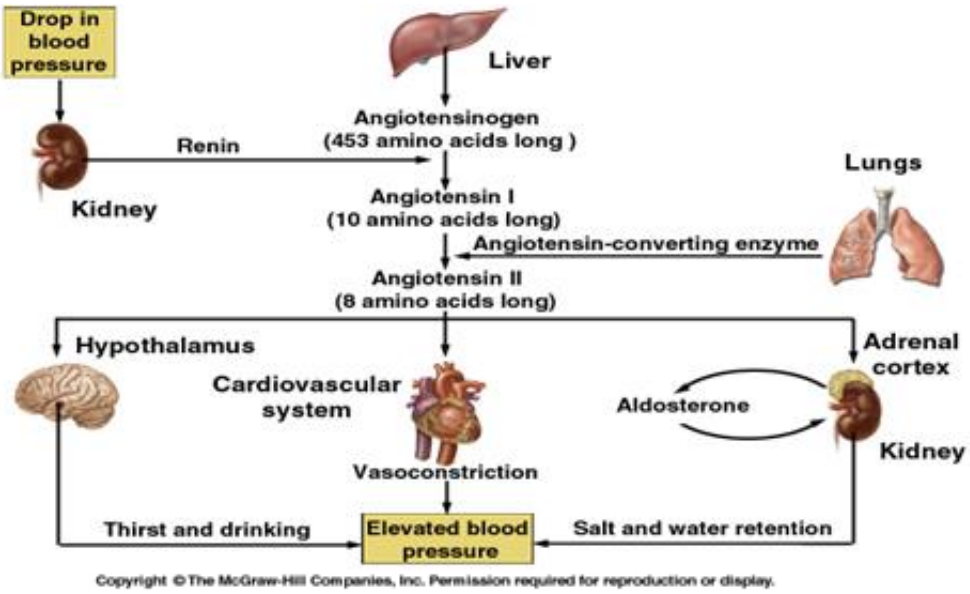

Figure 1. The different components involved in the RAAS pathway (17)

1.1.2. Angiotensin II

Angiotensin II, an important component of the RAAS, is used in many animal models especially rats and mice to induce hypertension. It mediates its effect through the action of 2 different GPCRs: angiotensin 2 – type 1 receptor (AT1-R) and angiotensin 2 – type 2 receptor (AT2-R), which are widely expressed in the heart, vessels, brain, and kidney (18).

AT1-R are more widely expressed in the cortex of a normal kidney in an adult than the AT-2R (19). The AT1-R binding site are primarily located in the glomeruli of the nephron. In contrast, the AT2-R are majorly found in the cortical blood vessels. Most of the vasoconstrictor effects of ANG II are mediated through its action on AT1-R. Angiotensin II binding to AT1-R activates the thirst centers within the brain and causes the release of ADH from the posterior pituitary gland to increase water reabsorption. It also mediates the release of catecholamines from the adrenal medulla and norepinephrine by the nerve terminal. It inhibits their reuptake, thereby prolonging their effect, and also increases cardiac contractility. All these physiological effects cause the blood pressure to increase (19).

The overexpression of AT1-R in the cardiomyocytes enhances the actions of Ang II and leads to cardiovascular remodeling and eventually to cardiac and vascular hypertrophy by increasing the cardiac afterload and vascular wall tension and the synthesis of the extracellular matrix (20).

AT2-R promotes cellular proliferation and apoptosis in the proximal tubular epithelial cells (21). AT2-R are also expressed in neonatal kidney tissues, suggesting a possible role in kidney development (22). Through the action on AT2-R, Angiotensin II leads to vasodilation, unlike its action on AT1-R which results in vasoconstriction.

AT2-R mediates its vasodilatory effect leading to the production of nitric oxide (NO) and guanosine cyclic 3',5'-monophosphate(cGMP) (23).

1.1.3 Kallikrein Kinin System (KKS)

The KKS involves a cascade of proteins in a metabolic pathway. Starting by kininogen, which is cleaved by the enzyme kallikrein into bradykinin-related peptides (24). This system has played an essential role in blood pressure control, vasodilation, smooth muscle relaxation, and inflammation (24).

KKS has a protective role against oxidative stress by decreasing reactive oxygen species and thereby preventing heart and kidney organ damage. The produced bradykinin-related peptides produce their effects through the action of 2 different receptors: bradykinin B receptor 1 (B1R) and bradykinin B receptor 2 (B2R). These receptors are GPCRS. B2R is ubiquitously expressed in the tissues, whereas B1R is expressed in response to stress conditions such as inflammation (25). Once these receptors are activated, they lead to the activation of downstream signals, which involve the rise in calcium concentration, the activation of phospholipase A2 and thus the generation of nitric oxide and prostacyclin.

The endothelial NO synthase (eNOS) enzyme is responsible for the production of NO. It oxidizes L-arginine to L-citrulline and Nitric Oxide in the presence of a cofactor (6R)-5,6,7,8-tetrahydro-L-biopterin (BH4) and Nicotinamide dinucleotide phosphate (NADPH). NO diffuses into the neighboring smooth muscle cell through a paracrine signal and activates guanylyl cyclase, leading to cGMP production. As for prostacyclin, it also diffuses into the neighboring SMC in parallel with NO but instead activates adenylyl cyclase, leading to cAMP production. Both pathways lead to the

relaxation of the SMC and provide anti-ischemic and anti-atherosclerotic effects on the SMC (25).

The bradykinin-related peptides are cleaved into inactive fragments by ACE, thereby inactivating the KKS; that is why the RAS counterbalances the KKS. The KKS serves as a protective system against oxidative stress and organ damage in the heart and the kidneys (25).

1.2 Hypertension and Vascular Damage

Cardiovascular damage in intima-media thickness, vascular stiffness, endothelial function and structure, low-grade chronic inflammation are functional and structural modifications observed in response to hypertension (26) (27). However, age, gender, sedentary lifestyles, and the physical state are risk factors that also play an essential role. Cardiovascular risk tends to increase in the presence of hypertension-mediated damage. Left ventricular hypertrophy (LVH) may result from an increase in the left ventricular workload. The left ventricle is characterized by the inability to pump blood efficiently and thickening walls, leading to arrhythmias and heart failure.

The thickness of the intima-media and the presence of carotid plaque also occur due to hypertension (26) (27). The increased blood pressure affects the morphology and the function of the vasculature, especially the arteries, by altering the arrangement of the elastic fibers. This alteration causes the deposition of collagen and calcium within the vascular wall (Weaver et al.) and the proliferation of the smooth muscles leading to arterial hypertrophy (28). Due to the stress generated by hypertension, the arterial walls' ECM is changed due to the alteration in the metalloprotease's activity.

These events trigger the release of a potent vasoconstrictor, endothelin, along with the release of inflammatory cytokines that eventually lead to endothelial dysfunction (28). All these mechanisms are depicted in the figure 2.

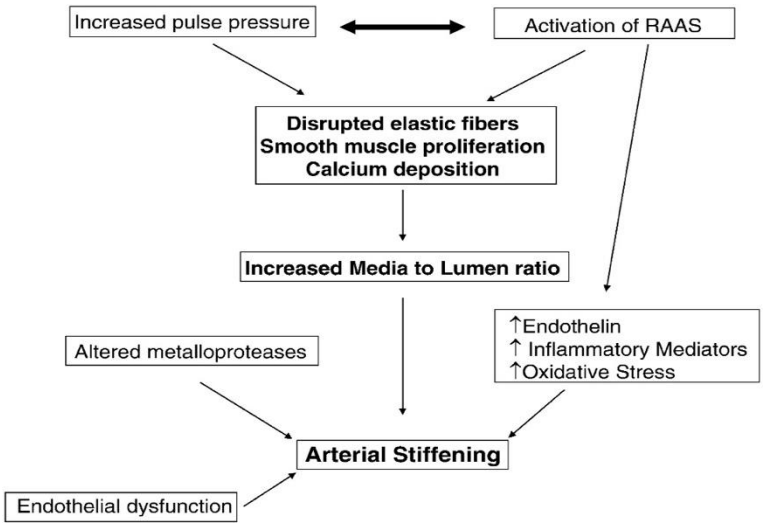

Figure 2. Changes in the vascular structure due to chronic hypertension (28)

1.2.1 eNOS and NO serving as vasoprotective agents

eNOS is a significant contributor against vascular diseases by generating vasoprotective and vasodilatory component NO (29). NO prevents platelet aggregation.

It has also been shown by Forstermann et al. that NO can inhibit the adhesion of leukocyte CD11/CD18 by preventing its adhesion to the surface of the endothelial cells. All of these suggest a possible protective role of NO and eNOS against atherogenesis (29).

1.2.2 eNOS and Oxidative Stress

It has been reported in hypertensive mouse models and other cardiovascular diseases that NADPH oxidase elevation leads to oxidative stress and ROS production (29). Angiotensin II-induced hypertension in a rat model led to increased vascular superoxide production via NADH/NADPH oxidase activation (30).

Under the effect of ROS, the bioavailability of cofactor BH4 is reduced. This affects NO production and leads to the generation of peroxynitrite ($ONOO^-$) leading to endothelial dysfunction. Endothelial dysfunction could be decreased by elevating eNOS expression leading to increased levels of NO. However, eNOS can also generate more superoxides when uncoupled, which suggests that eNOS might serve a role in the generation of more ROS in response to oxidative stress (figure 3).

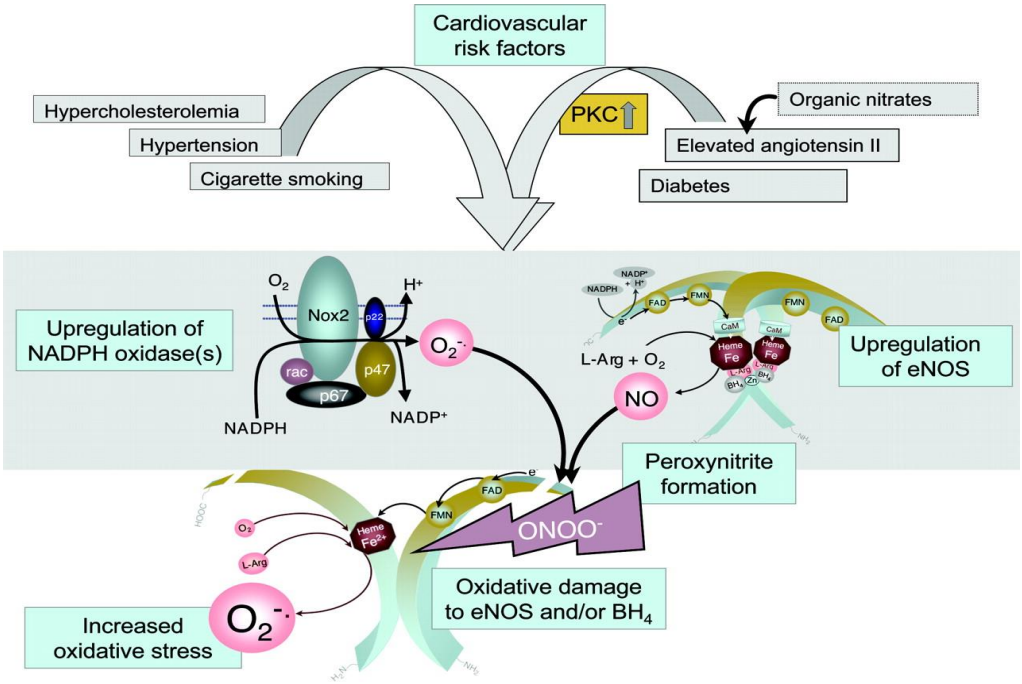

Figure 3. Endothelial dysfunction due to cardiovascular risk factors (29)

1.3 Hypertension, Inflammation, and Immunity

Itani et al. and other studies defined a significant role of the immune system in the pathogenesis of hypertension. The immunological memory, a major arm of adaptive immunity, acts as a defense mechanism against a previously encountered antigen. Once T-cells are introduced to a specific antigen, they expand. Most of these cells die by apoptosis, and those that remain are now referred to as memory T-cell (31). Those cells that remain in the periphery are termed effector T memory cells (TEM), and those that return to the secondary lymphoid organs are now called central T memory cells (TCM)

Immune cells such as T-cells and macrophages (or monocytes) tend to accumulate in the kidney and vessels due to hypertension, leading to damage. When these T cells infiltrate the kidney due to hypertension, they start secreting their inflammatory cytokines, leading to sodium retention, further aggravating the injury (31).

1.3.1 Immune Cells Involved in Hypertension

In a study by "Guzik et al." on RAG-$1^{-/-}$ mice that have no mature B and T lymphocytes, which were subjected to a chronic Ang II-infusion, hypertension was seen to be markedly blunted, and these mice had their endothelium-dependent vasodilation function preserved indicating the absence of ROS production (32,33). However, when transferring these mice with T cells only but not B cells, marked hypertension was observed similar to that noted in the wild-type mice after Ang II infusion. The mice with the transferred T-cells had their endothelial-dependent vasodilation disrupted, and exhibited a high levels of vascular superoxide following Ang II infusion. It was shown that after chronic Ang II infusion, detectable markers of activated effector T-cell were

found, including CD69, CCR5+and $CD44^{high}$ in the circulation with an elevation in chemokines level.

The adoptive transfer of CD8 cells to RAG-$1^{-/-}$ mice restored hypertension and mediated end-organ damage (3) which suggest that T-cells play a role in the pathogenesis of hypertension due to Ang II(4).

A hypertensive stimulus such as Ang II or salt intake promotes the proliferation and activation of T-cell by causing hypertension. Hence, targeting T-cells might serve as a therapeutic mechanism in hypertension (32, 33).

1.3.2 Role of Cytokines in Hypertension

As previously stated, T-cells release their inflammatory cytokines into the circulation and the kidneys as well. IL-17, a pro-inflammatory cytokine produced by TH_{17} cells, a subset of the CD4+ T cells population, plays a role in contributing to hypertension (33). CD8+ T can also secrete IL-17. IL-17A isoform contributes to hypertension (33). It has been found that in IL-$17A^{-/-}$ mice, blood pressure increased similarly to that of the wild type, however, it was not sustained. No vascular superoxides were produced, and the endothelial-dependent vasodilation was not impaired. IL-17A plays a deleterious role on the vessels through superoxide production and by causing the accumulation of inflammatory cells in the tissues after a hypertensive stimulus. Moreover, through the production of Ang II, the RAS system stimulates the production of tumor necrosis factor α (TNF α) and IL-6 (31). It has also been demonstrated by a study done on Dahl salt-sensitive rats with Ang II-induced hypertension that immune T-cells infiltrating to the kidneys of these rats produced TNF

α, IFN-gamma, and IL-17 cytokines, which lead to renal damage and a further increase in blood pressure (32)

Oxidative stress, hypertensive stimuli such as angiotensin II and salt intake, the release of cytokines, and shear stress activate NADPH oxidases which lead to the uncoupling of eNOS thus generating ROS.

ROS lead to salt and fluid retention in the kidneys, increasing the blood pressure and further exacerbating the condition (33). ROS plays a significant role in inflammation and tissue damage. It activates pro-inflammatory transcription factors such as Nrf2, NF-KB, and AP1, affecting the gene expression involved in adhesion molecules and chemokines, thus enhancing inflammatory responses (33,34).

Oxidative stress, which also plays a vital role in the pathogenesis of hypertension and other cardiovascular risk factors, alters endothelial cell permeability by increasing the entry of lipoproteins into the sub-endothelial space and their subsequent oxidation (34,35) ,thus leading to more ROS production and hence endothelial dysfunction.

1.3.3 Ang II Enhances the Immune Response

In 2018, the European Society of Cardiology and the European Society of hypertension (ESC/ESH) stated that hypertension is the second most common cause of chronic kidney disease after diabetes. Chronic hypertension causes the remodeling of the afferent arterioles of the nephrons due to the reduced blood flow to the kidney following hypertension and overstimulation of the SNS (36). When renin is over-produced by the juxtaglomerular (JG) cells, more Ang II levels are generated. Ang II leads to sodium reabsorption at the proximal and collecting duct level and further

elevates blood pressure and systemic vascular resistance (36). Figure 4 summarizes the different pathophysiologic mechanisms of hypertension in chronic kidney disease.

Ang II causes renal injury by increasing systemic and glomerular hypertension or ischemia due to the decreased renal blood flow. Ang II, as stated before, increases oxidative stress and the release of inflammatory mediators. The kidneys stimulate the release of profibrotic cytokine TGF-B, which causes inflammation and vascular and mesangial cell proliferation, and hypertrophy (37).

In a study performed on Sprague-Dawley rats fed a 4% salt diet to investigate the effect of two weeks of Ang II-infusion on the renal injury, using a servo-control technique to maintain a normal renal refusion pressure on the left kidney (38), found out that Ang II-induced hypertension promoted tubular necrosis, and interstitial fibrosis in the outer medullary region of the uncontrolled kidney glomerular injury was also observed in the outer cortex even in the servo-controlled method. Their study also indicated TGF-B and NFKB in regions of the interstitial fibrosis in the uncontrolled kidney, pointing out that it mediated its juxtamedullary injury by activating TGF-B and NFKB pathways (38).

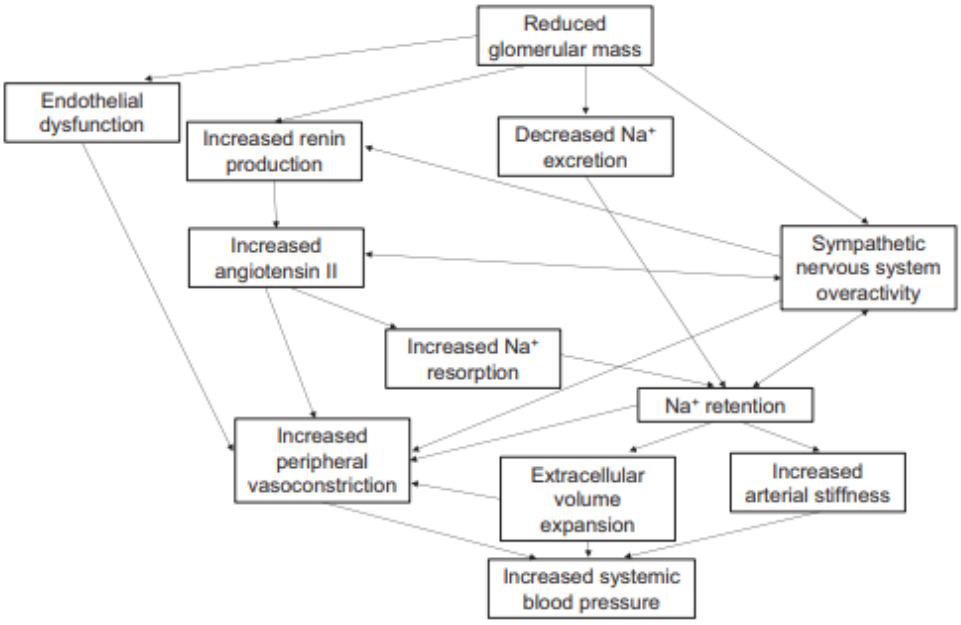

Figure 4. The pathophysiologic mechanisms of hypertension in CKD (36)

1.4 Phosphate, Hypertension, and Vessels

It has been shown for the past decade that different dietary approaches can modify blood pressure. One of the most important diets in decreasing high blood pressure is seen in the Dietary Approaches to Stop Hypertension (DASH) and the Mediterranean diet which include limiting the amount of sodium intake in food to low levels, adopting a diet rich in plant-based foods, low levels of saturated fats and limiting alcohol consumption (52).However, many studies have linked a possible role of phosphate in modulating blood pressure and exhibiting an effect on the vasculature (42, 43).

Many studies have been done to examine the role of phosphate in cardiovascular disease. In a study done to determine the relationship between serum phosphate levels and cardiovascular risk factors and metabolic syndrome components, it was found out that a high level of phosphate is associated with cardiovascular disease(42).However, a low level of phosphate is associated with metabolic syndrome(42).

In contrast, it has been stated that a high serum Pi level might lead to the increase in the activity of renin-angiotensin and thus cause hypertension through the release of FGF23 that allows vascular smooth muscle cells to uptake Pi and hence prolonged the arterial contraction and increased blood pressure (43).

Bozic et al. studied the effect of high levels of circulating phosphorous on the arterial blood pressure of healthy rats which were subjected to a high phosphate diet (1.2% P) for four weeks, showed an increase in blood pressure, which returned to normal after the addition of a phosphate binder (lanthanum Carbonate) in the diet (44). These rats also show an elevation in the expression and the activity of renin, Ang II levels, left ventricular hypertrophy, with an increase in PTH levels, all of which returned to normal after adding the phosphate binder to the diet (44).

A study performed on 13,444 participants to assess phosphorous intake with blood pressure levels and the risk of hypertension in 2 population-based studies showed that the amount of phosphorous ingested from dairy products exhibited an inverse relationship with blood pressure (45)).

Moreover, inorganic phosphate is considered an important agent to induce the trans-differentiation of the vascular smooth vascular muscle cells into osteoblast-like cells, thus causing the secretion of bone matrix proteins on the walls of the vessels and leading to vascular calcification (46).

These contrasting results illustrate how unclear the effect of phosphate on blood pressure and vasculature is. This requires additional research to investigate the role of phosphate and its consequences and if it serves as a therapeutic agent or a deleterious one over hypertension.

1.4.1 Phosphate, a general introduction

Phosphorous is the second most widely spread mineral in our body after calcium (39). Phosphate is the electrolyte that contains phosphorous minerals. Phosphorus, like calcium, plays an essential role as a building block for our teeth and bones as a "hydroxyapatite" mineral, where most phosphorous is present (39).

The normal range of phosphate in the serum is 2.5-4.5 mg/dL, and the RDA for the adults is 700 mg, and that for the children is 1200mg, but these values are usually exceeded through the diet (39). 90% of phosphate is present in the skeleton. The other amount is found in the intracellular compartment with a very small portion in the extracellular fluid with 10% in soft tissues, and less than 1 % is the serum (39). The amount of phosphate in our body comprises 1 % of the total human body weight (39). The phosphate concentration in the intracellular and extracellular compartment reflects the "only biological site accessible for phosphate measurement directly" (39). The serum/plasma form exists as inorganic phosphate (Pi), organic phosphate, and lipid phosphorous (40). The inorganic form, also known as the free phosphate form, is significantly absorbed in the gastrointestinal tract (80%) (41).

Regarding the organic form, which is organic esters and is organically bound in the food, only 40-60% is absorbed in the gastrointestinal tract. Two-third of the dietary phosphate is eliminated in the urine via the kidneys and the other portion in the feces (41). So, any excess phosphate is regulated and eliminated directly. An example of phosphate-rich food is pork, potatoes, bread, peanuts, baking powder, cola drinks. It is sometimes added to processed food as a preservative, such as meat and Ham. In cola drinks, phosphate is responsible for the brownish color because phosphate will interrupt a glycation reaction in this drink, so in the absence of phosphate, this reaction is not

interrupted and proceeds to give advanced glycation end products making the color of the cola drink pitch-black (41).The amount of phosphate in serum changes depending on different factors such as gender, age, diet, and the co-morbidity conditions of the person (39).

Phosphate is involved in nearly all biological processes. It is required in nucleic acid synthesis and metabolism, serving as the backbone of the nucleotide of DNA and RNA, muscle function, cellular signaling, energy metabolism as ATP where it can be stored and transferred as per the cell's need and when released, it is hydrolyzed into ADP to be utilized, and it is also involved in bone mineralization (40). It is also a part of the phospholipids that make up the cell membrane. It also has an important role in the central nervous system as it facilitates nerve conduction (39). It plays the role of a buffer in our blood to keep the pH of the blood balanced and regulated. The figure below summarizes the biological processes in which inorganic phosphate is involved in (39).

Any deficiency in phosphorous levels lead to abnormal bone mineralization, osteoporosis, muscle weakness, impaired leukocyte function, and other clinical diseases that reflect the importance of the participation of phosphate in vital biological processes

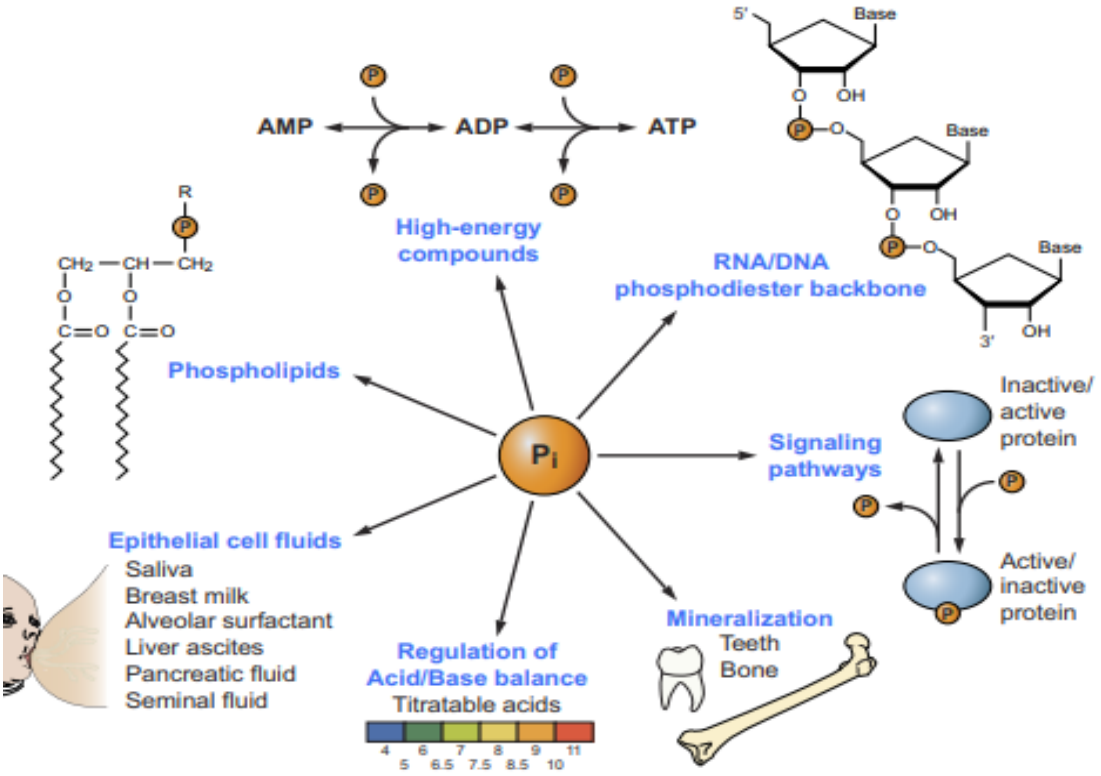

Figure 5. The biological processes of inorganic phosphate (39).

1.4.2 The regulation of Phosphate Homeostasis

Both the kidneys and the intestine play an important role in the absorption and the excretion of the Pi. Many hormones and regulators are involved in phosphate homeostasis, especially at the sites of absorption and reabsorption, such as Vitamin D, parathyroid hormone, and phosphatonins FGF23 (40). Vitamin D increases the intestinal absorption of Pi. In the kidneys, Pi reabsorption is influenced by PTH intervention at the level of the proximal convoluted tubule and the straight tubule (41).

Calcium is also involved in the homeostasis of phosphate; when calcium's concentration is elevated in the intestinal lumen, it causes the reduction of Pi intestinal reabsorption. In figure 6, when serum Pi drops due to low phosphate diet intake, PTH release is inhibited by an increase in the serum Ca concentration, which causes the reduction of the renal phosphate excretion to increase the serum Pi back to normal. In Parallel to that, when the serum Pi is low, the renal synthesis of 1,25-(OH)2 D3 is increased due to the stimulation of 25-hydroxyvitamin D3 1α-hydroxylase activity, which is expressed in the epithelium of the nephrons, to increase the intestinal absorption and the renal reabsorption of Pi (40). When serum Pi levels increase, the opposite occurs, as seen in the figure below.

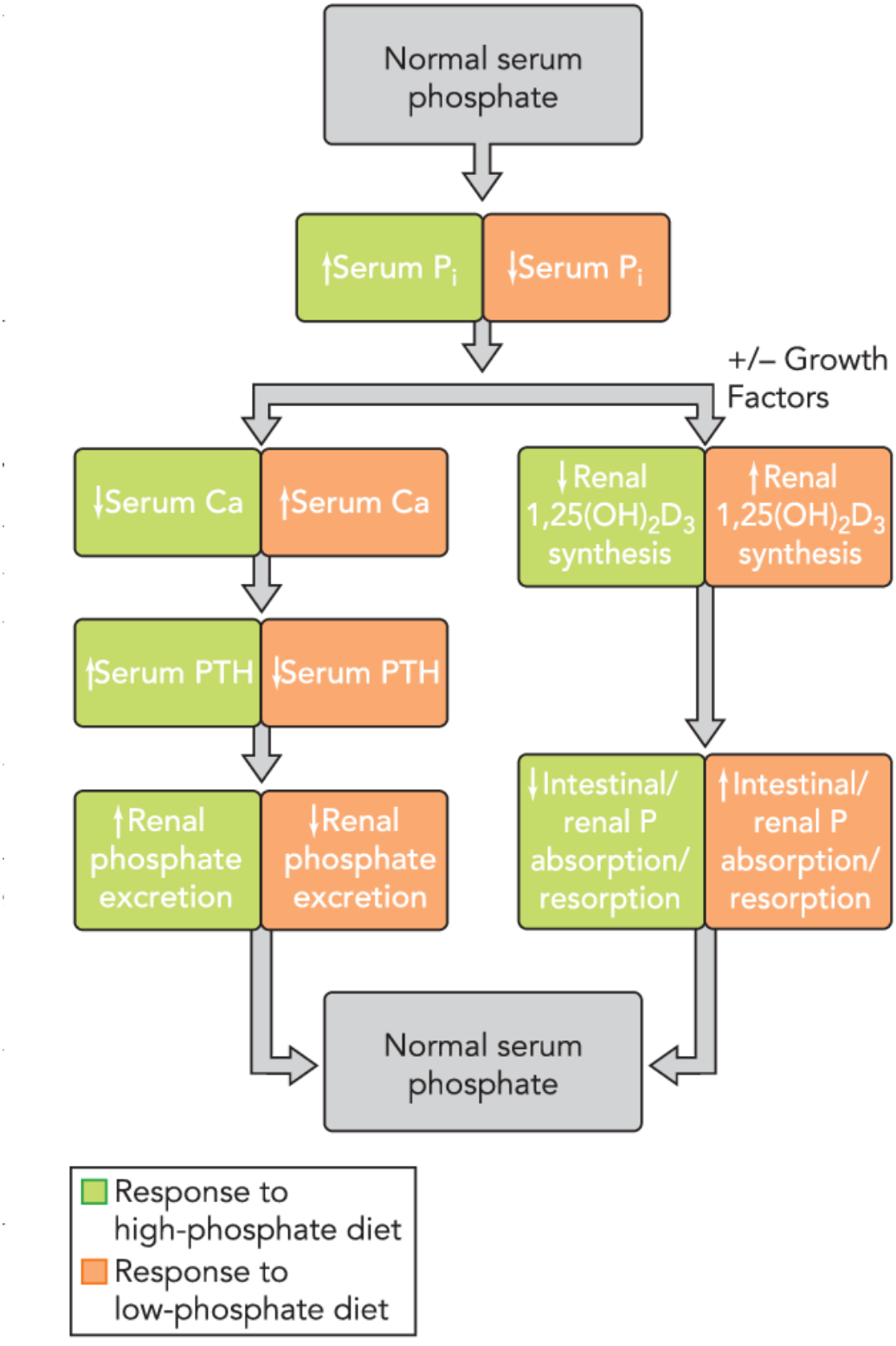

Figure 6. The adaptation to changes in the dietary phosphate (40).

Sodium-dependent phosphate co-transports of the SLC20 family and phosphate export protein (XPR1) also play a role in phosphate homeostasis, allowing the cell to take up phosphate (39). The presence of transports is crucial since phosphate cannot simply diffuse through the lipid bilayer of the cells due to its size, charge, and negative electrochemical gradient, so these transporters, which are expressed in every cell, sense and take up phosphate into the cell depending on its needs (39). The SLC20 family cotransporters located at the cellular level, such as SCL20A1 and A2, are involved in bone development and phosphate sensing. At the organism level, phosphate homeostasis is achieved by the sodium-dependent cotransporters of the SLC34 family (39). SCL34A1 and A3 are expressed at the kidney level, which is implicated in the body's phosphate content (39)

In the intestine, SLC34A2 is involved in the intestinal phosphate uptake and regulates phosphate concentration in epithelial fluids such as salivary glands, mammary glands, type 2 alveolar duct, and pancreatic ducts. Figure 7 shows the distribution of the SLC family's different subtypes in the human body.

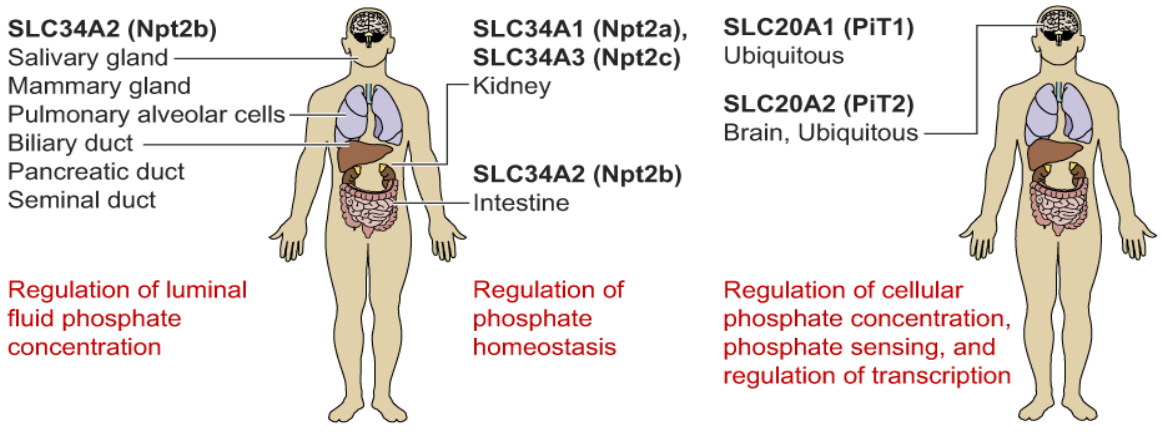

Figure 7.The distribution of the SLC family (39).

Another phosphate transporter in the SLC family is the SLC17 (type 1 sodium phosphate cotransporters); these transporters play a minor role in phosphate homeostasis in mammals because mutations in the SLC17 proteins did not result in overt abnormality in phosphate homeostasis (39).

Hernando et al. stated that abnormal phosphate homeostasis, either a decrease or an increase, results in an abnormal calcification, indicating an important role for phosphate in the regulation of mineralization. Any abnormality in the SLC family leads to many disorders; for example, an abnormality in the SLC20 protein expression and function results in abnormal bone structure and vascular calcifications (39).

An abnormality in the SLC34 protein increases or decreases phosphatemic disorders like rickets and pulmonary calcifications (39).

Phosphatonin molecules are also important in the regulation of phosphate. One of the most important players is fibroblast growth factor 23 (FGF23), a 251 amino acid protein (40). The addition of FGF23 in mice/rats led to the decrease in the activity of vitamin D3 hydroxylase and caused phosphaturia, indicating that FGF23 decreases serum Pi levels. Klotho receptor is important for the bioactivity of FGF23. It is a beta-glucuronidase receptor located in the cell membrane of the parathyroid gland and the kidneys where FGF23 exerts its effect (41).

It was seen that klotho knockout mice exhibited an identical phenotype to FGF23 knockout mice, indicating the importance of Klotho receptor presence for FGF23 to induce its action (40, 41).

The figure below shows that at low serum phosphate levels, calcium-sensing receptors (CaSR), located in the parathyroid gland, sense increased calcium serum levels and inhibit PTH release. Parallel to that, PiT1/PIT2 cotransporters present in the

bone prevent the release of FGF23. These two mechanisms signal the increase of phosphate reabsorption from the glomerular filtrate by increasing the expression/function of SLC34A1 (Npt2a) and SLC34A3 (Npt2c) in the kidney. In addition to that, the kidney also releases more 1,25-dihydroxy vitamin D to increase the intestinal absorption of phosphate from the diet through the increase in the expression or the function of SLC34A2 (Npt2b). These events cause an increase in serum phosphate levels. The opposite occurs in hyperphosphatemia or high serum phosphate levels, as seen in part B (39).

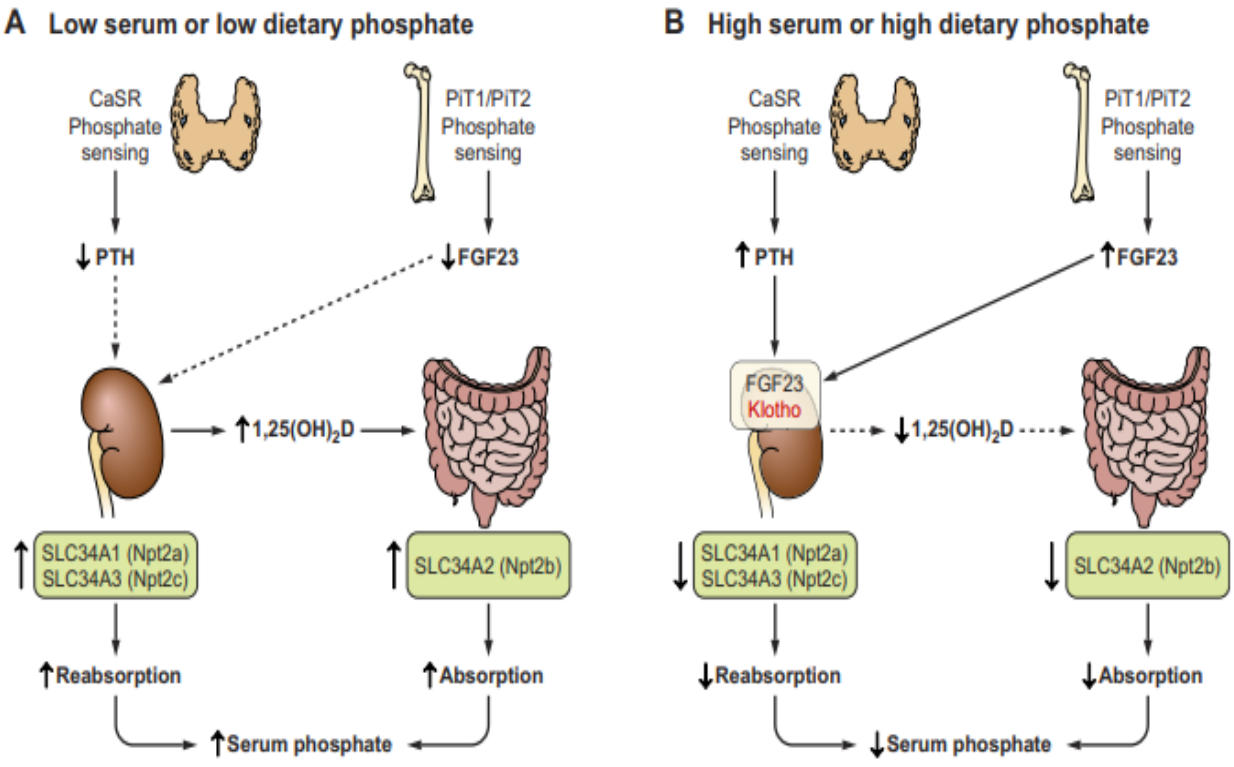

Figure 8. The regulation of phosphate homeostasis (39).

CHAPTER 2

OBJECTIVES

Many studies were performed to assess the role of phosphate in cardiovascular diseases, particularly the risk factor hypertension and its effect on various organs and vessels.

In order to test the effect of dietary phosphate on blood pressure and vasculature, we hypothesized that dietary phosphate intake alters sodium-dependent phosphate transporters' expression and activity, thus affecting the blood pressure response.

To test the above hypothesis, 3 specific aims were to be done. The first aim is to determine the effect of increasing doses of dietary phosphate of 0.15%and 1.5% on blood pressure in hypertensive mice. The second aim is to determine the effect of phosphate on vascular reactivity and function in hypertensive mice compared to normotensive ones. The third aim is to study the effect of dietary phosphate on the inflammation and infiltration of immune cells in vasculature in hypertensive mice...

CHAPTER 3

EXPERIMENTAL PROCEDURES

3.1 Animal Model and approval

This study included C57BL/6 male mice of approximately 12-weeks old obtained from the American University of Beirut Animal Care facility. The protocols and procedures performed on this study were approved by the Animal Care Facility Animal Ethics Committee and conducted following the IACUC Guidelines for the Ethical Use of Animals in research.

Mice were fed teklad diet and water *ad libitum* for 12 weeks before starting the experiment and for one week during the adaptation period and recording their baseline blood pressure. Mice were housed in cages at 25 °C on a 12 h light/dark cycle.

3.2 Study Design

The study design is presented in figure 9. A total of 72 C57BL/6 male mice were subjected to an adaptation period at the beginning of the protocol for 1 week followed by 1 week baseline BP recordings. During this period, mice were fed chow diet. Following that, mice were switched on a control diet containing 0.3% of phosphate for 1 in week in order to allow the mice to adapt to the food type and shape. Table 1 shows the food composition of each diet.

After this period, mice were divided randomly into 2 groups: Sham and Ang II groups. The Sham group was considered to be the control group. The Ang II groups were subjected to a subcutaneous miniosmotic pump implantation of ANG II infusion

(490 ng/kg/min) for 4 weeks to induced hypertension as seen in figure 9. During this period, mice were fed the control diet of 0.3% phosphate for 2 weeks.

Mice were then subjected to 2 weeks of phosphate diet with different percentages: a low phosphate diet of 0.15% and a high phosphate diet of 1.5%. However, some mice were kept under the control diet of 0.3% phosphate.

At the end of the protocol, mice were sacrificed by CO2 inhalation for further experimental studies.

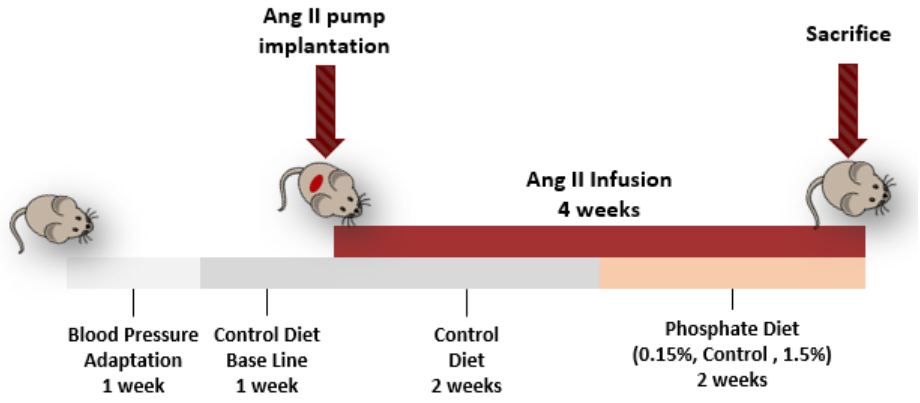

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Figure 9. The experimental design of the study.

Table 1. Diet composition of each food.

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 wt g	1000	1000	1000	1000

3.3 Blood Pressure Measurement

During the whole experimental study, blood pressure was measured via a non-invasive method. BP-2000 Blood Pressure Analysis System is an accurate, non-invasive tail-cuff method for measuring blood pressure for mice and rats. This system works by using photoplethysmography transmission, a technology that depends on infrared light to measure the volumetric variations of the blood circulation (47).

Figure 10 shows the machine Visitech system, which was used to record the blood pressure of the mice. First, the machine is turned on, and the mouse is placed on a 37 °C heated platform. A filter is placed on the mice to limit their movement and secure its place with its tail accessible to the cuff. The computer will display each run cycle, showing the systolic blood pressure, diastolic blood pressure, the average mean, and the pulse. The system reaches a total of 30 runs. Only valid measurements are included, whereas the software automatically excludes the false BP measurement.

Figure 10. Tail cuff non-invasive BP machine.

3.4 Single-cell Suspension Preparation

In this experiment, thoracic aortic vessels were isolated and used for analysis. The mouse thoracic aortas were harvested at sacrifice at the end of the protocol and cleaned from the perivascular adipose tissue deposited around it. The aorta was then analyzed for FACS analysis and vascular reactivity studies. A portion of the excised cleaned aorta was minced with fine scissors and digested by a solution of RPMI containing collagenase type A (ROCHE- 10103586001) and collagenase type B (ROCHE- 11088815001) placed in a gently rotating hybridization oven at 37°C for 40-45 minutes. After 40-45 minutes, the obtained sample is passed through a 70 μm placed on a falcon tube to eliminate unwanted and undigested debris. Following this step, the sample was centrifugated at 800G for 5 minutes at 4°C. A small ring of 2 mm approximately was introduced into the thoracic aorta after cleaning for vascular reactivity studies.

3.5 Flow Cytometry Analysis

Fluorescence-activated cell sorting, also known as FACS, is an immune-phenotyping technique that uses fluorescently specific labeled antibodies to sort and isolate the interested heterogeneous population for analysis and to differentiate between cell types. The aortic cells were washed with ice-cold PBS and stained with LIVE/DEAD fixable violet dead cell stain for 30 min at 4 °C in the dark. This stain is used to determine the viability of the cells. An amine-reactive can react with amines either at the cell surface or the cell's interiors. If the cells are alive, the dye can only respond to the cell-surface amine generating a less intense fluorescence.

The antibodies used for FACS are as follows: Brilliant Violet 510 -conjugated anti-CD45 antibody, peridinin chlorophyll protein-cyanin-5.5 -conjugated anti-CD3 antibody, allophycocyanin-cyanin-7 conjugated anti-CD4 antibody, phycoerythrin-cyanin-7-conjugated anti-CD8a antibody, APC-conjugated anti-CD44 antibody, and FITC anti-F4/80, and PE-conjugated anti-CD62L.The cells are incubated with the antibody cocktail for 30 min at 4°C covered by an aluminum foil due to light sensitivity. Flow cytometry analysis is performed on a BD FACS FACS Aria™SORP cell sorter, FACS Aria™SORP cell sorter, and the data analysis is performed by flowJo software.

3.6 Vascular Reactivity Studies

As shown in figure 11, Wire Myography is a technique that allows the assessment of vascular smooth muscle and vascular endothelium function by examining small vessels such as thoracic aortas and mesenteric vessels ranging from 60 μm-10 mm of internal diameter. The small vessels are dissected as rings and threaded into stainless wires, and secured to two supports. One support is attached to a micrometer to control the vessel's circumference, and the other is connected to a force transducer to measure the tension in the vessel.

The vessels used are kept alive in an oxygenated physiological salt solution (PSS) at 37°C. Thoracic aortas were collected and used for vascular reactivity studies in this experiment.

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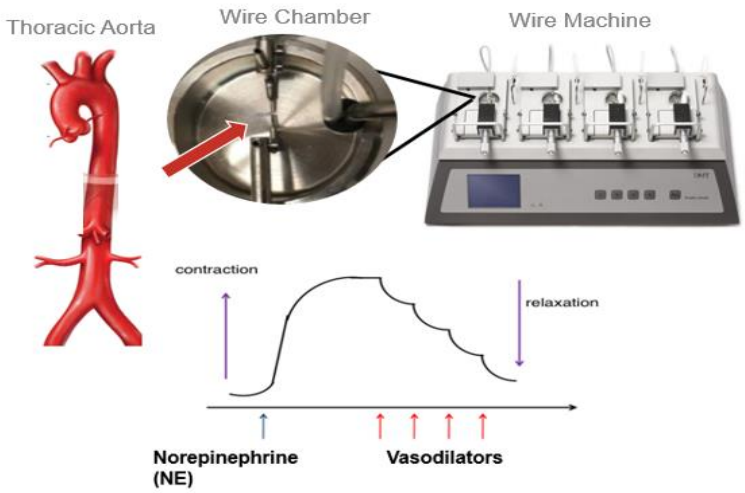

Figure 11. Vascular reactivity study.

3.7 Statistical Analysis

Data in this experiment are presented as the mean ± standard error of the mean. One-way and two-way ANOVA with multiple comparisons were employed.

CHAPTER 4

RESULTS

4.1 High Phosphate diet decreases high blood pressure

In order to measure the effect of phosphate diet on blood pressure, a tail-cuff non-invasive blood pressure machine was used to record the blood pressure in the Sham and hypertensive Ang II-infused mice that received 0.15 %, 0.3 % and 1.5 % phosphate diet.

Figure 12 represents the systolic blood pressure recorded during the whole protocol. At baseline, the systolic blood pressures of the sham and the hypertensive groups were similar, fluctuating between 112.75 mmHg and 123.83 mmHg.

Upon subjecting the Ang II group to angiotensin II pump implantation, the systolic blood pressure increased significantly to about 153.5- 158 mmHg two weeks after pump implantation. However, the systolic blood pressure in the sham control group remained similar to that recorded at baseline.

After 2 weeks from pump implantation, the two groups of mice were subjected to different phosphate diet percentages. 0.3% phosphate was considered the control group, 0.15% phosphate was considered the low phosphate diet group and 1.5% was considered the high phosphate diet group.

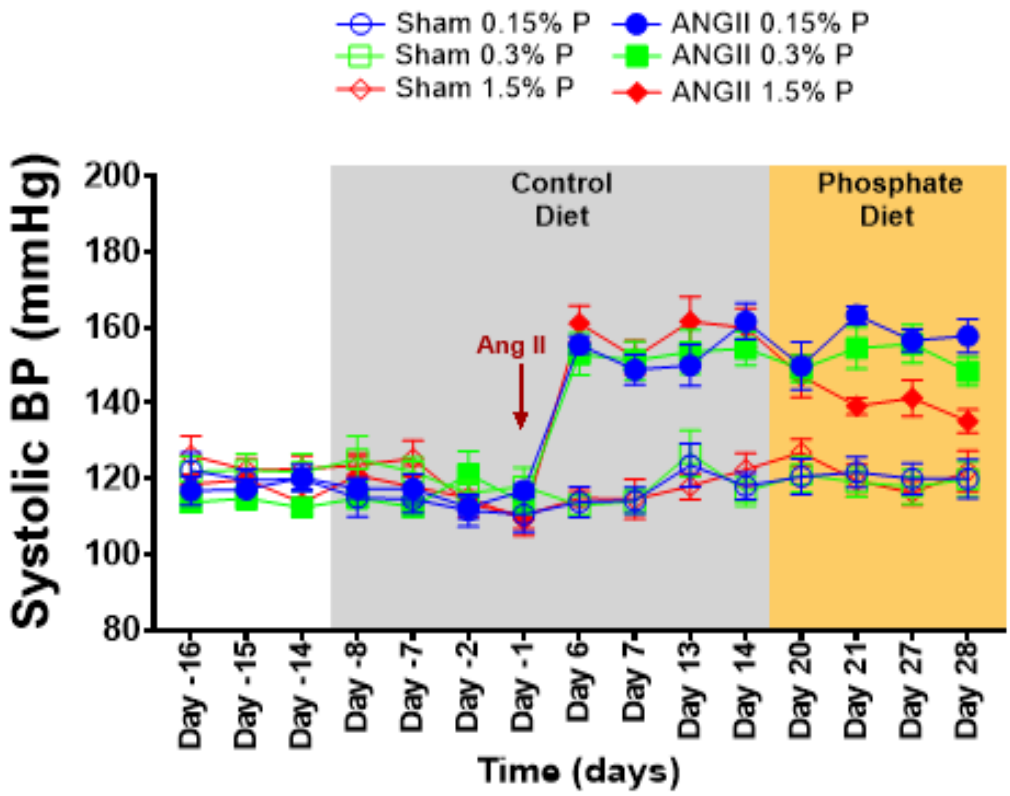

Figure 12. BP measurements in response to dietary phosphate intake in Ang II mice. Tail cuff SBP in response to 4 weeks angiotensin II infusion (490 ng/Kg/min) followed by phosphate intake. Data analyzed by 2 way ANOVA with repeated measurements, n=12-13 in each group.

At the end of the protocol, after 2 weeks of phosphate ingestion, the SBP of the Ang II group under 0.15% phosphate diet remained significantly higher than that of the sham group (157.79 ± 4.432 mmHg vs. 120 ± 5.12 mmHg, respectively. $p<0.0001$) (figure 13 A). Comparing the SBP of the hypertensive group under 0.3% phosphate to the sham group as seen in figure 13 B, the SBP in the hypertensive mice remained significantly higher than that of the sham group under 0.3% phosphate diet at the end of the protocol (148.4 ± 3.823 mmHg vs. 119.87 ± 4.561 mmHg, respectively, *$p<0.0001$).

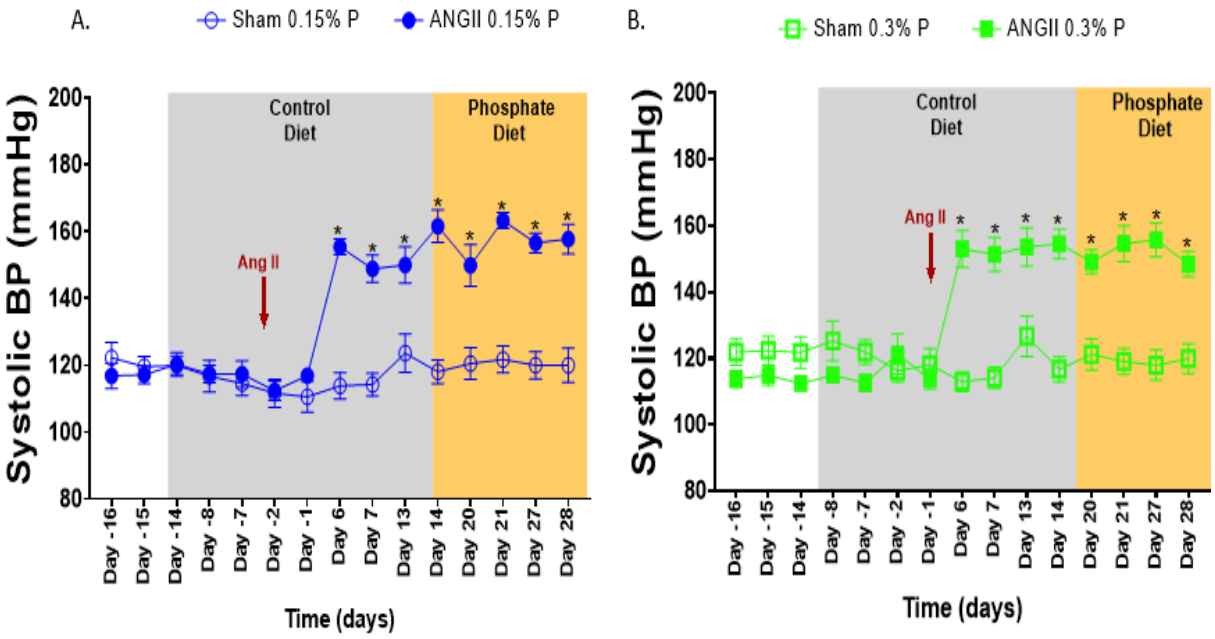

Figure 13. BP measurements in response to low and control dietary phosphate intake in Ang II mice. Tail cuff SBP in response to 4 weeks angiotensin II infusion (490 ng/Kg/min) followed by (A). 0.15% P intake and (B). 0.3% P intake. Data was analyzed by 2 way ANOVA with repeated measurements, n=12-13 in each group. *p<0.0001.

The SBP decreased significantly to a value of 135.2 mmHg in the hypertensive mice under the high phosphate diet (1.5%) after 2 weeks from pump implantation (figure 14). Comparing the SBP of the hypertensive mice with the sham group at the end of the protocol under the high phosphate diet (1.5%), there was no significance between the SBP values recorded (135.2 ± 3.076 mmHg vs 122 ± 5.308 mmHg).

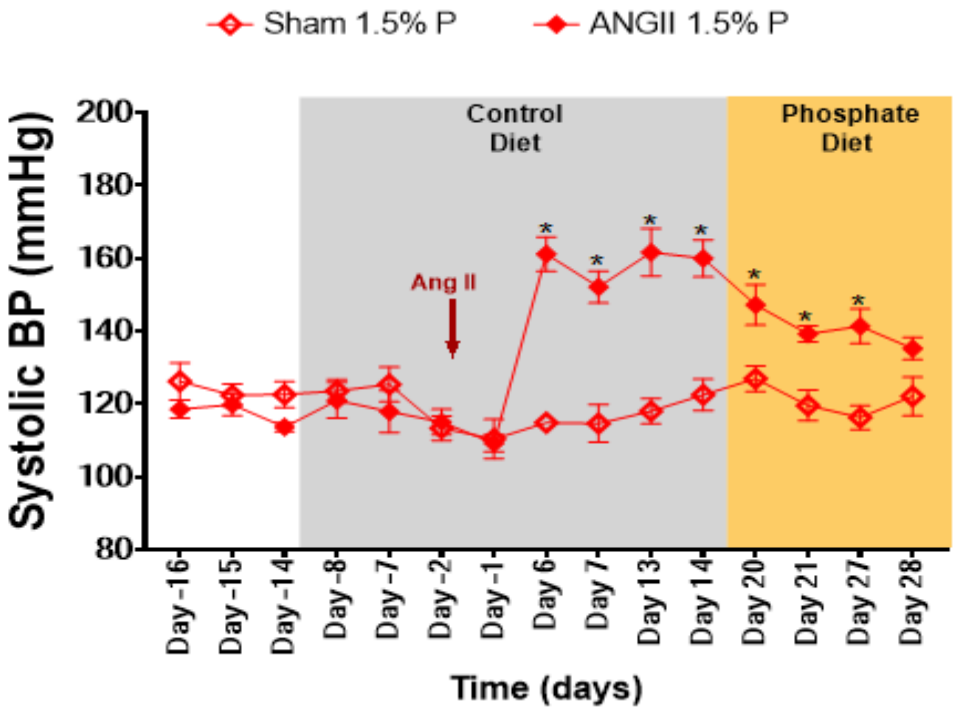

Figure 14. BP measurements in response to 1.5% high dietary phosphate intake in Ang II mice. Tail cuff SBP in response to 4 weeks angiotensin II infusion (490 ng/Kg/min) followed by 1.5% P intake. Data was analyzed by 2 way ANOVA with repeated measurements, n=12-13 in each group. *p<0.0001.

Similar results were seen in the diastolic blood pressure data. At baseline, the DBP in the sham and hypertensive mice were similar, fluctuating between 55-60 mmHg as seen in figure 15. After Ang II introduction, the DBP in the hypertensive mice increased significantly compared to the Sham group to approximately 84-91 mmHg. After phosphate introduction, the DBP of the hypertensive mice under the control diet 0.3% and the low 0.15% phosphate diet remained higher than that of the Sham group at the end of the protocol. The DBP of the hypertensive mice under 1.5% phosphate diet decreased to a value of (67.52 ± 3.213 mmHg) at the end of the protocol as seen in figure 16.

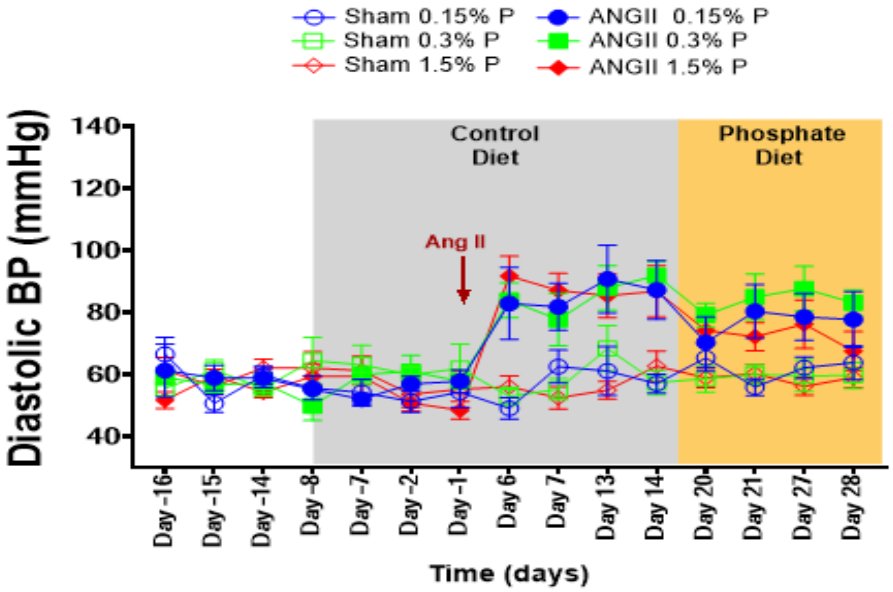

Figure 15. BP measurements in response to dietary phosphate intake in Ang II mice. Tail cuff DBP in response to 4 weeks angiotensin II infusion (490 ng/Kg/min) followed by phosphate intake. Data analyzed by 2 way ANOVA with repeated measurements, n=12-13 in in each group.

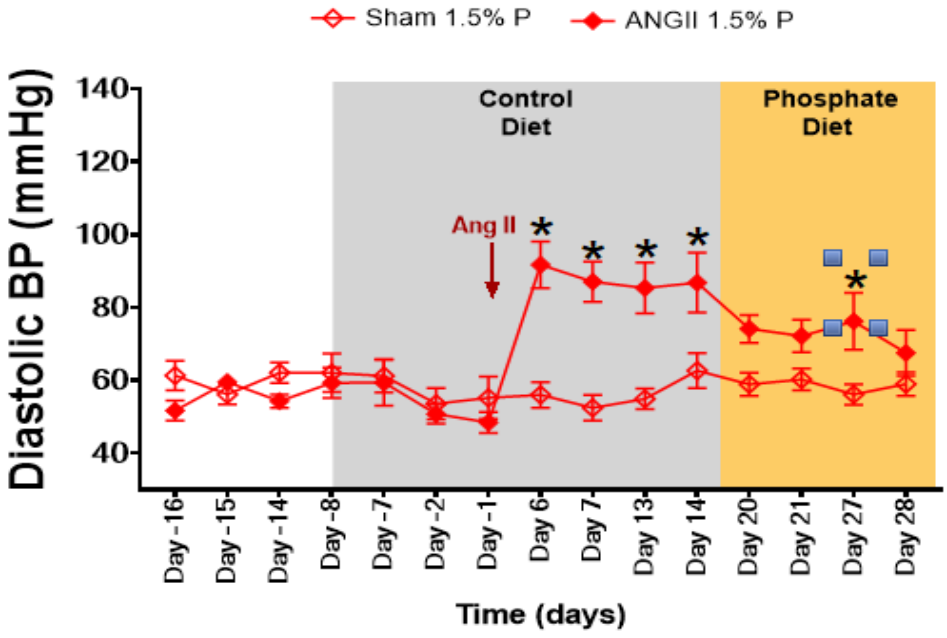

Figure 16. BP measurements in response to 1.5% high dietary phosphate intake in Ang II mice. Tail cuff DBP in response to 4 weeks angiotensin II infusion (490 ng/Kg/min) followed by 1.5% P intake. Data analyzed by 2 way ANOVA with repeated measurements, n=12-13 in each group. *p<0.0001.

4.2 High Phosphate diet affects endothelial-dependent vasodilation

To examine whether phosphate affects vascular reactivity and function, wire myography experiments were performed on thoracic aortas obtained after sacrifice. Vascular reactivity studies were performed to assess a tissue's basic properties and investigate the reactivity and function of the vascular endothelium and smooth muscle.

The thoracic aortas obtained from each diet group were subjected to increasing doses of Ach and SNP (Sodium Nitroprusside, NO donor) to assess the endothelium-dependent and independent vasodilation, respectively.

Following the pre-constriction with PE, vessels from the 1.5% phosphate of the control group vasodilated less to acetylcholine than the vessels from control (0.3%) and low (0.15%) phosphate of the sham groups (maximum percentage relaxation 59.5% vs. 94.7%, respectively) as seen in the figure 17 A.

The aortic vessels obtained from the Ang II-infused mice under high (1.5%) phosphate diet (maximum percentage relaxation 34.2%) also vasodilated less to acetylcholine compared with the vessels obtained from hypertensive Ang II-infused mice under low (0.15%) and control (0.3% P) (maximum percentage relaxation 78.4% vs. 69.6%, respectively) as seen in figure 17 B. There was a trend for a lower relaxation in response to Ach in both the normotensive and hypertensive Ang II-infusion mice under 1.5% phosphate diet.

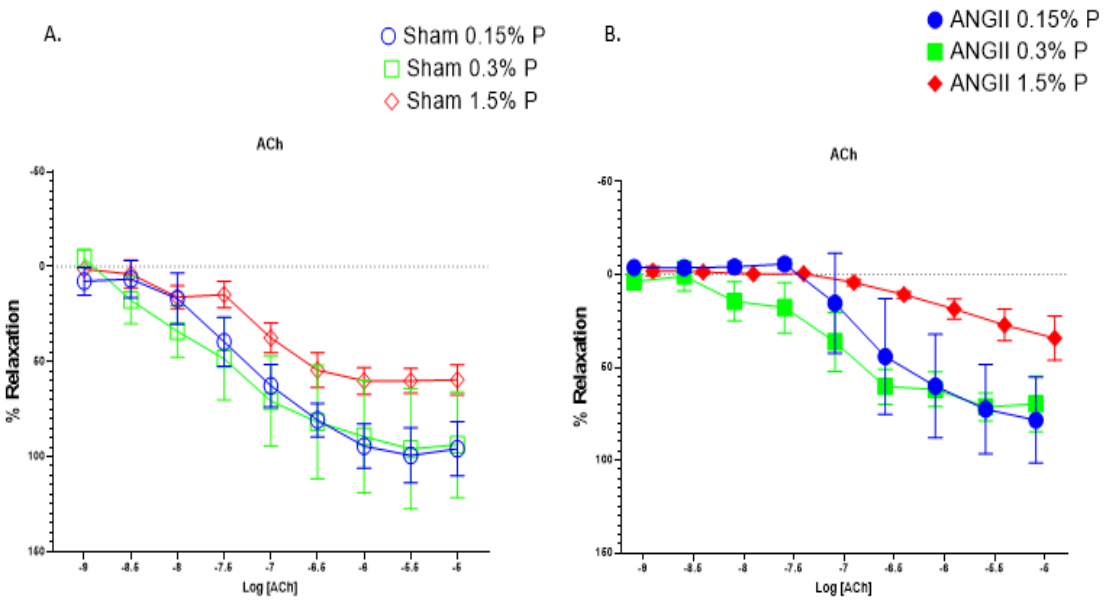

Figure 17.Concentration-response curves to acetylcholine (Ach) in isolated thoracic aortic rings of C57BL/6 (A) Sham and (B) Ang II-infused (490ng/Kg/min) mice under phosphate diet. Data expressed as mean ± SEM, n=3-7 in each group.

The results for the endothelium-independent vasodilator SNP are shown in figure 18. Comparing the curve of every diet group, it is seen that the curves are almost overlapping. The relaxation response to SNP was similar in both the sham and hypertensive mice under low, control, and high phosphate diets, with no significance.

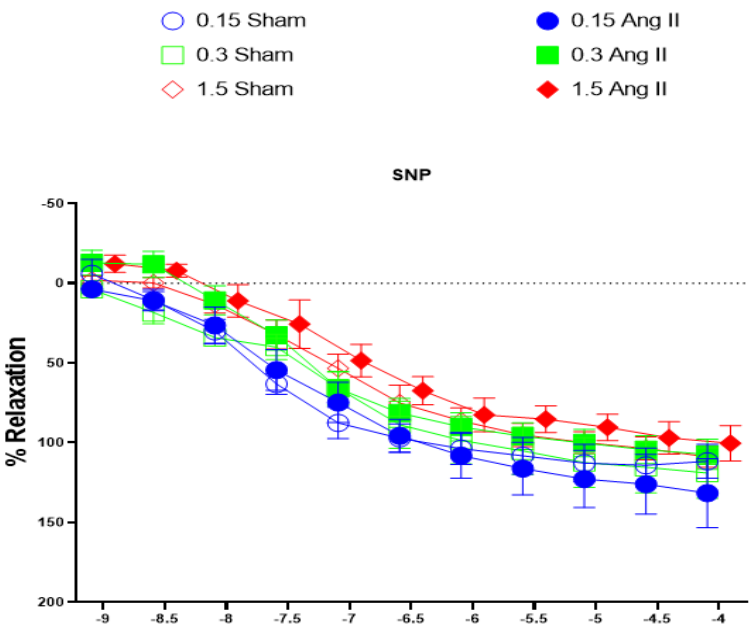

Figure 18. Concentration-response curves to sodium nitroprusside (SNP) in isolated thoracic aortic rings of C57BL/6 mice sham or angiotensin II infusion (490 ng/Kg/min) under phosphate intake. Data expressed as mean ± SEM, n=3-7 in each group.

4.3 High Phosphate exacerbates Ang II effect on vascular inflammation

Flow cytometry analysis was performed to assess whether phosphate affects the infiltration of immune cells in thoracic aortas in response to hypertensive stimuli.

The results show that the extent of inflammation and immune cells infiltration in thoracic aortas was further exacerbated in the Ang II-infused mice under the 1.5% phosphate diet. There was a trend for a higher number of total leukocytes (CD45+ cells) (figure 19 A) and specifically total T lymphocytes (CD3+ cells) (figure 19 B) in the thoracic aortas of the Ang II-infused mice compared to the sham group, with being the highest in the Ang II-infused mice under 1.5% phosphate diet. The Ang II-infused mice under 1.5% phosphate diet exhibited a higher total T lymphocytes memory cells (CD3+

Tm) (figure 19 C). More CD3+ Tm cells were significantly present in the 1.5% phosphate group compared to the 0.15% group in the hypertensive Ang II-infused mice (*p<0.05).

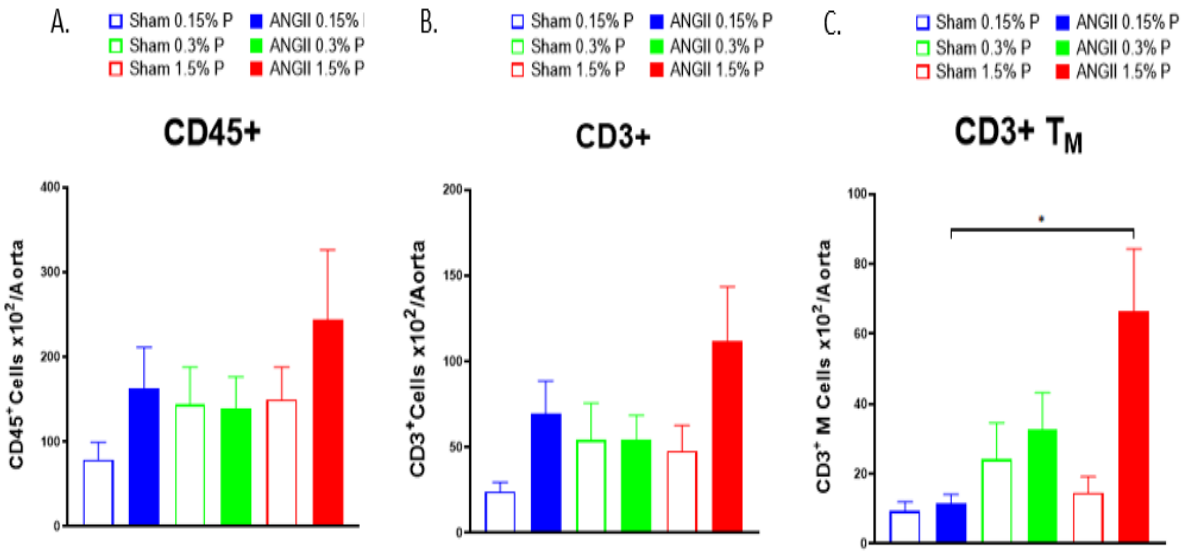

Figure 19. Effect of phosphate on leukocyte infiltration into the thoracic aorta. Summary data for aortic accumulation of the absolute number of (A) Total leukocyte (CD45+), (B) Total T cells (CD3+), (C) Total memory T cells (CD3+ Tm) in response to either sham or Ang II infusion (490 ng/Kg/min). Data are expressed using one-way ANOVA, n=6-11 per group.*P<0.05.

Moreover, there was a trend for a higher number of helper T cells (CD4+) in the Ang II-infused mice under the high phosphate diet (1.5%) (Figure 20).

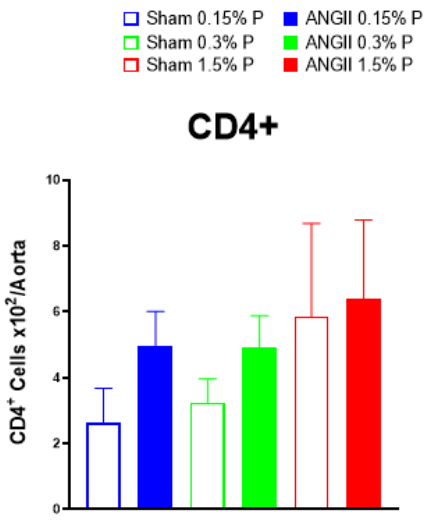

Figure 20. Effect of phosphate on CD4+ T-cell infiltration into the thoracic aorta. Summary data for aortic accumulation of the absolute number of CD4+ T-cell in response to either sham or Ang II infusion (490 ng/Kg/min). Data are expressed using one-way ANOVA, n=6-11 per group.

Subsequently, cytotoxic T cells (CD8+) present in the hypertensive mice were higher compared to the sham group. The Ang II-infused mice under the low (0.15%) and control (0.3%) phosphate diet groups exhibited a trend for a lower number of CD8+ cells compared to the high phosphate diet group (1.5%) as seen in figure 21 A. Regarding memory cytotoxic T cells, a sparse number of central memory CD8+ cells (CD8+ TCM) were detected in the thoracic aortas of all the sham groups compared to the hypertensive ones. More effector memory cytotoxic cells (CD8+ TEM) were detected in the hypertensive groups compared to the sham groups (figure 21 B). The Ang II-infused mice under the high phosphate diet (1.5%) exhibited a significantly higher level of CD8+ TEM cells compared to the low (0.15%) and control (0.3%) diet groups.

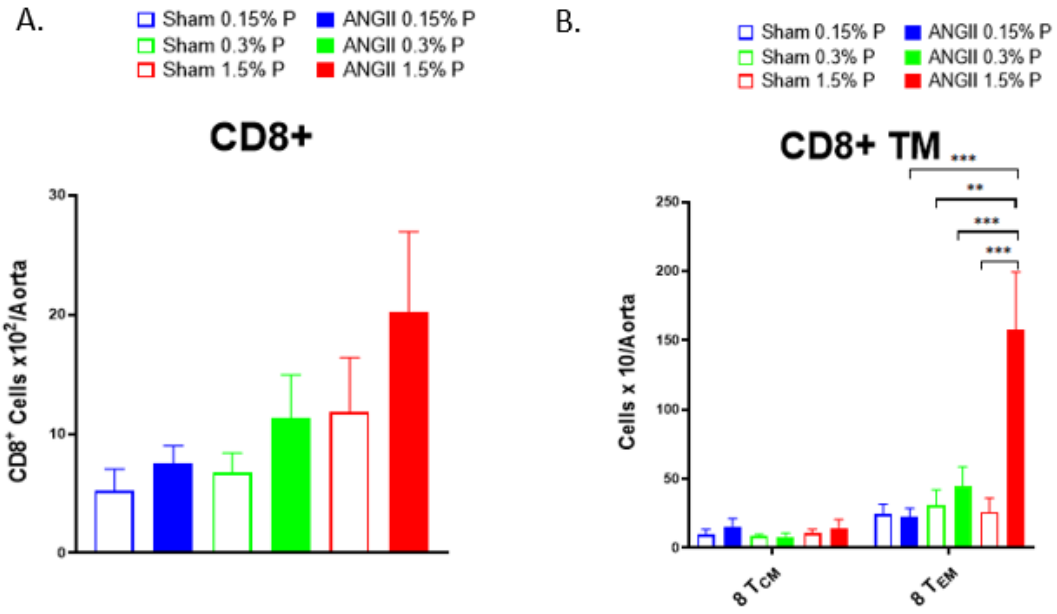

Figure 21.Effect of phosphate on CD8+ T-cell infiltration into the thoracic aorta. Summary data for aortic accumulation of the absolute number of (A) CD8+ T-cell, (B) memory CD8+ T-cell in response to either sham or Ang II infusion (490 ng/Kg/min). Data are expressed using one-way ANOVA, n=6-11 per group. **p<0.01, ***p<0.001.

CHAPTER 5

DISCUSSION

In order to investigate the effect of phosphate on blood pressure, different increasing doses of phosphate were used, ranging from 0.15% being the lowest percentage, 0.3% the control percentage diet, and 1.5% being the highest percentage of phosphate diet.

Looking at the SBP and the DBP results, low (0.15%) and (0.3%) control phosphate had a minimal effect on blood pressure in sham and Ang II-infused mice.

The high phosphate diet (1.5%) reduced the high blood pressure in the hypertensive Ang II-infused mice after 2 weeks from pump implantation to normal levels similar to that recorded in the sham group under the high phosphate diet (1.5%). The SBP decreased from 160 mmHg (2 weeks after Ang II) to 135.3 mmHg at the end of the protocol. The total marked decrease was 24.8 mmHg. There was no significant difference between the SBP recorded between the hypertensive and sham mice at the end of the protocol (135.2 vs 122 mmHg, respectively). Similarly, DBP in the Ang II-infused mice decreased from 86.9 to 67.9 mmHg at the end of the protocol, marking a total decrease of 19 mmHg. The high phosphate diet did not affect blood pressure in the sham group. Thus, the high phosphate diet (1.5%) significantly lowers the blood pressure after Ang II infusion.

Our results suggest a possible protective role of 1.5% phosphate in decreasing high blood pressure following hypertensive stimuli. The exact biological mechanism on how dietary phosphorous reduces BP is still unclear. One possible mechanism is that phosphate intestinal and renal reabsorption via Sodium-Phosphate coupled co-transports

are decreased due to the high levels of sodium and phosphate. The availability of high levels of sodium and phosphate ions causes either the decrease in the expression and/or the activity of sodium-phosphate cotransporters, especially those expressed in the kidneys, leading to increased levels of sodium and phosphate excretion in the urine or thus decreasing blood pressure after Ang II-infusion.

Another possible mechanism is that under the effect of a high phosphate diet (1.5% P), FGF23, secreted from osteoblasts and osteocytes, is released and targets the kidneys through activating klotho receptor, which is expressed prominently on the distal convoluted tubules and ubiquitously in the proximal convoluted tubules, causing a decrease in the expression of sodium-phosphate cotransporters (48). This might in return, decrease the influx of both phosphate and, most importantly Na+, thus causing a decrease in blood pressure in a hypertensive model followed by high phosphate diet intake.

In contrast to our findings, different studies suggest that high doses of phosphate increase blood pressure, stating it as a deleterious agent on blood pressure, rather than a protective one. For example, Bozic et al. showed an increase in blood pressure in healthy rats subjected to 4 weeks of high phosphate intake of 1.2% (44). Also, supporting their findings, it was stated that a high serum Pi level might lead to the increase in the activity of renin-angiotensin and hence the development of hypertension through the increase of FGF23 that allows vascular smooth muscle cells to uptake Pi and hence prolonged the arterial contraction and increased blood pressure (43).

Given that phosphate had a protective role in lowering blood pressure following hypertensive stimuli, vascular reactivity and function were also assessed. After plateau contraction had been reached by PE, acetylcholine was added to assess endothelium-

dependent vasodilation and thus the presence of an intact and functional endothelium. As seen in the results, the high phosphate diet (1.5%) in the sham group exhibited a lower relaxation percentage of 59.57% in response to Ach, compared to the low (0.15%) and control (0.3%) phosphate diets (average of 94.76%).

Based on what has been reported in literature, Ang II reduces the relaxation response to Ach to 70% (34). Also, the high phosphate diet (1.5%) in the Ang II-infused mice exhibited a lower relaxation percentage of 34.2% in response to Ach, compared to the low phosphate diet (78.4%) and the control phosphate diet (69.6%). Although not statistically significant, there was a trend for a lower relaxation in response to Ach in both the sham and hypertensive Ang II-infused mice under the high phosphate diet.

Both the control and Ang II mice had similar relaxation responses to SNP with no significant influence between them, indicating that the endothelium-independent vasodilation is functional and that the high phosphate diet targets the endothelium rather than the smooth muscle.

A functional endothelium is characterized by the ability to produce and release NO. Under normal physiological conditions, NO is continuously produced from L-arginine under the effect of endothelial nitric oxide synthase (eNOS) in the presence of cofactor BH4 and NADPH. It then diffuses to neighboring smooth muscle cells to activate (GC) and leads to cGMP production, thus causing relaxation.

One possible mechanism of action of phosphate on affecting the endothelial-dependent vasodilation is through affecting NO pathway production. In accordance to our results, Stevens et al. indicated that the endothelial-dependent vasodilation was impaired when resistance vessels were exposed to high phosphate. They pointed out that the high phosphate concentration disrupted the whole pathway of NO production,

which resulted in decreased NO and cGMP levels and total reduction of total and phospho-eNOS expression responsible for generated NO (49). This mechanism might have occurred in the thoracic aortas obtained from the control and Ang II groups under the effect of the high phosphate diet, where the high phosphate diet of 1.5% targeted the endothelium causing a possible dysfunction, thus a less relaxation response to Ach.

Given that different players are involved in the NO pathway, phosphate might have had a deleterious effect on one or more important players implicated in the NO production, thus causing impairment in the endothelial-dependent vasodilation.

In addition to that, it was found that when exposing bovine aortic endothelial cells to a phosphorous load, there was an increase in the production of ROS, which depended on phosphorous influx by sodium-phosphate dependent transporters (specifically type III sodium-dependent phosphate transporters 1 and 2 encoded by SCL20A1 AND SCL20A2, respectively.), and decreased the availability of NO by the inhibitory phosphorylation of eNOS, also they found out that the high phosphorous load inhibited endothelium-dependent vasodilation of rat aortic rings (50).

For eNOS to be active, it needs to be phosphorylated by Akt at serine 1176 amino acid residue in the presence of Ca2+-Calmodulin and thus activates the enzyme and leads to NO production. However, in the absence of Ca2+-Calmodulin, PKC phosphorylates eNOS at the threonine Thr 497 amino acid residue and leads to the inhibition of eNOS activity and hence NO is no longer produced. It was suggested in previous studies that the high phosphorous load might enhance the phosphorylation at Thr 497 amino acid residue of eNOS by PKC, thus inhibiting eNOS activity and decreasing NO production. In addition to that, the presence of ROS caused a decrease in

the bioavailability of NO by converting NO into ONOO-. These events, which are depicted in figure 22, eventually lead to endothelial dysfunction (50).

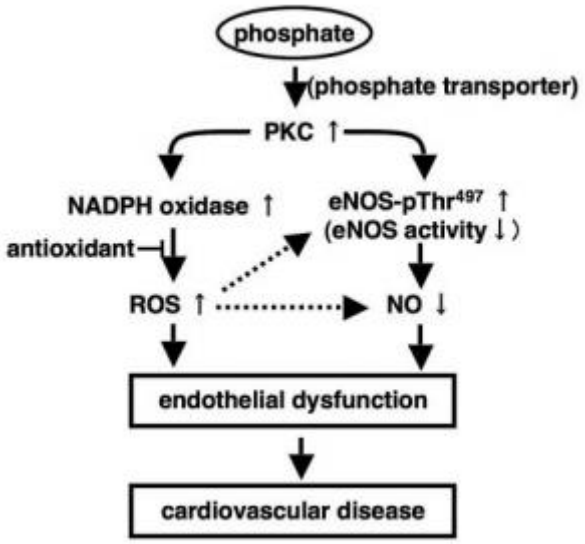

Figure 22. The possible pathway of phosphate-mediated endothelial dysfunction (50).

In this study, we focus on the adaptive immunity. Since accumulating evidence suggest that the immune system is involved in the pathogenesis of hypertension (31,33,34), flow cytometry was performed on thoracic aortas collected from all the diet groups of normotensive and hypertensive mice. A higher number of CD45, CD3, CD4 and CD8 cells were present in the hypertensive mice group compared to the sham group with being the most present in the Ang II-infused mice under the high phosphate diet. The Ang II-infused mice under the 1.5% phosphate showed a much higher significant accumulation of memory CD8 cells than that in the low and control phosphate groups of the hypertensive mice, suggesting that the high phosphate diet is further exacerbating the effect of Ang II in activating the immune system and thus leading to more immune

cells infiltration and vascular inflammation and causing a possible injury to the thoracic aortas.

Over the past decade, it has been noted that inflammation is linked to endothelial dysfunction. Oxidative stress is the basic etiology of the alteration in the arterial wall (51). ROS availability alters the NO pathway at the arterial wall, leading to a decrease in the NO production, Arginine and ATP. This subsequently causes a damage followed by inflammation and dysfunction to the endothelium (51). The endothelial cells prevent vascular injury and limit clot formation in areas where hemostasis is important to restore vascular integrity. When platelets and leukocytes adhere and infiltrate to the endothelium, this leads to extravasation of white blood cells to the site of injury and causes more inflammation. So, any dysfunction to the endothelium-dependent regulatory system leads to decreased levels of NO production. This will cause an injury to the endothelium and thus subsequent infiltration of immune cells. When endothelial cells are no longer intact, CD8 cells infiltrate under the effect of repeated hypertensive stimuli of Ang II on AT-1R and cause further inflammation and damage (49).

The Ang II-infused mice under the high phosphate diet showed the highest number of immune cells accumulation, suggesting more infiltration of immune cells. This group also showed a possible impairment in the endothelial-dependent vasodilation, suggesting an explanation that due to the loss of the endothelial integrity, immune cells were able to infiltrate more and thus cause vascular inflammation.

High phosphate seems to be implicated in vascular inflammation and endothelial dysfunction, especially after repeated hypertensive stimuli.

Figure 23 represents a graphical presentation of the working model showing the obtained results in this project, where high phosphate diet of 1.5% had a protective role in reducing blood pressure in Ang II-infused mice. However, the high phosphate diet exhibited a negative effect on the endothelial-dependent vasodilation in both sham and Ang II-infused mice and a further vascular inflammation to the thoracic aortas of the hypertensive mice and thus causing a subsequent vascular damage.

It's still unclear how phosphate diet can be deleterious on the vasculature which was seen in this experiment and previous studies done to investigate the role of phosphate on the vasculature (49, 50) and at the same time be beneficial in reducing a hypertensive blood pressure. Nitric oxide levels might have been decreased and this would cause blood to consequently increase, the overall effect seen in the mice is the decrease in blood pressure following high phosphate diet ingestion. These two curious and contradictory results suggest that high phosphate diet is performing these effects by different mechanism of actions through targeting discrete components involved in the endothelial function and high blood pressure, which still requires further investigation.

CHAPTER 6

CONCLUSION

In conclusion, as seen in figure 23 which represents the working model of this experimental study, a high dose of phosphate (1.5%) decreases SBP by 24.8 mmHg and DBP by 19 mmHg, following hypertensive stimuli, making high dietary phosphate a protective agent on blood pressure in hypertension.

However, given that high dietary phosphate might have induced a possible endothelial dysfunction via disruption of the endothelial-dependent vasodilation and vascular inflammation, suggests that high phosphate might serve as a deleterious agent over the vasculature.

Whether phosphate is beneficial or not still requires more research experiments and analysis to fully understand its effect on the vasculature and whether its consumption should be limited.

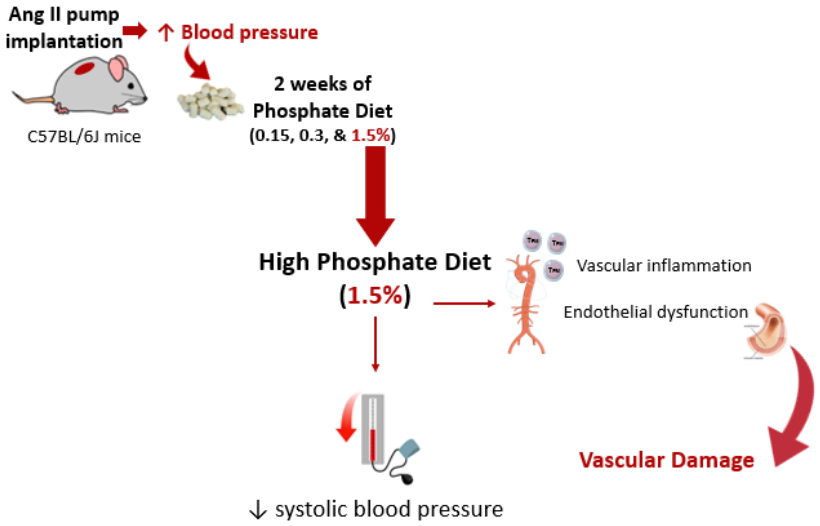

Figure 23. The working model of the study.

CHAPTER 7

LIMITATIONS AND FUTURE DIRECTIONS

The exact mechanism on how a high phosphate diet is lowering blood pressure following hypertensive stimuli and why a high dose of phosphate targets high blood pressure and not a normotensive one is still unclear.

Further investigation, specifically on the expression of sodium-dependent phosphate transporters needs to be done to reveal the exact mechanism. The expression of these transporters can be screened in the kidneys and endothelium mainly. Urine sodium levels and serum phosphate levels can also be measured to assess whether their excretion is causing the reduction in blood pressure.

In addition to that, a low-infused Ang II model can be used to examine whether the levels of Ang II itself are causing the decrease in blood pressure after phosphate diet ingestion.

The absence of significance in the Ach response curve in the 1.5% phosphate diet, indicates a trend of a decrease in the relaxation response rather than a significant one. This requires an additional increase in the sample size.

How endothelial dysfunction occurred in the groups subjected to a 1.5% phosphate diet is yet unclear. In order to know the exact mechanism, the NO pathway should be investigated. This includes investigating whether eNOS is present, coupled and functional, and phosphorylated at active or inhibitory sites. In addition to that, NO and ROS levels should be assessed in the aortas. Further vascular reactivity studies will be performed on resistant mesenteric vessels.

Vascular inflammation needs to be assessed if it is correlated to endothelial damage. Screening of adhesion molecules can be a sign of immune cells infiltration due to the loss of endothelial integrity.

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