AMERICAN UNIVERSITY OF BEIRUT

DETERMINING THE POSTPRANDIAL METABOLITES AND HORMONAL STATUS AFTER THE INGESTION OF PHOSPHORUS PRELOAD IN OVERWEIGHT AND OBESE SUBJECTS

by
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AN ABSTRACT OF THE THESIS OF

Marwa Osmat Hassan for Master of Science
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Title: Determining the postprandial metabolites and hormonal status after ingestion of a phosphorus preload in overweight and obese subjects.

In the literature, the positive effect of phosphorus preload on glucose, insulin, and appetite was previously reported and is encouraging; however, the gut hormones response after phosphorus preload was never tested. The objective of this study was to assess the impact of phosphorus on blood glucose, insulin, and appetite hormones (GLP-1 and GIP) in overweight and obese subjects.

This is a single blind cross over design study whereby each subject presented twice to the facility to take either the treatment or the placebo. Seven subjects were enrolled (BMI>25kg/m^2) of which four were males and three were females. After an overnight fast, the subjects were given a glucose solution (75g) together with phosphorus pills (500mg) or placebo pills. Serum levels were tested for insulin, phosphate, glucose, GLP-1, GIP, triglycerides, total cholesterol, HDL, and LDL at different time intervals: time 0, 15, 30, 60,120, 180, and 240 minutes.

The results showed no change in the levels of insulin, glucose, GIP, triglycerides, total cholesterol, HDL, and LDL when comparing the phosphorus group to the placebo group. However, in the phosphorus test group the serum phosphate levels increased significantly at 120 minutes and GLP-1 levels were significantly higher.

In this study the phosphorus preload did not cause any change in glucose and insulin responses after ingestion of a glucose solution, which is not in line with the previous findings. It was hypothesized that this response is due to the presence of insulin resistance in those overweight and obese subjects. The increase in serum phosphorus and the rise in GLP-1 levels show promising result and open the door for future research since long term ingestion of phosphorus in overweight and obese individuals could be able to impact more insulin sensitivity than just one preload.

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ABBREVIATIONS

%	Percentage
/	Per
<	less than
x	multiplied by
2, 5-AM	2, 5-anhydro-D-mannitol
ß	Beta
^{o}C	Degree Celsius
ACC	Acetyl-CoA carboxylase
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
AUBMC	American University of Beirut Medical Center
Bcl	B-cell lymphoma
BMI	Body mass index
cAMP	Cyclic adenosine monophosphate
CeA	Central nucleus of the amygdale
CPT-1	carnitine palmitoyl transferase 1
CREB	cAMP response element-binding protein
CRU	Clinical Research Unit
dl	Deciliter
DPP-4	Dipeptidyl peptidase-4
EPAC2	exchange protein activated by cAMP

ERK1/2	Extracellular signal regulated kinase
et al.	et allii (and others)
FFA	Free fatty acids
FFM	Fat free mass
FGF 23	Fibroblast growth factor 23
FoxO1	Forkhead box protein O1
GIP	Glucose-Dependent Insulinotropic polypeptide
GLP-1	Glucose-like peptide 1
GLP-1R	Glucose-like peptide 1 receptors
g	Grams
h	hour
$H_2PO_4^-$	Dihydrogen phosphate
HbA1c	Glycosylated hemoglobin
HDL	High density lipoprotein
HPO_4^{2-}	Monohydrogen phosphate
IRS-2	Insulin receptor substrate 2
Kcal	Kilocalories
Kg	Kilogram
LDL	Low density lipoprotein
LPL	lipoprotein lipase
mg	Milligram
min	Minute
ml	Milliliter
mmHg	Millimeter of mercury
mmol	Millimoles

NF- κB	Nuclear factor kappa B
nM	Nanomole
OGTT	Oral glucose tolerance test
oz	Ounce
Pdx-1	Pancreas duodenum homeobox 1
pg	Picogram
PKA	Protein kinase A
PKB	Protein kinase B
pmol	picomoles
PO_4^{3-}	Phosphate
PTH	Parathyroid hormone
RDA	Recommended dietary allowance
RPM	Rounds per minute
SEM	Standard error of the mean
TCA	tricarboxylic acid
TG	Triglycerides
TORC2	Transducer of regulated CREB activity 2
UL	Tolerable Upper Intake Level
VLDL	Very low density lipoprotein
WHO	World Health Organization

CHAPTER I

INTRODUCTION

Obesity is becoming a great health concern due to its increased prevalence. It was estimated by the World Health Organization (WHO) that 1.6 billion people worldwide were overweight and 400 million adults were obese in 2005 and that the levels in 2015 will increase reaching 2.3 billion for overweight adults and at least 700 million for obese adults (Nguyen and El-Serag, 2010). Similarly, diabetes prevalence is also on the rise from 171 million in year 2000 to 366 million in 2030 (Wild et al., 2004). Therefore, both obesity and diabetes are now considered global epidemics.

Obesity increases the risk of developing diabetes, heart disease, hypertension, stroke, different types of cancer, and digestive diseases (Nguyen and El-Serag, 2010). In addition, obesity increases the risk of death by 2 to 3 folds even in obese non-smokers (Adams et al., 2006).

Obesity is caused by several factors: environmental, genetic, and human behavior. Other than the decrease in physical activity, there is an increased trend toward the production of high density processed food and an increased trend toward eating readymade meals, processed foods, and fast food away from home. This is causing a major change in peoples' lives whereby people are consuming less nutritious foods raising the concern to a wide range of health conditions (Popkin et al., 2012; Nguyen and El-Serag, 2010). Dietary behavior is shifting towards high consumption of refined carbohydrates, added sugars and fats and less towards consumption of legumes, vegetables, and coarse grains which are more nutritious (Popkin et al., 2012).

Obesity is usually accompanied by hyperinsulinemia, hypertension, hypertriglyceridemia, and low serum HDL which make up the components of the metabolic syndrome (Haglin, 2001). Overweight, obese, and diabetic individuals have a low serum phosphate level (Haglin, 2001; Ditzel and Lervang, 2009). Furthermore, there is a positive correlation between serum phosphate and the metabolic syndrome (Gudmundsdottir et al., 2008). Phosphate has been known to be involved in carbohydrate metabolism ever since 1925 (Bolloger and Hartman, 1925). Phosphate is important for carbohydrate metabolism given that it stimulates enzymes needed in glycolysis (Uyeda and Racker, 1965b; Ditzel and Lervang, 2010), in oxidative phosphorylation and tricarboxylic acid cycle (TCA cycle) for ATP production (Kemp and Brindle, 2012; Ditzel and Lervang, 2010). Hypophosphatemia is associated with impaired glucose metabolism, increased insulin resistance, and decrease in insulin production (DeFronzo and Lang, 1980; Haap et al., 2006; Xie at al., 2000). There is an increased risk of developing type 2 diabetes in obese individuals, partly due to the fact that overeating and overconsumption of carbohydrate rich food cause a decrease in serum phosphate leading to increased risk of diabetes (Haglin, 2001). Other reasons for hypophosphatemia could be the increased consumption of low phosphorous food, the unbalanced diet, trans-cellular shift, and increased phosphate excretion (Haglin, 2001).

Both GLP-1 and GIP have been shown to improve insulin production, decrease insulin resistance, and improve glycemia (Seino et al., 2010) which is similar to the effect of phosphorus. The objective of this research is to study the effect of phosphorus on glucose levels, insulin levels, lipid profile, and most importantly on two incretin hormones: Glucose-like peptide 1 (GLP-1) and Glucose-Dependent Insulinotropic polypeptide (GIP).

CHAPTER II

LITERATURE REVIEW

A. Phosphorus

Phosphorus is an essential element and the sixth most abundant mineral in the body (Blumsohn, 2004). The body content of phosphorus is 700 g in a 70 kg average adult (Amanzadeh and Reilly, 2006). It is present mainly in the bone and teeth (85%), in addition to being present in the soft tissues (14%) and extracellular fluid (1%) (Amanzadeh and Reilly, 2006). There are two forms of phosphorus: organic and inorganic. The important intracellular organic phosphate stores are adenosine triphosphate (ATP), the body's main energy reservoir, and 2,3-diphosphoglycerate, an isomer which decreases oxygen affinity to hemoglobin and thus enhances its availability and delivery to the tissues (Gaasbeek and Meinders, 2005; Kalantar-Zadeh et al., 2010). The inorganic forms exist mainly as HPO_4^{2-}, $H_2PO_4^-$, and $PO4^{3-}$ (Gaasbeek and Meinders, 2005). Phosphorus is vital for the normal functioning of all cells since it is needed in phosphorylation reactions, for the structural component of the cell membrane (phospholipids) (Kalantar-Zadeh et al., 2010), for production of nucleic acids, and for urinary buffering (Amanzadeh and Reilly, 2006).

1. Dietary phosphorus:

Dietary phosphate is usually present in foods that contain high levels of protein such as meat, fish, and dairy (Kalantar-Zadeh et al., 2010) - 12 to 16 mg of phosphorus is present per gram of protein (Ritzl, 2005). The average diet provides 800-1500 mg of phosphate per day (Gaasbeek and Meinders, 2005). From the ingested phosphorus, 40 to

80 % is absorbed in the intestine. Phosphate is also present in plant sources such as beans, peas, seeds and nuts which is less than 50 % bioavailable as compared to the animal sources since it is mostly in the form of phytate (Kalantar-Zadeh et al., 2010). Inorganic phosphate is also a component of many food additives which are added to processed food and is readily absorbed (more than 90 %) which may contribute up to 1000mg/day of phosphorus in the American diet (Kalantar-Zadeh et al., 2010). According to Food and Nutrition Board Institute of Medicine, the recommended dietary allowance (RDA) for men and women above 19 years of age is 700 mg/day and tolerable upper intake level (UL) is 4 g/day (1997).

Table 1: Food sources of phosphorus

Food source	**Phosphorus content (mg/100g)**
Almonds	476
Beef	178
Cashew	463
Cheddar cheese	483
Chicken breast	181
Crab	194
Egg(whole)	168
Kidney beans	141
Lamb	189
Lentils	180
Lima beans	112
Milk	94
Mozzarella Cheese	497
Salmon	261
Soybeans, cooked	234
Tuna, canned in oil	294
Walnuts	327

(Kalantar-Zadeh et al., 2010)

2. Regulation of Phosphate:

The kidneys and the small intestine are the two major organs that regulate phosphorus balance in the body (Amanzadeh and Reilly, 2006). Around 70 % of the dietary phosphorus is absorbed via the small intestine, particularly through the duodenum and jejunum via an active sodium dependent channel that is stimulated by 1, 25-dihydroxyvitamin D_3 (active pathway) and via a passive mechanism of phosphate ions diffusion (Gaasbeek and Meinders, 2005). The kidneys are the most important organs for regulating serum phosphate in the body making phosphorus excretion equivalent to phosphorus absorption at the level of the intestine (Gaasbeek and Meinders, 2005). The kidneys can reabsorb up to 80 % of the filtered phosphate, so low phosphorus intake leads to complete reabsorption of phosphate by the kidneys whereas high phosphorus diet results in decreased reabsorption (Amanzadeh and Reilly, 2006). The normal range of serum phosphorus is 2.5 to 4.5 mg/dl (Kalantar-Zadeh et al., 2010).

Many hormones are also involved in regulating serum phosphate: parathyroid hormone (PTH), 1,25-dihydroxyvitamin D_3 , and Fibroblast growth factor-23 (FGF-23). PTH is secreted by the parathyroid gland. It is stimulated when serum phosphate concentrations are elevated, thus increasing phosphate excretion by the kidneys leading to hypophosphatemia (Bergwitz and Juppner, 2010). PTH leads to an elevation in 1, 25-dihydroxyvitamin D_3 production followed by an increase in FGF-23 which is stimulated by the rise in vitamin D (Bergwitz and Juppner, 2010). FGF-23 is another hormone involved in phosphate homeostasis. FGF-23 is secreted by the osteocytes in bones (Quarles, 2008). It functions as a phosphaturic agent by binding to the FGF receptor in the presence of klotho protein; thus, inhibiting phosphate reabsorption in the kidney and inhibiting the 1α-hydroxylase to produce the active form of vitamin D (1, 25-dihydroxyvitamin D3) (Quarles, 2008). Both dietary phosphate and 1,25-

dihydroxyvitamin D_3 increase FGF-23 production (Bergwitz and Juppner, 2010). Moreover, FGF-23 has been also shown to suppress PTH production and secretion causing a reduction in serum phosphate and calcium (Bergwitz and Juppner, 2010). In addition, vitamin D also affects serum phosphate levels by increasing its intestinal absorption. It is influenced by PTH and FGF-23 whereby PTH upregulates vitamin D production and FGF-23 downregulates it. Nevertheless, vitamin D also acts to stimulate FGF-23 production and to inhibit PTH secretion (Bergwitz and Juppner, 2010; Quarles, 2008). Additionally, the thyroid hormone has been also shown to increase phosphorus reabsorption by the proximal tubule (Amanzadeh and Reilly, 2006). Accordingly, phosphate regulation is a complex process requiring an integrated and interrelated network of hormonal pathways.

3. Phosphate and glucose :

Phosphate is important for carbohydrate metabolism. It stimulates hexokinase and phosphofructokinase enzymes that are needed in glycolysis (Uyeda and Racker, 1965b; Ditzel and Lervang, 2010). Phosphate does not only stimulate glycolysis but it has been shown to counteract the glycolysis inhibition caused by glucose-6-phosphate (which inhibits hexokinases) and ATP (which inhibits phosphofructokinase) (Uyeda and Racker, 1965a). In addition, phosphorus is needed in the oxidative phosphorylation and tricarboxylic acid cycle (TCA cycle) for ATP production (Kemp and Brindle, 2012; Ditzel and Lervang, 2010).

In 1921, Fiske was the first to determine a relationship between glucose ingestion and phosphate retention (Amanzadeh and Reilly, 2006). Since 1925, phosphate has been shown to be involved in carbohydrate metabolism (Bolloger and Hartman, 1925). Glucose injection in dogs caused a drop in serum phosphate

concentration reaching very low levels after 2 h of glucose infusions together with urinary phosphate excretion (Pollack et al., 1934), but, in the same study, dogs made diabetic by pancreatectomy had no change in phosphate levels when glucose was injected until insulin was supplied. Similar results were obtained by Markowitz (1926) whereby depancreatised dogs had no change in urine phosphate excretion when glucose was administered due to the absence of insulin. Other preliminary studies on normal animals showed that administration of insulin and glucose separately or together caused a decrease in serum phosphate concentration, increase in muscle phosphate concentration (Harrop and Benedict, 1923), and a decrease in urinary phosphate excretion (Sokhey and Allan, 1924; Harrop and Benedict, 1923) compared to diabetic dogs without insulin that had high levels of phosphorus excretion (Sokhey and Allan, 1924).). Berthelay et al. (1984) also observed a decrease in serum phosphorus, decreased phosphorus excretion, and increased calcium excretion with 75 g of a glucose load (Berthelay et al., 1984). These experiments indicate that there is an intracellular shift of phosphate in the body: phosphate retention in response to insulin or after carbohydrate ingestion which usually occurs in the muscle and not in the liver (Pollack et al., 1934; Harrop and Benedict, 1923), where inorganic phosphate and glucose are transported intracellularly in a similar manner for glycogen production (Harrop and Benedict, 1923; Bolloger and Hartman, 1925).

On the other hand, phosphate infusion has also been shown to improve glucose uptake into the cells (Nowicki et al., 1996) and insulin sensitivity (Khattab at al., 2011). Serum phosphate was negatively correlated with 2 h blood glucose and positively correlated with insulin sensitivity when measured in 881 non-diabetic subjects undergoing oral glucose tolerance test (OGTT) (Haap et al., 2006). Similar results were obtained by DeFronzo and Lang (1980) whereby hypophosphatemia was associated

with impaired glucose metabolism and decreased insulin sensitivity. One explanation of those findings is that hypophosphatemia leads to impaired glucose tolerance through causing insulin resistance (Haap et al., 2006). Hypophosphatemia induced by a phosphate deficient diet in male Sprague-Dawley rats also lead to a decrease in insulin concentration, an increase in plasma glucose levels, and a decrease in liver glycogen (Xie et al., 2000). Low serum phosphate levels also inhibit phosphorylation of carbohydrate intermediates in glycolysis and gluconeogenesis (Xie et al., 2000). According to their findings, phosphate deficient diet in the fed state led to an increase in cAMP levels producing a decrease in pyruvate kinase, an increase in phosphoenolpyruvate,, a de-inhibition of fructose-1,6-bisphosphatase, and an upregulation of glucose-6-phosphatase all of which resulted in increased glucose production due to increase in gluconeogenesis and glycogenolysis causing hyperglycemia (Xie et al., 2000). Similar findings were obtained by Ellam et al. (2011) whereby Apolipoprotein E knockout mice fed on low phosphate diet had a 4 fold increase in insulin resistance when compared to other diets higher in phosphate.

Phosphate depletion leads to decreased insulin production by the pancreatic cells since phosphate depletion increases the influx of ionized calcium into the cytosol increasing the basal ionized calcium (Fadda et al., 1991) which inhibits mitochondrial oxidation and ATP production. The low ATP levels lead to impairment in calcium ATPase and sodium potassium ATPase; thus decreasing the extrusion of calcium out of the cell, increasing further the intracellular basal calcium levels and causing an impaired insulin secretion (Levi et al., 1992). Usually glucose enters the cell through a facilitative transporter, undergoes glycolysis and thus causes ATP production which leads to opening of calcium channels, increasing intracellular calcium levels and triggering exocytosis of insulin (Novak, 2008). Consequently, ATP production and the rise in

intracellular calcium levels (the calcium entering the cell versus the basal level of calcium) are the causes of insulin secretion by the pancreatic cells. Therefore, if the basal calcium level is already higher, there is a smaller rise in calcium levels, and a lower ATP production, as mentioned earlier, and thus lower insulin levels are produced (Fadda et al., 1991; Levi et al., 1992). In summary, phosphorus is needed postprandially for the phosphorylation of metabolites and the production of ATP which is an important component of carbohydrate metabolism (Mattar et al., 2010; Gudmundsdottir, 2008); therefore, low serum phosphorus leads to impaired glucose utilization and insulin secretion and thus increases insulin resistance (Gudmundsdottir, 2008).

4. Phosphate's relationship to appetite and food intake:

The liver is one of the primary organs involved in the control of the feeding behavior through ATP production from the oxidation of glucose and various other metabolites by the TCA cycle and oxidative phosphorylation (Friedman, 1995). ATP has been shown to activate the sodium pump creating a membrane potential at the level of the vagus nerve (linking the brain to the liver); thus generating the signal for satiety (Langhans, 1996; Friedman, 2007). Therefore, a reduction in ATP has been shown to trigger feeding behavior (Friedman, 1995).

Fructose administration to rats or humans caused an accumulation of fructose-1-phosphate, a depleting of phosphate, and consequently a decreased ATP content (Nishi et al., 1989; Mattar et al., 2010). Fructose is directly taken by the liver and phosphorylated to fructose-1-phosphate, thus using the ATP, trapping phosphorus, and reducing the levels of both ATP and inorganic phosphate (Mattar et al., 2010). The administration of 2,5-anhydro-D-mannitol (2,5-AM), a fructose analogue, to rats caused an increase in food intake which was the result of a reduction in hepatic ATP levels

through phosphate trapping; hepatic ATP reduction was inhibited in rats injected with sodium phosphate since phosphate is needed for ATP regeneration (Rawson and Friedman, 1994). Another study by Tordoff et al. (1991) also showed that 2, 5-AM administration caused an increase in food intake, but hepatic vagotomy reduced the effect of the fructose analogue in stimulating food intake. This also shows that hepatic ATP levels and the signal through the vagus nerve affect satiety signals.

A study by Obeid et al. (2010) showed that phosphorus preload decreased food intake at a subsequent meal. Subjects were asked to consume a solution of sucrose, fructose, glucose, or just water with or without phosphorus, and then they were offered a pizza meal after 80 minutes of consuming the preload. The effect was a decreased energy intake which ranged between 25 % and 35%. This also shows how phosphorus levels affect satiety and energy intake.

5. Phosphate and the metabolic syndrome:

Obesity has been shown to be accompanied by hyperinsulinemia, hyperglycemia, hypertension, and hypertriglyceridemia which indicates the presence of the metabolic syndrome (Haglin, 2001). It has been suggested that disturbed serum phosphate levels may play a role in development of the metabolic syndrome since phosphate is needed in carbohydrate metabolism (Park et al., 2009). Serum phosphate was shown to be negatively correlated with the BMI in 881 non-diabetic subjects (Haap et al., 2006). A cross-sectional study of 2752 obese patients (between the year 1986 and 1996 admitted to the education center in North Sweden) showed that serum phosphate correlated negatively with BMI , blood pressure (systolic and diastolic), and blood glucose, but correlated positively with serum calcium, magnesium and HDL (Haglin, 2001). A similar relationship was observed by Gudmundsdottir et al (2008) who found a

negative correlation between serum phosphate and the metabolic syndrome in middle aged men followed over 20 years. Another study evaluated the serum phosphate in the metabolic syndrome where 2265 patients participated in a 4 week education program of diet and physical activity and were followed up after 6 months, one year, and one and a half year (Haglin et al., 2001). At baseline, both men and women with low serum phosphate had higher BMI, systolic blood pressure, and lower serum calcium. In addition, only men had a negative correlation between serum phosphate and blood glucose. After the intervention, weight reduction caused a significant increase in serum phosphate, a significant decrease in glucose, and a significant decrease in triglycerides (Haglin et al., 2001).

6. Phosphate and Obesity:

Serum phosphate was shown to be negatively correlated with BMI (Haglin et al., 2001; Haap et al., 2006). Obese children between 6 and 12 years, had a lower serum phosphate level as compared to controls (Celik and Andiran, 2011). In addition, women had an increase in serum phospate levels after weight loss (Haglin et al., 2001). This correlation could be attributed to overconsumption of energy with a low nutrient diet or low protein consumption (Haglin et al., 2001).

Different studies showed that hepatic ATP stores and ATP replenishment was inversely correlated with body mass index (BMI) (Cortez-Pinto et al., 1999; Nair et al., 2003).A hypothetical model states that there is a relationship between energy intake, energy storage, hepatic ATP, and energy expenditure (Friedman, 1995). Energy intake usually increases in response to an increase in energy expenditure which is translated through a decrease in hepatic ATP level initiating a signal for eating (Friedman, 1995). In obesity, the metabolic state usually directs fuels in the body towards fat storage and

not towards fuel oxidation for ATP production which traps the fuels mainly for storage (Friedman, 1995). Thus, hyperphagia results from increased fat storage and decreased ATP production (Friedman, 1995; Wlodek and Gonzales, 2003).

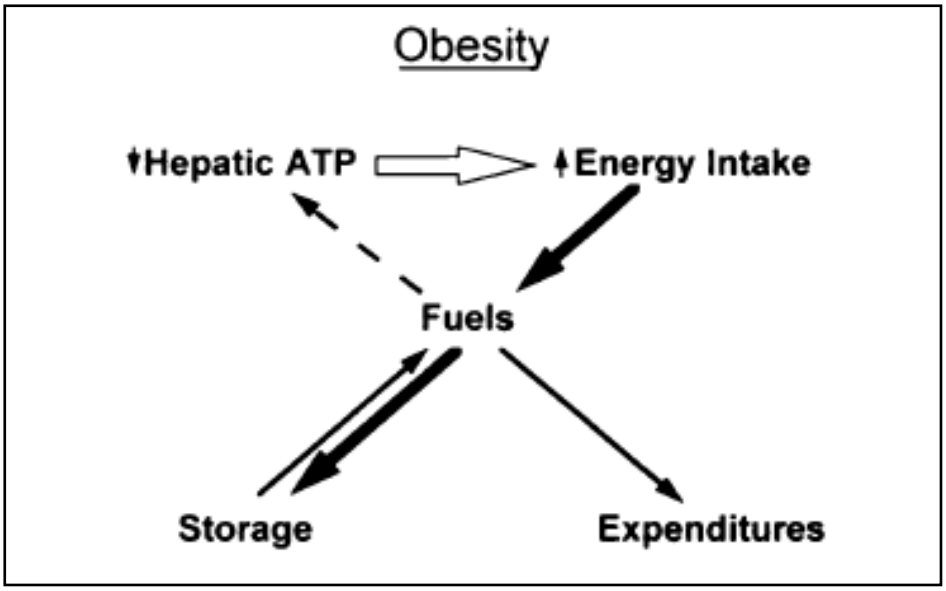

Figure 1:Hypothesized mechanism for increased energy intake associated with increased energy deposition during the development of obesity (Friedman, 1995)

The reason for this shift towards fat storage (illustrated in Figure 2) is due to the inhibition of aconitase enzyme (which converts citrate to isocitrate in the tricarboxylic acid cycle), due to increased inflammation and elevated cytokines; thus inhibiting ATP production. The accumulation of citrate in the cytosol leads to its conversion to acetyl-CoA and malonyl-CoA leading to fatty acid synthesis. In addition, high concentrations of acetyl-CoA carboxylase (ACC) and malonyl-CoA inhibit carnitine palmitoyl transferase 1 (CPT1) which decreases beta-oxidation (Wlodek and Gonzales, 2003). Therefore, decreased ATP levels increase the appetite signaling, leading to increased food intake, and shift of fuel for fat storage, consequently increased fat storage and thus increased inflammatory factors. This results in a vicious circle that perpetuates obesity whereby inflammatory markers lead to increased fat production and the increased adipose tissue leads to increased inflammation (Figure 3) (Wlodek and Gonzales, 2003).

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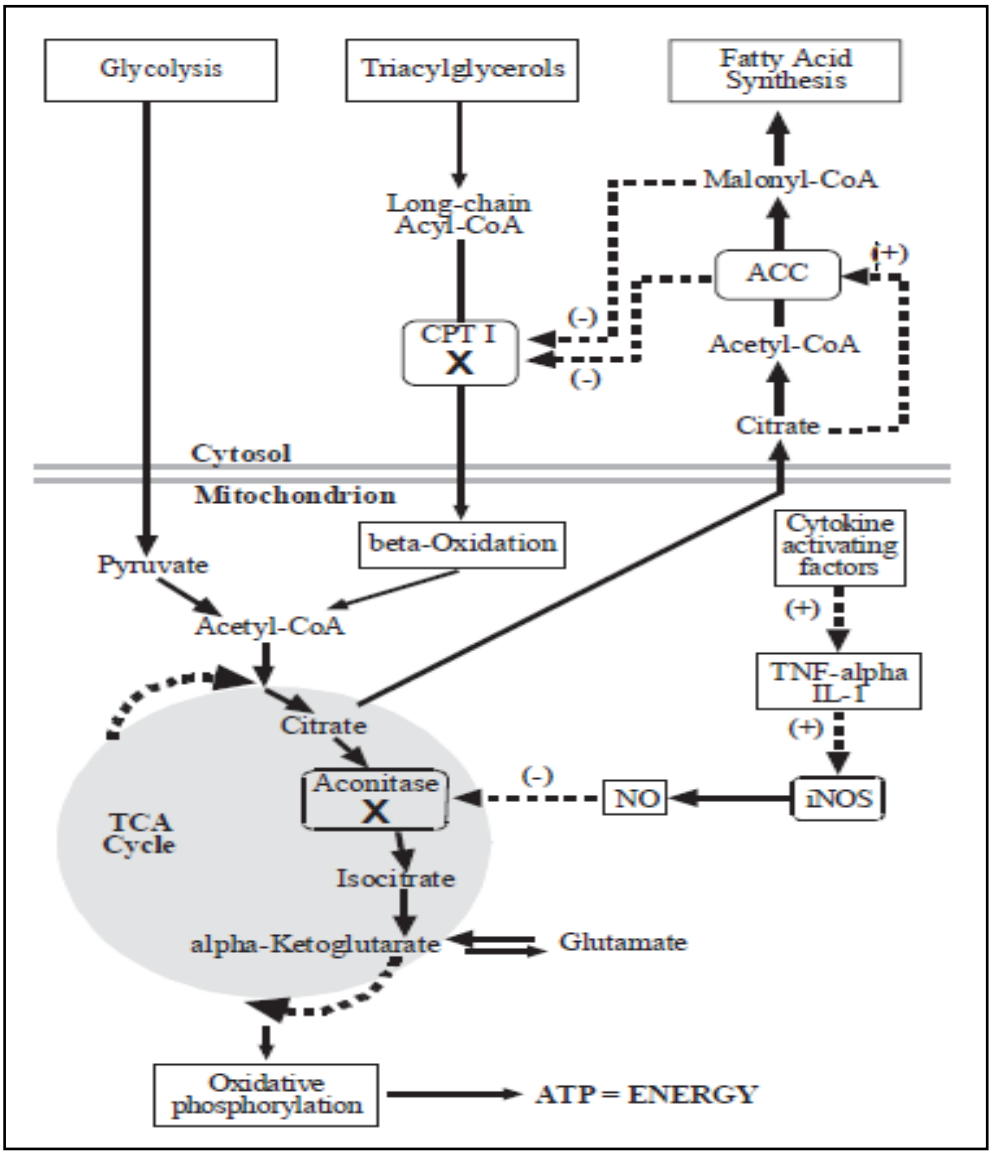

Figure 2: Energy metabolism in obesity

(Wlodek and Gonzales, 2003)

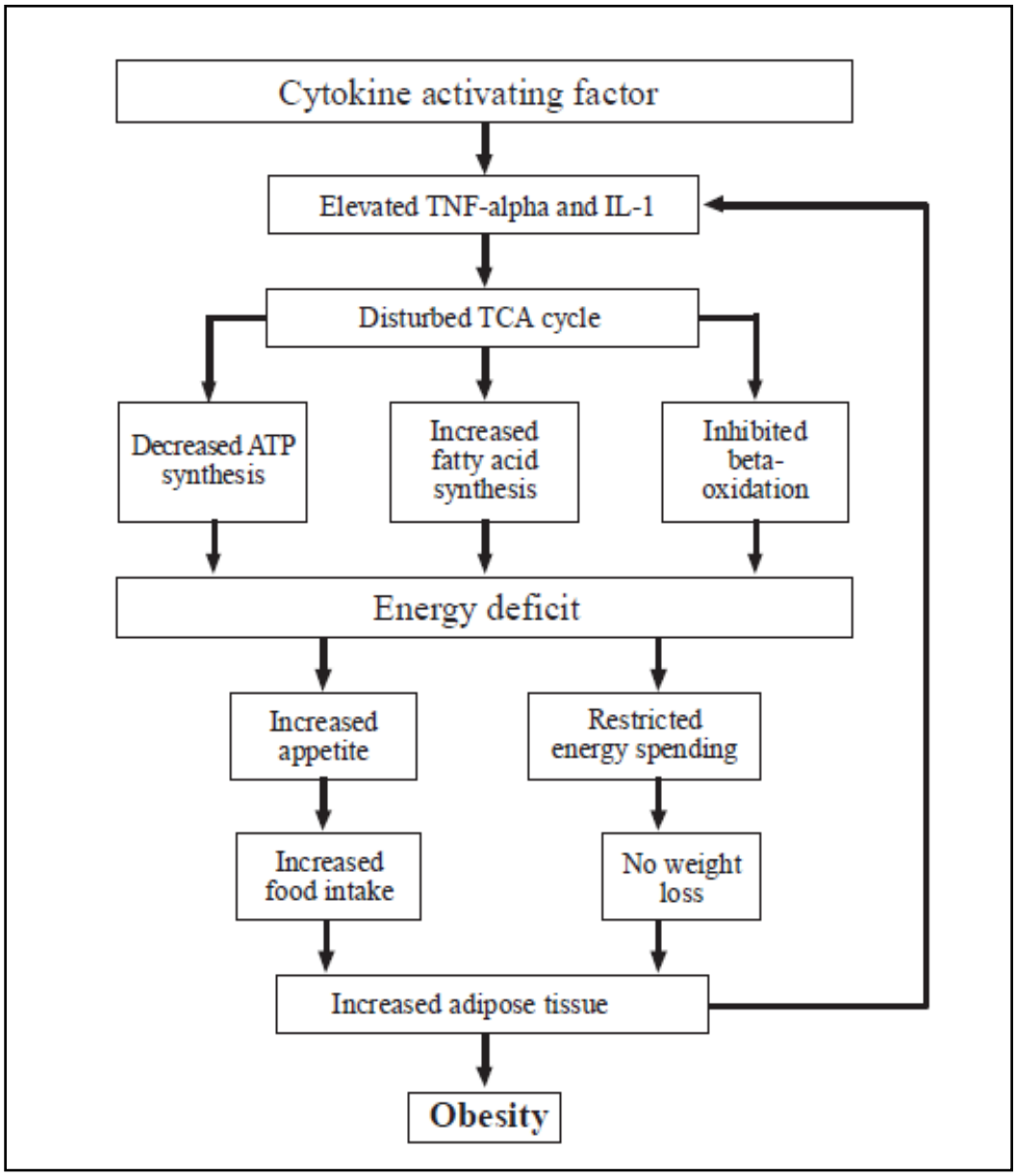

Figure 3: Vicious circle of events leading to obesity

(Wlodek and Gonzales, 2003)

7. Phosphate and Diabetes:

Diabetes is usually caused by insulin resistance combined with impaired insulin production leading to hyperglycemia. As previously mentioned, hypophosphatemia is correlated with insulin resistance, decreased insulin production, and glucose intolerance. Both type 1 and type 2 diabetic patients are found to have hypophosphatemia (Ditzel and Lervang, 2010).

A study performed on healthy, young, lean, insulin-resistant offspring of patients with type 2 diabetes showed impaired mitochondrial phosphorylation, 30% reduction in mitochondrial ATP production, and an 80% increase in intramyocellular lipid content when compared to matched controls (Petersen et al., 2004).Similar results were obtained by Peterson et al. (2005) whereby there was a 90% increase in intramyocellular lipid content in insulin resistant offsprings. In addition, insulin stimulated increase in intramyocellular phosphate concentration and ATP production were blunted in insulin resistant offsprings as compared to controls (Peterson et al., 2005). Thus, insulin resistant offsprings of parents with type 2 Diabetes have mitochondrial dysfunction with impaired insulin that affects phosphorus transport and ATP synthesis.

Investigators have also found an increased phosphate excretion in diabetic subjects with decreased plasma concentrations particularly in those with uncontrolled blood glucose levels (Ditzel and Lervang, 2009). A study comparing 26 children with type1 diabetes versus 28 healthy children found that fasting urinary phosphate excretion was 3 times higher in the diabetic group and that the phosphate excretion was correlated with glucose excretion and blood glucose levels (Ditzel et al., 1982). Another study performed on 28 subjects with diabetes mellitus showed a decrease in phosphate excretion and an increase in serum phosphate when patients were hospitalized for

diabetes control (Nagasaka et al., 1995). In summary, serum phosphate levels are negatively correlated with blood sugar levels (Ditzel and Lervang, 2009).

8. Phosphate and dyslipidemia:

Dyslipidemia is a component of the metabolic syndrome. Park et al. (2009) studied 46,798 healthy subjects over 20 years of age and found that serum phosphate is positively correlated with HDL, LDL, and total cholesterol, and negatively correlated with triglyceride. This study demonstrated an increased risk of cardiovascular disease hypothesizing that high phosphate levels could lead to deposition of calcium phosphate in the vascular smooth muscle cells (Park et al., 2009). Similarly, serum phosphate was found to be positively correlated with HDL and negatively correlated with triglycerides (Haglin, 2001). Another study by Dhingra et al. (2007) demonstrated a positive association between serum phosphate and total cholesterol to HDL ratio in individuals without kidney disease or cardiovascular disease. It is also hypothesized that serum phosphate affects the phospholipids in the liver thus causing a disturbed lipid profile (Haglin, 2001) and that increased serum phosphate causes an increase in PTH which causes increased interleukin-6 production (proinflammatory markers) (Dhingra et al., 2007). Apolipoprotein E knockout mice fed an atherogenic diet with different phosphate contents ranging from low to high resulted in an aortic atheroma after 20 weeks in the high phosphorus group, thus indicating an increased risk of atherosclerosis (Ellam et al., 2011). Serum phosphate was associated with increased risk of heart failure and myocardial infarction in 4127 participants followed over 60 months who had a previous myocardial infarction, are dyslipidemic, but with normal kidney function (Tonelli et al., 2005). Evaluating 3368 Framingham Offspring study participants over 20 years showed a 55 % increased cardiovascular disease risk in individuals with serum phosphorus

levels higher that 3.5mg/dl (Dhingra et al., 2007). In conclusion, several studies associated higher levels of serum phosphate and phosphate supplementation, even when the serum phosphate levels were still within normal levels, to an increased risk of cardiovascular disease.

9. Phosphate and hypertension:

As mentioned previously, hypophosphatemia is associated with high systolic and diastolic blood pressure (Haglin, 2001). A cross-sectional study including 4680 subjects revealed that a lower blood pressure was observed in those with higher phosphorus intake (Elliott et al., 2008). Another cross-sectional cohort study including 13444 participants also found an inverse relationship between phosphorus intake and blood pressure (systolic and diastolic) whereby a 500mg higher phosphorus intake per day was associated with a 1.1mmHg lower systolic blood pressure (Alonso et al., 2010). During hypophosphatemia, ATP production decreases, calcium excretion increases, but intracellular calcium levels increase together with intracellular sodium levels. This mechanism together with hyperglycemia and hyperinsulinemia (observed during hypophosphatemia) lead to decreased sodium excretion and thus increased blood pressure (Haglin, 2001). In addition, the increased intracellular calcium level has been shown to cause increased vascular resistance. Nevertheless, the increased calcium excretion leads to a decrease in plasma ionized calcium which has long been shown to be inversely related to mean blood pressure (Ljunghall et al., 1987; Hunt et al., 1991).

10. Phosphate and thermogenesis:

Thirty six obese females were given a low fat calorie restricted diet (1000 Kcal per day) together with a calcium and potassium phosphate tablet or a placebo tablet. The

results showed a significant increase in resting metabolic rate (Kaiuba-Uscilko et al., 1993). Nazar et al. (1996) also achieved a 12 to 19 % increase in resting metabolic rate when he supplemented overweight women with a mineral tablet containing calcium, potassium and sodium phosphate. A double blind crossover study on 15 obese and 15 lean postmenopausal healthy women consuming 100 ml of orange juice with or without a mineral supplement (of potassium, magnesium, and phosphate (39mmol)) caused a 6.3 % increase only in obese subjects taking phosphate with no increase in lean subjects (Jaedig et al., 1994). So, obese individuals respond with an increased postprandial thermogenesis after being treated with phosphate and thus a low metabolic rate with an imbalance in phosphate and carbohydrate leads to the increased plasma glucose, increased BMI, and increased triglycerides (Haglin, 2001).

B. Incretins:

Incretins are hormones released from the gastrointestinal tract in response to consumption of food, mainly carbohydrates and fat. They are called incretins because they increase the glucose dependent secretion of insulin from the pancreatic ß-cells (Weaver et al., 2008). The two major incretins are glucagon-like peptide 1 (GLP-1) and glucose dependent insulinotropic peptide (GIP). Oral glucose load produced a higher insulin response than intravenous injection of glucose due to the incretin effect of GIP and GLP-1 that are produced from the small intestine (Seino et al., 2010).

GIP and GLP-1 are rapidly cleaved by dipeptidyl peptidase-4 (DPP-4) and thus are deactivated (Song and Wolfe, 2007; Seino et al., 2010; Silva and Bloom, 2012). Nevertheless, total forms of GLP-1 and GIP should be measured and not

only the active forms to be able to analyze and study their secretion and processing in vivo (Seino et al., 2010).

1. Glucose-like peptide 1

GLP-1 is a 31-amino acid hormone secreted from the L cells of the lower intestine and colon in response to meal ingestion (Seino et al., 2010). The half life of GLP-1 is 2 minutes since after its secretion it gets degraded by DPP-4, thus rendering it biologically inactive (Silva and Bloom, 2012). GLP-1 is produced from the cleavage of proglucagon. The glucagon gene, which encodes 158 amino acids, is cleaved by convertases that are tissue specific. In the L cells of gut and in the CNS, proglucagon is cleaved to GLP-1, whereas in the pancreatic α cells, proglucagon cleavage yields glucagon (Gallwitz, 2012; Silva and Bloom, 2012).

In addition to being present in the pancreatic ß cells, GLP-1 receptors (GLP-1R) are also present in the heart, central nervous system, kidney, lung, gastrointestinal tract, adipose tissue, adrenal cortex, bone, thyroid, and bone (Phillips and Prins, 2012). This allows GLP-1 to have both a central and a peripheral role.

a. GLP-1 incretin effect and beyond:

GLP-1 is considered an incretin hormone due to its normoglycemic actions. It stimulates the secretion of insulin from the pancreatic ß cells and inhibits the secretion of glucagon from the pancreatic α cells (Gallwitz, 2012), in addition to favoring hepatic glycogen storage (Knauf et al., 2005). GLP-1 infusion intravenously, accompanied by a meal thereafter, has been shown to decrease gastric emptying, decrease fasting blood sugar, decrease postprandial blood sugar, and abolishes the postprandial increase in triglycerides (Meier et al., 2006). The insulinotropic effect of GLP-1 is illustrated in figure 4. GLP-1 binds to its receptor GLP-1R (G coupled receptor) on the pancreatic ß

cells activating adenylate cyclase leading to production of cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP) (Baggio and Drucker, 2007; Seino et al., 2010). cAMP in turn leads to activation of protein kinase A (PKA) and exchange protein activated by cAMP (EPAC2) which causes an increase in intracellular calcium influx elevating cytosolic calcium levels and thus leading to enhanced insulin granule exocytosis from ß cells (Baggio and Drucker, 2007; Seino et al., 2010). This pathway contributes to increase insulin secretion in response to glucose. The increase in calcium levels also generates an increase in mitochondrial ATP synthesis causing closure of potassium channels, thus delaying the action potential which in turn affects insulin exocytosis into the plasma (Baggio and Drucker, 2007). Activation of cAMP/PKA pathway and elevation of calcium levels also lead to increased insulin gene transcription and biosynthesis (Baggio and Drucker, 2007).

Usually Type 2 Diabetes develops due to insulin resistance and a decrease in ß cell mass which results from ß cells apoptosis. Apoptosis is most probably caused by the increased cytokines, increased plasma glucose and free fatty acid levels (Cornu et al., 2009). GLP-1 has been shown to protect against ß cells apoptosis through increased cAMP formation, increasing protein kinase A activation which migrates to the nucleus leading to increased phosphorylation of the transcription factor CREB (Cornu et al., 2009; Seino et al., 2010). The activated PKA results in dephosphorylation and nuclear import of the transducer of regulated CREB activity 2 (TORC2). In the nucleus, CREB and TORC2 form a complex promoting gene transcription of anti-apoptotic gene bcl-2 and inhibiting caspase 3 thus inhibiting apoptosis (Seino et al., 2010). In addition, binding of GLP-1 to its receptor leads to increase in insulin receptor substrate 2 (IRS-2) and extracellular signal regulated kinase (ERK1/2) originating in proliferation of ß cells (Seino et al., 2010) and inhibiting their apoptosis (Cornu et al., 2009). GLP-1 has been

also shown to increase pancreas duodenum homeobox 1 (Pdx-1) gene transcription which is essential for pancreatic development, ß cells functioning, insulin gene transcription and secretion (Baggio and Drucker, 2007). GLP-1 also play its proliferative and cytoprotective role by inhibiting the transcription factor forkhead box protein O1 (FoxO1) (Baggio and Drucker, 2007; Seino et al., 2010). GLP-1 was also shown to protect ß cells against gluco-lipotoxicity by activation of protein kinase B (PKB) and bcl-1 antiapoptotic gene in addition to enhancement of nuclear factor kappa B (NF- κB) DNA binding activity (Buteau et al., 2004).

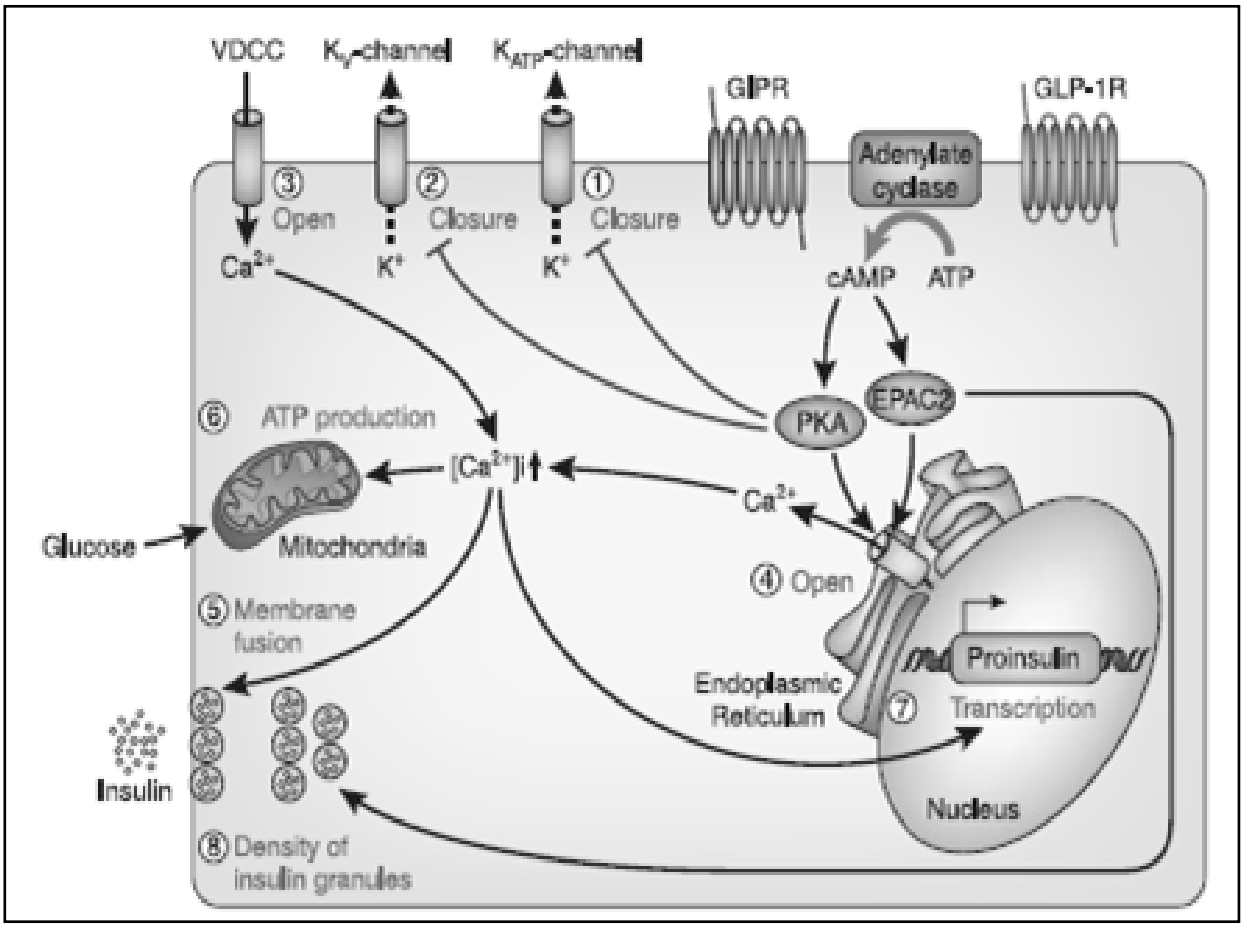

Figure 4: Molecular insulinotropic mechanisms of GIP and GLP-1
(Seino et al., 2010)

b.GLP-1 affecting hunger, appetite, and gastric emptying

Despite the blood glucose lowering effect, GLP-1 administration has been shown to suppress appetite and energy intake. GLP-1 was administered at a rate of 50 pmol/ kg.h post a fixed breakfast to 20 healthy subjects causing an enhanced satiety and a 12 % reduction in energy intake at the ad libitum lunch compared to placebo (Flint et al., 1988). A similar experimental design was performed on 12 diabetic males and the results also showed that GLP-1 (at a dose of 1.5 $pmol \cdot kg^{-1} \cdot min^{-1}$) reduced consumption by 29 % in the ad libitum meal, decreased hunger, increased fullness, lowered blood glucose, increased insulin, and reduced glucagon levels (Gutzwiller,1999). Another experiment whereby GLP-1 was administered at a physiological dose (at a rate of 45 pmol/kg of fat-free mass (FFM) x h) also showed suppression of hunger and prospective food consumption in addition to a decrease in gastric emptying rate (Flint et al., 2001). When GLP-1 was administered subcutaneously for 6 weeks at a rate of 4·8 $pmol.kg^{-1}$ min^{-1}, it showed a decrease in levels of blood glucose, HbA1c (1.3% decrease), free fatty acids, a decrease in gastric emptying, body weight (1.9kg), and appetite (Zander et al., 2002).

Liraglutide, a GLP-1 receptor agonist, was subcutaneously injected to diabetic subjects for 4 weeks causing weight loss and decreased gastric emptying (Horowitz et al., 2012). Liraglutide was also given to eighteen diabetic subjects once daily for 3 weeks, followed by a wash out period then a once daily placebo administration and the results showed a decrease in fasting and postprandial glucose, an increased fasting and postprandial insulin, and a delay in gastric emptying (Flint et al., 2011). Liraglutide was given once daily at a dose of 1.8 mg for 26 weeks showing similar results: a decrease in fasting and postprandial blood glucose levels, a decrease in HbA1c, and weight loss (Buse et al., 2009).

c. GLP-1 acts on the central nervous system

GLP-1 is considered a hormone when secreted by the L cells of the intestines and a neuropeptide when secreted by the brain, specifically in the nucleus of the solitary tract (Knauf et al., 2005; Cabou, 2008). In addition to being produced by cerebral cells (Knauf et al., 2005), GLP-1 appears to cross the blood brain barrier by simple diffusion with no inhibition of influx (Kastin et al., 2002). It is also found to be present in the brainstem, hypothalamus, and thalamus (Goke et al., 1995). GLP-1 has a central role in suppression of food intake. Kinzig et al. (2002) injected GLP-1 in the lateral ventricle, the fourth ventricle, and the central nucleus of the amygdale (CeA). Infusion of GLP-1 in the lateral and the fourth cerebral ventricle caused a dose dependent decrease in food intake, unlike its administration in CeA where food intake was not affected; in addition, GLP-1 administration into the lateral ventricle and CeA caused taste aversion (Kinzig et al., 2002).

To assess the action of central GLP-1 receptors, intracerebroventricular administration of GLP-1 or GLP-1 antagonist was performed together with oral glucose or intravenous glucose infusion (Burmeister et al., 2012). The results showed a higher insulin level and a lower glucose level with central GLP-1 administration together with oral glucose solution. The effect of GLP-1 was not manifested with intravenous glucose administration. Blocking the GLP-1R with its antagonist had no effect on the insulin or on the glucose. These results demonstrate that central GLP-1R activation lead to decreased glucose levels only when glucose is ingested orally not when administered intravenously (Burmeister et al., 2012). Another study performed by Knauf et al (2005) showed that intacerebroventricular administration of exendin-4 (GLP-1 agonist), during hyperglycemia, inhibited muscle glucose utilization and increased insulin secretion thus causing an increased hepatic glycogen storage. In addition, intracerebroventricular

administration of GLP-1 leads to a decrease in hepatic triglycerides (Burmeister et al., 2012). Similar results were obtained by Parlevliet et al. (2010) wereby GLP-1 administration caused a significant increase in glucose infusion to maintain euglycemia and GLP-1R antagonist diminished this effect by 62%. Likewise, GLP-1 administration led to a decrease in plasma triglycerides and VLDL-TG production during hyperinsulinemia (Parlevliet et al., 2010). Usually the signal transmission from the brain to peripheral tissues is performed by the vagus nerve (Cabou et al., 2008).

GLP-1 also has a neuro-protective role. It has been shown to delay Alzheimer's disease (D'Amico et al., 2010), protect neurons against oxidative injury and thus apoptosis (Perry et al., 2003), stop the progression and reverse the lesions established in Parkinson's disease (Harkavyi et al., 2008), and promoted neurogenesis and normalized dopamine imbalance in the in the substantia nigra in Parkinson's disease (Bertilsson et al., 2008).

d. GLP-1 and the cardiovascular system

A number of studies demonstrated GLP-1 to have a cardio-protective role, but this cardio-protective effect of GLP-1 is still exploratory and less established than its anti-diabetic effect (Sun et al., 2012). GLP-1 plays a similar mechanism on the heart myocytes whereby it inhibits cellular apoptosis and increases cell proliferation (Ussher and Drucker, 2012). A meta analysis pooling six trials revealed that exenatide (GLP-1 agonist) treatment over six months had an effect on significantly decreasing systolic blood pressure but not diastolic blood pressure when compared with placebo (Okerson et al., 2010). GLP-1 administration to healthy subjects caused an increase in forearm blood flow indicating positive effects on endothelium-dependent vasodilatation (Basu et al., 2007). This beneficial effect was observed when GLP-1 was infused with

acetylcholine and Glimepiride (sulfonylurea medication) or acetylcholine and placebo, but not with acetylcholine and glyburide (another sulfonylurea medication) (Basu et al., 2007). When GLP-1 was infused to 12 diabetic subjects with coronary artery disease, it caused an improvement in endothelial function but no improvement in insulin sensitivity (Nystrom et al., 2004). A meta-analysis of 45 randomized controlled trials, with publication dates ranging from 2002 till 2011, did not show significant results regarding the cardiovascular benefit, but showed highly significant results with respect to increased glycemic control (Sun et al., 2012). Two month old GLP-1R knockout mice had a decreased resting heart rate and elevated left ventricular and diastolic pressure compared with controls. The 5 month old GLP-1R knockout mice had a smaller heart size and thicker walls of the septum and posterolateral myocardium (Gros et al., 2003). The Liraglutide Effect and Action in Diabetes (LEAD) studies observed a 2.1 to 7.7 mmHg reduction in systolic blood pressure of 4,456 patients from 40 different countries and 600 different sites (Phillips and Prins, 2012).

GLP-1 also has an anti-atherosclerotic effect. A study conducted by Derosa et al. (2010) studied the effect of giving exenatide (GLP-1 agonist) or glibenclamide (antidiabetic drug sulfonylureas) to diabetic patients after 3, 6, 9, and 12 months.. Exenatide caused a decrease in body weight, insulin resistance, inflammatory markers, fasting plasma glucose, glycosylated hemoglobin, and postprandial plasma glucose. Glibenclamide also caused a decrease in fasting plasma glucose, glycosylated hemoglobin, postprandial plasma glucose, but on the contrary caused an increase in body weight and inflammatory markers. This shows that GLP-1 caused a decrease in inflammation thus playing a cardioprotective role (Derosa et al., 2010). Similarly, treatment of mice with exendin-4 (a GLP-1 receptor agonist) for 28 days increased slightly HDL levels, suppressed significantly the inflammatory response in the

macrophages, reduced monocyte adhesion to the endothelium, and suppressed atherosclerogenesis (Arakawa et al., 2010). The experimental study performed by Cabou et al. (2008) also revealed a decrease in reactive oxygen species and an increase in glutathione antioxidant when exendin-4 was infused into the brains of mice.

e.Other roles of GLP-1:

Research has shown a role of GLP-1 in bone formation, but insufficient data are present so far. GLP-1 receptors are expressed on bone marrow stromal cells and immature osteoblasts, but not on mature osteoblasts (Walsh and Henriksen, 2010). GLP-1 receptor agonist stimulates the calcitonin expression in the thyroid cells and GLP-1 receptor knockout mice developed osteopenia and increased bone fragility as well as bone resorption (Yamada et al., 2008). Wild-type female mice treated with DPP-4 inhibitor significantly had improved vertebral volumetric bone mineral density and trabecular architecture (Kyle et al., 2011). This reveals the role of GLP-1 in affecting the bone metabolism, but the exact mechanism is still unknown.

In summary, GLP-1 improves glycemia, insulin resistance, decreases the body mass index, inhibits pancreatic and cardiac cell apoptosis, decreases atherosclerosis, decreases inflammation, and improves cardiac function, decreases appetite, and decreases gastric emptying (Ussher and Drucker, 2012).

2. Glucose-Dependent Insulinotropic polypeptide

Glucose-Dependent Insulinotropic polypeptide (GIP) is a 42 amino acid peptide hormone synthesized and released from the intestinal K cells, specifically the duodenum and jejunum in response to the ingestion of glucose or fat (Song and Wolfe,

2007). The half life of GIP is 5 to 7 minutes since similar to GLP-1, after its release from the K-cells, it gets degraded by DPP-4, thus rendering it biologically inactive (Song and Wolfe, 2007; Seino et al., 2010). GIP receptors are present in different organ tissues such as the heart, stomach, brain, pancreas, enterocytes, adipocytes (Usdin et al., 1993), and bone (Bollag et al., 2000) which indicates that GIP has functions in different tissues and organs. GIP was first named as "gastric inhibitory polypeptide" since it has the ability to inhibit gastric acid secretion, but then it was found to possess an insulinotropic action, and thus was named "glucose dependent insulinotropic polypeptide" (Yip and Wolfe, 2000).

a. Insulinotropic action of GIP:

GIP acts on the pancreatic ß cells to stimulate insulin release (Seino et al., 2010). GIP increases not only insulin secretion but also increases the transcription of proinsulin and biosynthesis of insulin in the pancreatic ß cells (Nie et al., 2012; Baggio and Drucker, 2007). GIP antagonist inhibited insulin release by 72% (Tseng et al., 1996). Another study done by Tseng et al. in 1999 showed that GIP antagonist caused a decrease in insulin secretion by 54% and a decrease in glucose absorption by 15% 20 minutes after a glucose meal. There is also evidence that GIP affect insulin sensitivity as well since incubation of GIP with rat adipocytes caused an increase in insulin sensitivity and increased receptor affinity to insulin (Starich et al., 1985). GIP was observed to be the major physiological incretin since it was found to be responsible for 80% of the incretin induced insulin release (Weaver et al., 2008). In addition, to improve insulin production and sensitivity, GIP decreases ß cell apoptosis and increases beta cell proliferation (Baggio and Drucker, 2007). GIP has been shown to have similar activation pathway as GLP-1 on insulin exocytosis in pancreatic ß cells by stimulating

cAMP production, elevating intracellular calcium, and activating PKA (Figure 4) (Baggio and Drucker, 2007; Seino et al., 2010, Figueiredo et al., 2010). Hence, CREB, ERK1/2 and Bcl-2 gene (an anti-apoptotic gene) are activated (Baggio and Drucker, 2007; Seino et al., 2010) in addition to EPAC2 which leads to downregulation of bax, a proapoptotic gene (Baggio and Drucker, 2007). Consequently, GIP not only activates insulin release from pancreatic ß cells but also promotes proliferation and inhibits apoptosis of pancreatic cells.

b. GIP and Obesity

GIP has been shown to play a role in the pathogenesis of obesity. GIP secretion was found to be higher in obese subjects compared to matching healthy subjects (Roust et al., 1988). GIP mRNA levels were found to increase after glucose and fat administration, 4 folds and 2.5 folds respectively where as prolonged fasting caused a 44 % decrease in GIP mRNA (Higashimoto et al., 1995). This reveals that GIP synthesis and secretion increase in response to fat and glucose administration while food deprivation leads to a decrease in GIP synthesis and secretion (Higashimoto et al., 1995).

GIP has been shown to increase lipoprotein lipase (LPL) release. LPL is the enzyme responsible for clearing triacylglycerol from the circulation, thus generating fatty acids for storage within the adipocyte (Song and Wolfe, 2007). A randomized cross-over study done by Murphy et al. (1995) showed that a test meal with 80 g of fat lead to a significantly higher levels of GIP than a 20 g fat test meal. In addition, LPL levels and triacylglycerol levels were significantly higher with the 80 g fat meal although there was no difference in insulin response between the different test meals. This shows that GIP causes an increase in LPL and affects lipoprotein homeostasis in

response to a high fat meal (Murphy et al., 1995). This was also demonstrated by older experimental studies whereby GIP caused an increase in LPL activity when incubated for 2 h with cultured preadipocytes (Eckel et al., 1979). It was speculated that the increase in LPL activity is a mechanism for clearing triglycerides from the circulation after a meal (Eckel et al., 1979). A study by Kaushik et al. (2006) showed that GIP caused an increase in glycerol levels (increased lipolysis) and increased FFA reesterification (decreased FFA release) thus hypothesizing that GIP promotes triglyceride synthesis thereby contributing to pathogenesis of obesity.

In addition, treatment of human subcutaneous adipocytes with GIP (100 nM) in the presence of insulin (1 nM) resulted in approximately 3.0-fold increases in LPL activity, compared to basal and GIP together with insulin was shown to increase LPL gene expression and transcription in human adipocytes (Kim et al., 2010). The incubation in the presence of GIP and insulin together caused a more significant increase in LPL activity compared to either hormone alone (Knapper et al., 1995). GIP has been shown to inhibit glucagon stimulated lipolysis, increase glucose transport into the adipocytes, and increase conversion of glucose to lipids (Hauner et al., 1988). GIP alone caused a decrease in fatty acid incorporation into adipose tissue, but in the presence of insulin, it enhanced the insulin effect of increasing fatty acid incorporation into the adipose tissue (Beck and Max, 1983). Through increasing LPL activity, GIP (in the presence of insulin) has been shown to increase clearance of triglycerides postprandially (Nie et al., 2012). Oben et al. (1991) also showed that GIP, similar to insulin, promotes fatty acid synthesis in the rat adipose tissue.

In addition, GIP has been shown to increase intestinal glucose absorption (Tseng et al., 1999) and increase GLUT-4 in the membrane of fat cells (Song and Wolfe, 2007), thus contributing to obesity. Tseng et al. in 1999 showed that GIP

antagonist caused a decrease in insulin secretion by 54% and a decrease in glucose absorption by 15% 20 min after a glucose meal.

GIP has been shown to contribute to obesity and adiposity since in GIP receptor knockout mice, a high fat diet caused no weight gain, no insulin resistance, no increase in visceral fat, and no liver steatosis all of which were observed in control mice fed a high fat diet (Getty-Kaushik et al., 2006). Similar results were obtained by Miyawaki et al. in 2002 whereby mice fed a high fat diet lead to hypersecretion of GIP, increased visceral and subcutaneous fat deposition and insulin resistance. Conversly, mice lacking the GIP receptor were protected from obesity and insulin resistance (Miyawaki et al., 2002). GIP receptor deficient mice on high fat diets showed similar energy intake compared to control mice, but increased energy expenditure, and a decreased respiratory quotient indicating that fat is the main source being used for energy (Seino et al., 2010). This reinforced a relationship between GIP, over nutrition, and obesity.

Therefore, excessive nutrient ingestion could lead to GIP hypersecretion which causes increased nutrient intake by the adipocytes, thus increasing the fat storage which in turn leads to obesity, insulin resistance, and diabetes (Kaushik et al., 2006).

c. Other roles of GIP:

GIP has anti-atherogenic effect in diabetic and non-diabetic mice. GIP was shown to reduce the surface area of the atherosclerotic lesion, decrease the plaque size, and the macrophage infiltration in the aorta (Nogi et al., 2012). In addition, the foam cell formation was significantly suppressed by 50 % in GIP infused diabetic mice compared to vehicle infused counterpart (Nogi et al., 2012). GIP induced suppression of foam cell formation in macrophages of diabetic and non- diabetic mice was significant,

15% and 35% respectively (Nogi et al., 2012). In addition, GIP was able to decrease reactive oxygen species generation and block the advanced glycation end products that play a major role in vascular injury (Ojima A. et al., 2012).

Bone mineralization was also found to be regulated by GIP. GIP receptors messenger RNA were found to be present in normal rat bone osteoblasts and osteocytes and in osteoblast-like cell lines. Furthermore, GIP has been shown to inhibit apoptosis of osteoblasts (Seino et al., 2010). GIP also increases osteoblast number and activity and prevents PTH-induced osteoclast activation (Walsh.and.Henriksen, 2010).All of the previous findings indicate that GIP is involved in bone formation through its action on osteoblasts (Bollag et al., 1999). It increases osteoblastic activity and inhibit osteoclastic activity (Phillips and Prins, 2012).

GIP also has a central role. GIP receptor mRNA is expressed in several areas of the brain such as the cerebral cortex, the hippocampus, and the olfactory bulb (Usdin et al., 1993). In addition, exogenous GIP delivery, in vivo and in vitro, caused hippocampal stem cell proliferation (Nyberg et al., 2005). GIP also plays a role in neuronal protection and repair after injury (Buhren et al., 2009). An increase in GIP mRNA expression of the dorsal root ganglia and the ventral part of the spinal cord was observed after sciatic nerve crush in rats (Buhren et al., 2009). Moreover, GIP receptor knockout mice had impairment in axonal regeneration after 14 days when compared to wild type mice (Buhren et al., 2009). The involvement of GIP and its receptor in neurogenesis, neuroprotection, and cell proliferation (Holscher and Li, 2008) show a potential involvement of GIP in neurological diseases such as Parkinson's and Alzheimer (Figueiredo et al., 2010).

CHAPTER III

MATERIALS AND METHODS

A. Subjects

This study is a single blind randomized cross over design in which subjects act as their own controls. It was approved by the Institutional Review Board committee at American University of Beirut. Seven overweight and obese subjects (four males and three females) with a body mass index (BMI) greater than 25 were recruited. They were all asked to sign an informed consent. Participants were chosen to be of the age range 18 to 45 years, non-diabetic, with glycosylated hemoglobin (HbA1c) less than 6.4 %. Subjects with kidney diseases, diabetes, and/or any individuals taking medications that affect glucose metabolism or body weight were excluded from the study.

B. Study design

The study was performed in the Clinical Research Unit (CRU) at the American University of Beirut Medical Center (AUBMC). Participants were asked to present to the facility twice fasting for at least 8 hours before the test, once to take phosphorus and another time to take placebo pills (Table 2 shows the compositions of the tablets). Once they arrived, an intravenous catheter was placed and blood was collected (time zero). Then, subjects were asked to take 4 tablets of either phosphorous (500 mg) or placebo together with a 75 grams glucose solution (orange flavor). Subsequently, blood was collected at 15minutes, 30 minutes, 60 minutes, 120 minutes, 180 minutes, and 240 minutes (a total of 6 blood withdrawals, 10 ml each). The collected blood samples were drawn into a centrifuge tube that contains no anticoagulant, stored in a refrigerator at

4^{o}C (until the end of the experiment), then centrifuged at 4^{o}C at 3000 RPM for 15 minutes. The serum separated was stored in aliquots at -80 ^{o}C. The serum was used to test GLP-1, GIP, insulin, phosphorus, glucose, HDL, LDL, and triglycerides.

Table 2: Composition of the phosphorus and placebo tablets.

Phosphorus tablets		**Placebo tablet**	
Ingredients	**Weight (mg)**	**Ingredients**	**Weight (mg)**
Potassium phosphate monobasic (KH2PO4)	189.4	Micro crystalline cellulose	300
Potassium phosphate dibasic(K2HPO4)	349.5	Calcium carbonate	200
Dicalcium phosphate	108	Stearic acid	160
Microcrystalline cellulose	50	Magnesium stearate	15
Stearic acid	50	Croscarmellose sodium	20
Magnesium stearate	10	Silicon dioxide	20
Croscarmellose sodium	10		
Silicon dioxide	5		

C. Analytical procedure

1.Insulin, GLP-1, and GIP

Serum insulin, GLP-1, and GIP levels were tested for each blood collection using the ELISA kit from Millipore Corporation, Billerica, MA, USA.

- The insulin test was done in a microwell plate. The test is based on two monoclonal antibodies that bind to insulin: one which is immobilized in the plate and the second which is a horseradish peroxidase conjugate. After the insulin in the serum binds to the antibodies, 3,3',5,5'-Tetramethylbenzidine followed by a stop solution are added whereby a yellow color forms and thus the absorbance is read. To obtain the concentration levels in the serum, the concentration of the controls (which are present in the kit) and the measured absorbance of the control (tested in the microwell plate) are used to derive an equation for calculating the concentrations.

- GLP-1 and GIP were also tested in a microwell plate. Two antibodies were used, one which is a monoclonal antibody and the other which is a polyclonal antibody, to capture the molecule. Then, the horseradish peroxidase conjugate was used in the presence of 3,3',5,5'-Tetramethylbenzidine, and followed by a stop solution whereby the results (absorbance) were read using a spectrophotometer. To obtain the concentration levels in the serum, the concentration of the controls (which are present in the kit) and the measured absorbance of the control (tested in the microwell plate) are used to derive an equation for calculating the concentrations.

2.HbA1c

HbA1c was measured once with the participant's first visit and at time zero. The blood withdrawn was analyzed at the Department of Pathology and Laboratory

Medicine at AUBMC using the BioRad Variant Hemoglobin Analyzer which uses the high-performance liquid chromatography method.

3.Triglycerides, cholesterol, HDL, LDL, total phosphate, glucose

Triglycerides, glucose, cholesterol, HDL, LDL, total phosphate were tested using the Virtos machine 350 by Ortho-Clinical Diagnostics, Johnson and Johnson Company. Three methods are used by this machine: potentiometric reaction, colorimetric reaction, and immune-rate reaction.

4. HOMA-IR

Insulin sensitivity was calculated using the homeostasis model assessment (HOMA) equation: Insulin (mU/L) x glucose (mmol/L)/22.5. HOMA levels >2.5 is a cutoff point for insulin resistance (Keskin et al., 2005).

D. Statistical analysis

The results were analyzed by the Minitab 16. Paired t-test was used to compare the levels of each collection point between phosphorus and control. One way ANOVA using Fisher method was used to analyze the presence of a statistical difference within the same treatment. The General Linear Model (GLM) was used to further analyze the difference between the two different groups and the difference at each blood collection. Then, the mean change (the change from baseline) and the area under the curve (AUC) were also calculated to determine significance using paired t-test. P values less than 0.05 is considered statistically significant.

CHAPTER IV

RESULTS

A. Subjects characteristics and baseline parameters.

Seven subjects were recruited for this study, 2 being overweight and 5 being obese. The characteristics of the subjects are present in Table 3. High insulin levels were detected in 5 subjects and insulin resistance was detected in 4 participants at baseline. The baseline levels of total cholesterol, LDL, HDL, and triglycerides were also taken on each visit (Table 4). Triglyceride levels were above normal in 4 subjects.

B. Blood Parameters

1.*Serum phosphate levels*

The serum phosphate levels were significantly different at different time intervals within the same test group (Table 5). In the phosphorus treatment, phosphate levels at time 240 was significantly different and significantly higher than the phosphate levels at time 15, 30, 60, and 120. In the placebo treatment, phosphate levels at time 60, 120, and 180 were significantly different and lower than phosphate levels at time 0 and 15. Phosphate levels at time 30 and 120 were also significantly different in the placebo treatment group whereby the levels at time 120 were significantly less than the levels at time 30. The levels at time 240 in this group were not significantly different to any of the phosphate levels within this same group.

The results showed that serum phosphate decreased after consuming the glucose solution. However, in the phosphate treatment group, serum phosphate started

to increase at time 120, but it continued to decrease in the placebo treatment group (Table 5 and Figure 5).

The serum phosphate taken at different time intervals showed that phosphate levels were higher in the phosphorus treatment versus the placebo treatment. Serum phosphate levels were significantly higher in the phosphorus test group at 120, 180, and 240 minutes (Table 5 and Figure 5).

The mean phosphate change was also significantly higher at time 120, 180, and 240 in the phosphate treatment compared to the placebo treatment (Figure 6). The mean change showed that the serum phosphate level in the both test groups started to decrease after glucose consumption; however the serum phosphate in the phosphorus test group started to increase after time 60 but only reached significance at time 120. The serum phosphate level in the placebo test group dropped significantly at time 120 and picked up slightly at time 180 and 240.

2.*Glucose and Insulin levels.*

The glucose levels taken at the different time intervals are presented in table 6 and figure 7. The glucose levels were significantly different within the same test group at different time intervals whereby it peaked 30 minutes after taking the glucose solution and started decreasing after 60 minutes. There was no statistical significance between the placebo group and the phosphorus group at any time interval.

The serum insulin levels were not significantly different across the different treatments (Table 7). The levels within the same treatment group were significantly different having a similar trend to glucose, peaking at time 30 and decreasing after time 60 (Figure 9). Time 15, 30, 60, and 120 minutes are significantly different than the levels at time 0 and 240 within the same test group (Table 7). In the placebo test group,

the insulin levels at time 180 is significantly different than the levels at time 15, 30, 60, and 120 whereas in the phosphorus test group, insulin levels at time 180 was significantly different to time 30 and 60 (Table 7).

Mean change of serum glucose and insulin did not show any significance at any time interval when comparing the placebo group to the phosphorus group (Figure 8 and 10). Glucose and insulin both peaked at 30 minutes and decreased after 60 minutes of glucose ingestion. Glucose and insulin both followed a similar trend.

Table 3: Subject characteristics

	Mean ± SEM	**Minimum**	**Maximum**	**Number of participants with levels above normal (n=7)**
Age (yrs)	27.43 ± 3.04	21	45	--------
Weight (kg)	92.20 ± 4.52	77.5	105	--------
Height (cm)	1.71 ± 0.03	1.6	1.84	--------
BMI (kg/m^2) *Overweight* *Obese*	31.33 ± 1.18	26.5	36.1	 2 5
HbA1c (%)	5.0 ± 0.20	4.3	5.9	--------
Insulin (µIU/mL)	42.2 ±18.65	2.09	140.58	5
Fasting blood glucose (mg/dl)	90.79 ± 4.14	80	115	0
HOMA	9.8 ± 4.53	0.62	30.99	4

Values are means ± standard error of the mean
Homeostasis model assessment (HOMA)

Table 4: Baseline levels of the lipid profile

	Mean ± SEM	**Number of participants with levels above normal (n=7)**
Total Cholesterol (mg/dl)	188.6 ± 15.95	1
LDL (mg/dl)	115.85± 17.8	1
HDL (mg/dl)	37.71 ± 3.41	--------
Triglycerides (mg/dl)	175.45± 45.25	4

Values are means ± standard error of the mean

Table 5: Serum phosphate levels after the placebo and phosphorus treatment

Phosphorus (mg/dl)	Time 0	Time 15	Time 30	Time 60	Time 120	Time 180	Time 240	p value
Placebo test	3.8286± 0.0969 [a]	3.700± 0.115[a]	3.571± 0.151[a,b]	3.314± 0.130[b,c]	3.186± 0.150[c]	3.30± 0.129[b,c]	3.529± 0.149[a,b,c]	0.014ǂ
Phosphorus test	3.8571 ± 0.0782[a,b]	3.757± 0.125[b]	3.629± 0.115[b]	3.557± 0.111[b]	3.729± 0.121[b]	3.914± 0.122[a,b]	4.2± 0.199[a]	0.025ǂ
p value	0.604	0.618	0.522	0.108	0.005*	0.003*	0.018*	

Values are means ± standard error of the mean

Means on the same row with different letters are significantly different

* p value <0.05, values are significantly different for the placebo test versus the phosphorus test at each time interval using paired t-test.

ǂ p value <0.05, values are significantly different between the different time intervals within the same test group using one way ANOVA

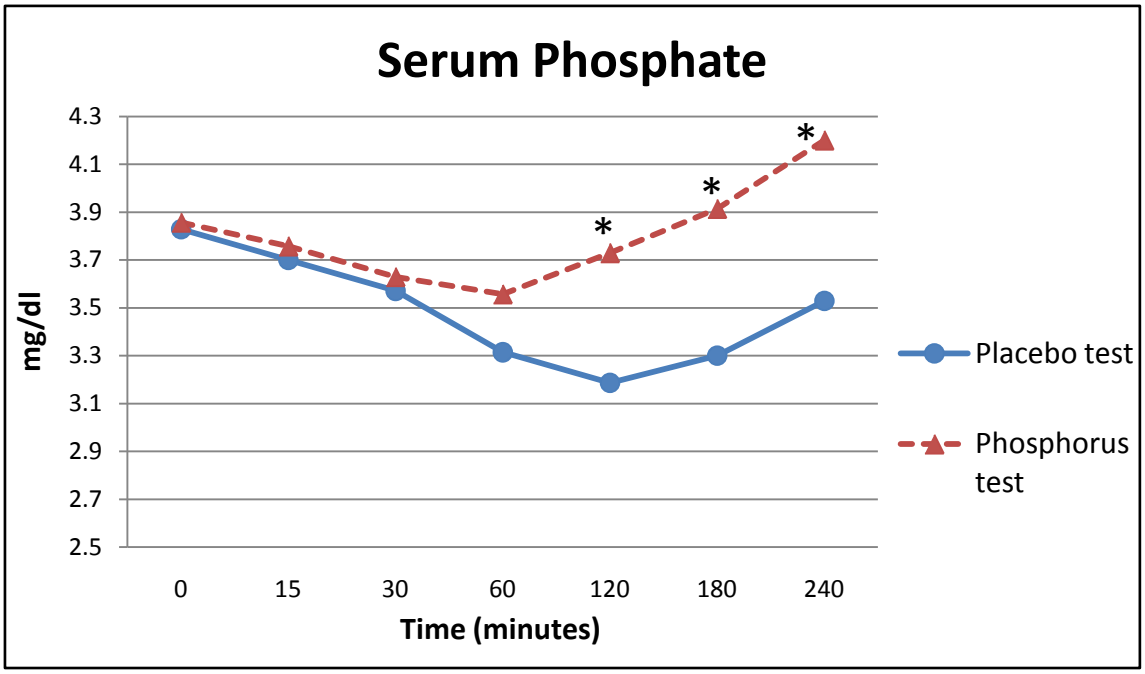

Figure 5: Serum phosphate curve

* values are significantly different between the phosphorus group and the placebo group at $p<0.05$ using paired t-test.

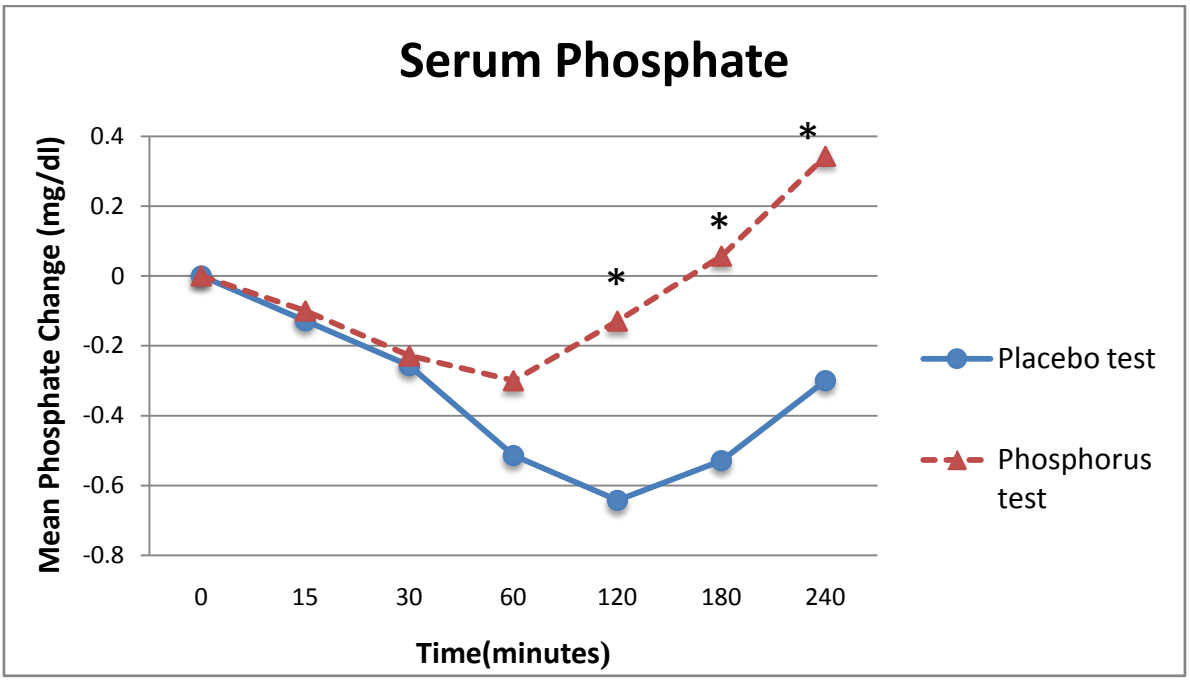

Figure 6:Mean phosphate change

*values are significantly different between the phosphorus group and the placebo group at $p<0.05$ using paired t-test

Table 6: Glucose levels after the placebo and phosphorus treatment

Glucose (mg/dl)	Time 0	Time 15	Time 30	Time 60	Time 120	Time 180	Time 240	p value
Placebo test	91.57 ± 3.88 ^{d,e}	121 ± 7.31 ^{b,c}	152.9± 10.0 ^{a}	146.6± 15.1 ^{a,b}	107.43 ±9.51^{c,d}	80.57± 8.19^{e}	70.29± 4.10 ^{e}	< 0.001 ǂ
Phosphorus test	90 ± 4.4^{c,d}	125.14 ± 8.61^{a,b}	144.6± 14.7 ^{a}	135± 19.3^{a,b}	106.1± 11.2 ^{b,c}	79.29± 6.89^{c,d}	73.43± 4.55^{d}	< 0.001 ǂ
p value	0.704	0 .684	0.444	0.435	0.924	0.931	0.369	

Values are means ± standard error of the mean

Means on the same row with different letters are significantly different using Fisher method

ǂ p value <0.05, values are significantly different between the different time intervals within the same test group using one way ANOVA.

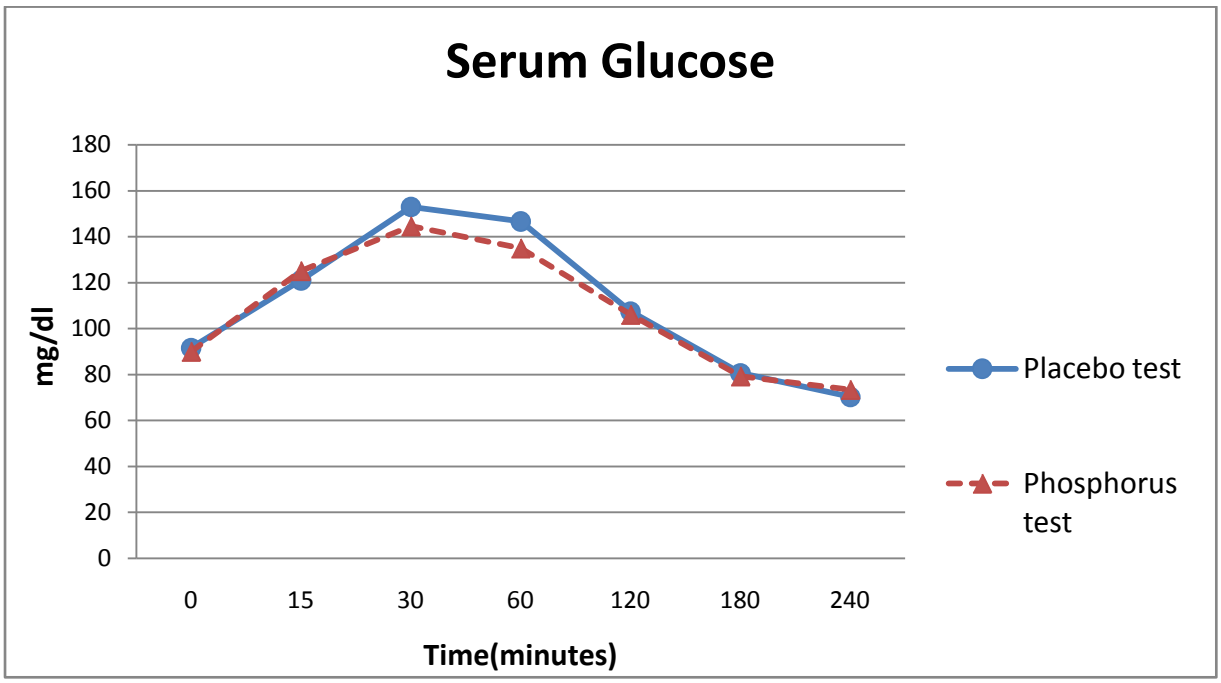

Figure 7: Serum glucose curve
* values are significantly different between the phosphorus group and the placebo group at $p<0.05$ using paired t-test.

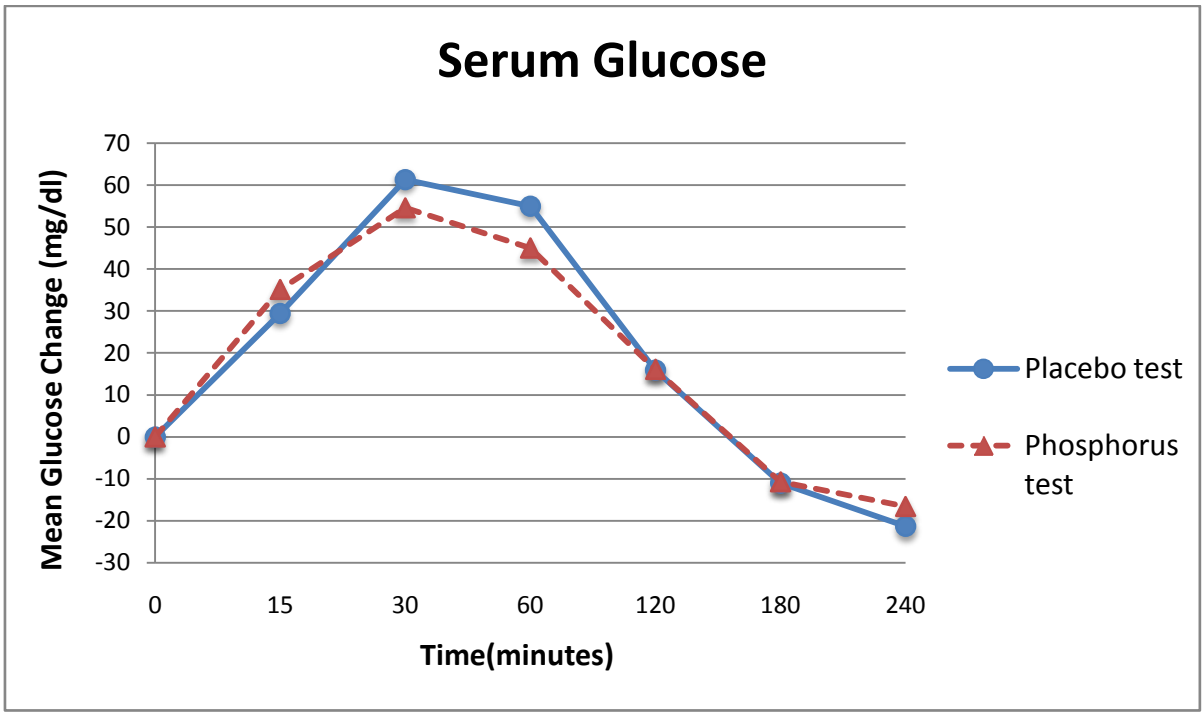

Figure 8: Mean glucose change
* values are significantly different between the phosphorus group and the placebo group at $p<0.05$ using paired t-test.

Table 7: Insulin levels after the placebo and phosphorus treatment

Insulin (µIU/mL)	Time 0	Time 15	Time 30	Time 60	Time 120	Time 180	Time 240	p value
Placebo test	35.9 ± 14.8[b]	111.7 ±22.0[a]	140.1 ±20.2[a]	139.7 ± 17.3[a]	119.2 ± 28.2[a]	44.3 ± 18.8[b]	21.3 ± 10.7 [b]	< 0.001ǂ
Phosphorus test	48.5 ±22.5 [c]	117.2± 22.0[a,b]	146.9 ±15 [a]	144.4 ± 17.8 [a]	117.1± 24.9 [a,b]	59.9 ±24.2 [b,c]	31.2 ±22 [c]	0.001ǂ
p value	0.472	0.714	0.640	0.817	0.935	0.504	0.469	

Values are means ± standard error of the mean

Means on the same row with different letters are significantly different using Fisher method

* p value <0.05, values are significantly different for the placebo test versus the phosphorus test at each time interval using paired t-test.

ǂ p value <0.05, values are significantly different between the different time intervals within the same test group using one way ANOVA

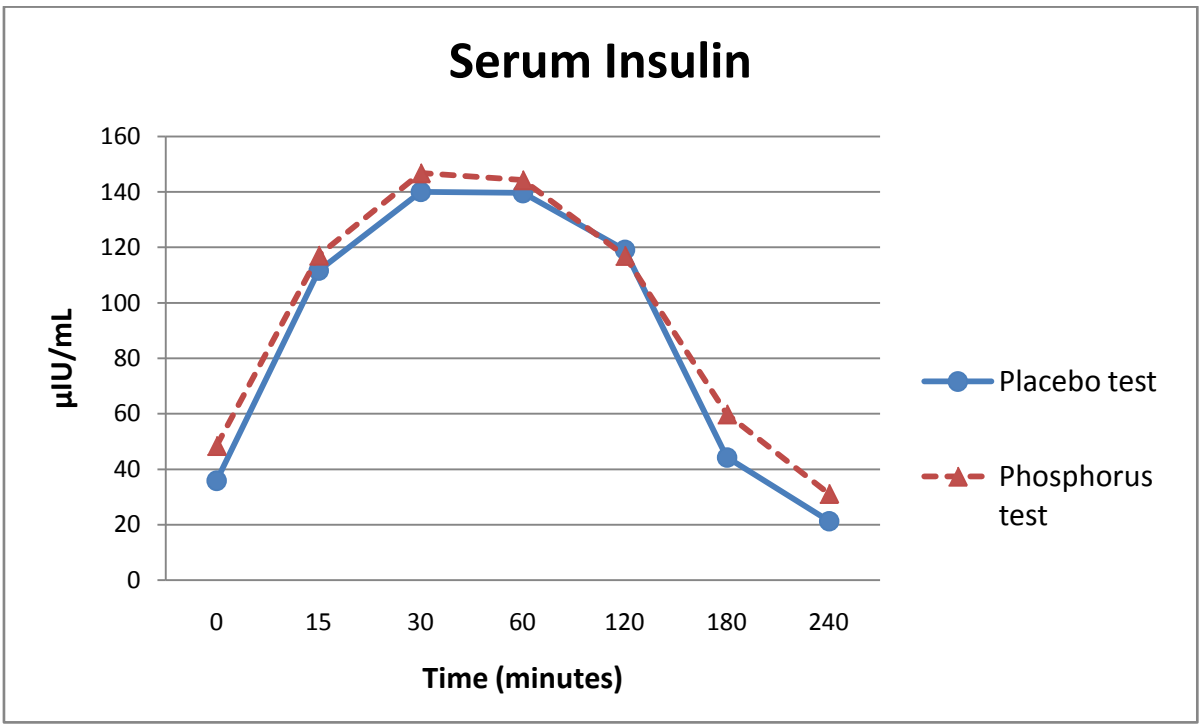

Figure 9: Serum insulin curve
* values are significantly different between the phosphorus group and the placebo group at $p<0.05$ using paired t-test.

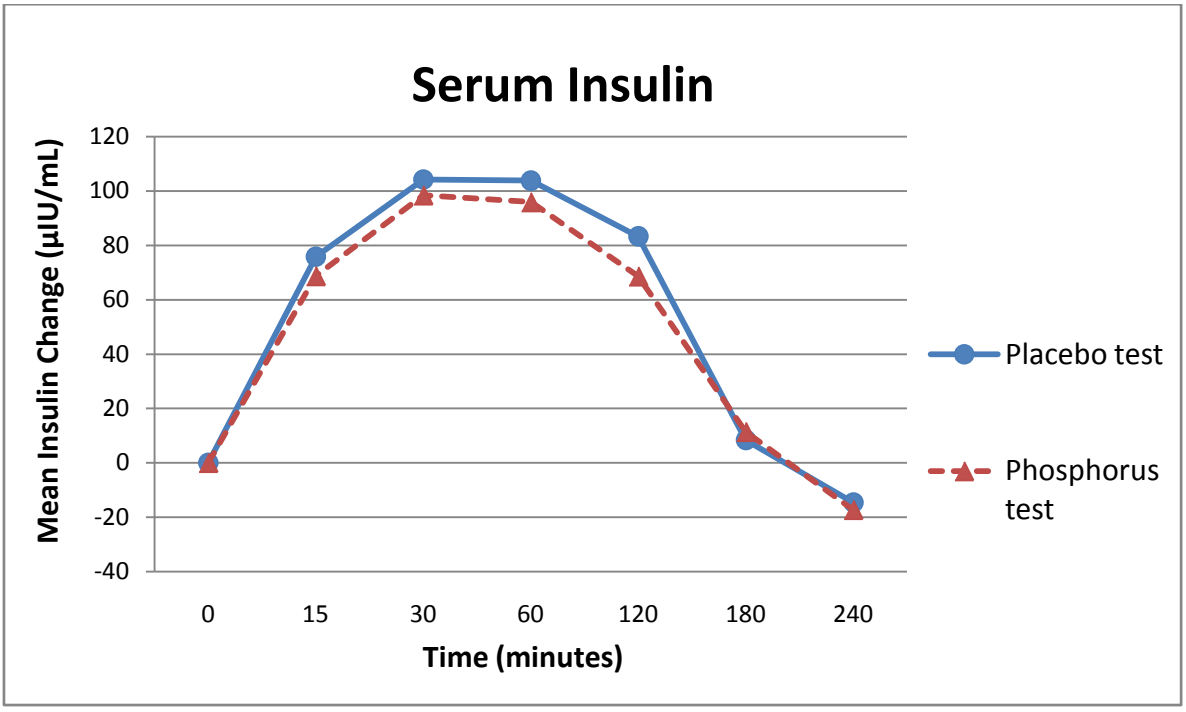

Figure 10: Mean insulin change
* values are significantly different between the phosphorus group and the placebo group at $p<0.05$ using paired t-test.

3.*Lipid profile*

No significant difference was noted in the levels of serum triglyceride, HDL, LDL, and total cholesterol between the two different groups neither over time intervals nor across the whole treatment period (Table 8).

4.*GLP-1 and GIP*

GLP-1 did not show any statistical significance within the same treatment group (Table 9). In the placebo group, GLP-1 levels at time 0, 15, and 30 minutes were significantly higher than time 120 and the serum GLP-1 levels at time 15 was also significantly higher than the levels at time 120 and 180. In the phosphorus group, the GLP-1 levels were significantly higher at time 30 compared to time 120 and 240.The levels at time 15 were also significantly higher than time 240. The GLP-1 levels at time 120 were significantly less than the GLP-1 levels at time 30 within the same test group in both groups. This shows that GLP-1 dropped after glucose ingestion. According to the GLP-1 curve and mean change of GLP-1(Figure 11 and 12), serum GLP-1 reached its peak at 30 minutes in the phosphorus group and at 15 minutes in the placebo test group and then dropped thereafter.

Similarly, serum GLP-1 did not show any significance when comparing the two treatment groups at the different time intervals. Nevertheless, GLP-1 across the different test groups approached significance at time 30 (p value of 0.051) with its values being higher in the phosphorus test group (Table 9).

Although the mean change of GLP-1 in the phosphorus treatment test was higher than the placebo treatment test, the mean change did not reach statistical significance (Figure 12).

The serum GIP levels within the same test group showed significant changes at different time intervals after glucose ingestion (Table 10). The serum GIP levels within the same group at time 15, 30 and 60 minutes in both treatment groups are significantly different than the levels at time 0,180,and 240 minutes. In the placebo group, GIP levels at time 120 were significantly different to the levels at time 15, 30, 180, and 240 whereas the levels in the phosphate test group at time 120 were significantly different than time 240. GIP peaked at 15 minutes post glucose ingestion in the phosphorus group and at 30 minutes post glucose ingestion in the placebo group, then decreasing significantly thereafter at 120, 180, and 240 minutes in the placebo group and at 180 and 240 minutes in the phosphorus group (Table 10 and Figure 13).

Serum GIP levels were not significantly different when comparing the placebo group to the phosphorus group. Similarly, mean change of serum GIP did not show any significance at any time interval (Figure 14)

Table 8: Lipid profile after the placebo and phosphorus treatment

		Time 0	Time 15	Time 30	Time 60	Time 120	Time 180	Time 240	p value
Triglyceride (mg/dl)	**Placebo test**	172± 42.2 [a]	157± 36[a]	159.1± 34.2[a]	161.7± 32.8[a]	142.6 ±32.4[a]	137.4± 31.2[a]	142.0± 32.5[a]	0.991
	Phosphorus test	178.9 ±48.3[a]	179.9 ±44.5[a]	178± 40 [a]	174.1± 38.6 [a]	128.9 ±32 [a]	111± 28.9 [a]	111.7± 24.6 [a]	0.599
	p value	0.803	0.390	0.441	0.578	0.416	0.149	0.080	
Total Cholesterol (mg/dl)	**Placebo test**	187.1 ±14.6[a]	182.0 ±15.5[a]	183.7± 14.0 [a]	183.7± 14.3 [a]	177.9± 14.9 [a]	182.3± 14.9 [a]	181.9± 13.7 [a]	1
	Phosphorus test	190.1 ±17.3[a]	189.9 ±15.2[a]	180.0± 12.1 [a]	185.6± 15.3 [a]	179.4± 12.7 [a]	179.7± 12.3 [a]	184.6± 13.6 [a]	0.995
	p value	0.696	0.269	0.697	0.760	0.829	0.776	0.698	
LDL (mg/dl)	**Placebo test**	115.3 ±15.5[a]	113.9 ±15.6	114.1± 15.0 [a]	113.9 ± 14.9 [a]	112.3± 14.6 [a]	117.6± 14.8 [a]	116.0± 14.4 [a]	1
	Phosphorus test	116.4 ±20.1[a]	115.9 ±18.8[a]	107.4± 16.1 [a]	113.0± 18.1 [a]	115.6± 14.5 [a]	118.6± 13.3 [a]	122.7± 15.2 [a]	0.998
	p value	0.830	0.662	0.321	0.889	0.528	0.881	0.287	
HDL (mg/dl)	**Placebo test**	37.71 ±3.50[a]	36.57 ±3.36[a]	37.57 ±3.79[a]	37.57± 3.40 [a]	37± 3.30 [a]	37.57 ± 3.26 [a]	37.29± 3.26 [a]	1
	Phosphorus test	37.71 ±3.33[a]	38.14 ±2.94[a]	36.86 ± 2.79 [a]	37.71 ± 2.88 [a]	38.29 ± 3.15 [a]	38.43 ± 3 [a]	39.43 ± 2.93 [a]	0.999
	p value	1.000	0.587	0.808	0.953	0.638	0.768	0.472	

Values are means ± standard error of the mean

Means on the same row with different letters are significantly different (Fisher method)

* p value <0.05, values are significantly different for the placebo test versus the phosphorus test at each time interval using paired t-test.

ǂ p value <0.05, values are significantly different between the different time intervals within the same test group using one way ANOVA

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Table 9: Serum levels of GLP-1 after placebo and phosphorus treatment

GLP-1 (pM)	Time 0	Time 15	Time 30	Time 60	Time 120	Time 180	Time 240	p value
Placebo test	47.18±7.12^{a,b}	50.25±6.64 ^{a}	47.19±9.19^{a,b}	36.09±7.27^{a,b,c}	23.98 ±2.93 ^{c}	30.33±5.19^{b,c}	34.55±7.02 ^{a,b,c}	0.066
Phosphorus test	54.22±7.12 ^{a,b,c}	54.93± 5.84 ^{a,b}	61.13±9.33 ^{a}	45.51±6.39^{a,b,c}	38.73±6.91^{b,c}	41.63± 9.97^{a,b,c}	33.03±7.2 ^{c}	0.138
p value	0.351	0.623	0.051	0.073	0.089	0.317	0.806	

Values are means ± standard error of the mean

Means on the same row with different letters are significantly different using Fisher method.

* p value <0.05, values are significantly different for the placebo test versus the phosphorus test at each time interval using paired t-test.

ǂ p value <0.05, values are significantly different between the different time intervals within the same test group using one way ANOVA

Table 10: Serum levels of GIP after placebo and phosphorus treatment

GIP (pg/mL)	Time 0	Time 15	Time 30	Time 60	Time 120	Time 180	Time 240	p value
Placebo test	154.8±58.6^{c,d}	355.5±46.4^{a}	376.5 ±33.1^{a}	338.0± 32.7 $^{a, b}$	232.9±34.2^{b,c}	118.5±27.2^{d}	55.7±14.7^{d}	<0.001
Phosphorus test	198.3±75.4 ^{b,c,d}	430.5 ±68.3 ^{a}	402.2 ±50 ^{a}	351.1± 45.8 $^{a, b}$	291.4±83.7 ^{a,b,c}	166.9±30.6^{c,d}	81.2±25.8^{d}	0.001
p value	0.128	0.345	0.442	0.730	0.589	0.304	0.307	

Values are means ± standard error of the mean

Means on the same row with different letters are significantly different using Fisher method.

* p value <0.05, values are significantly different for the placebo test versus the phosphorus test at each time interval using paired t-test.

ǂ p value <0.05, values are significantly different between the different time intervals within the same test group using one way ANOVA

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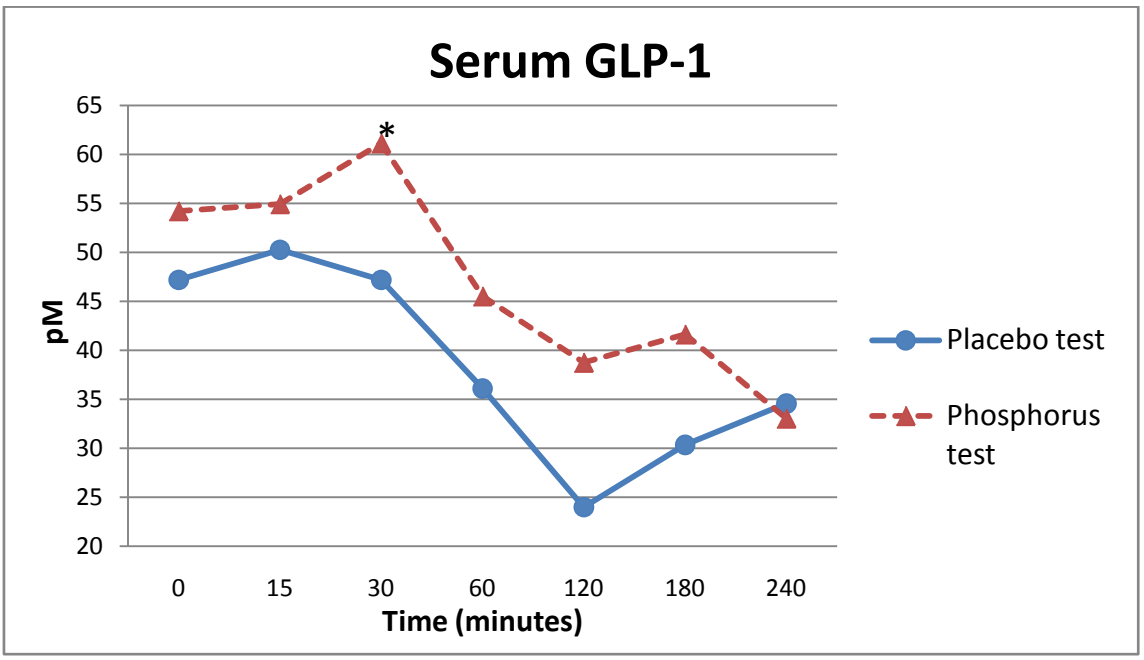

Figure 11: Serum GLP-1 curve
* values are significantly different between the phosphorus group and the placebo group at $p<0.05$ using paired t-test.

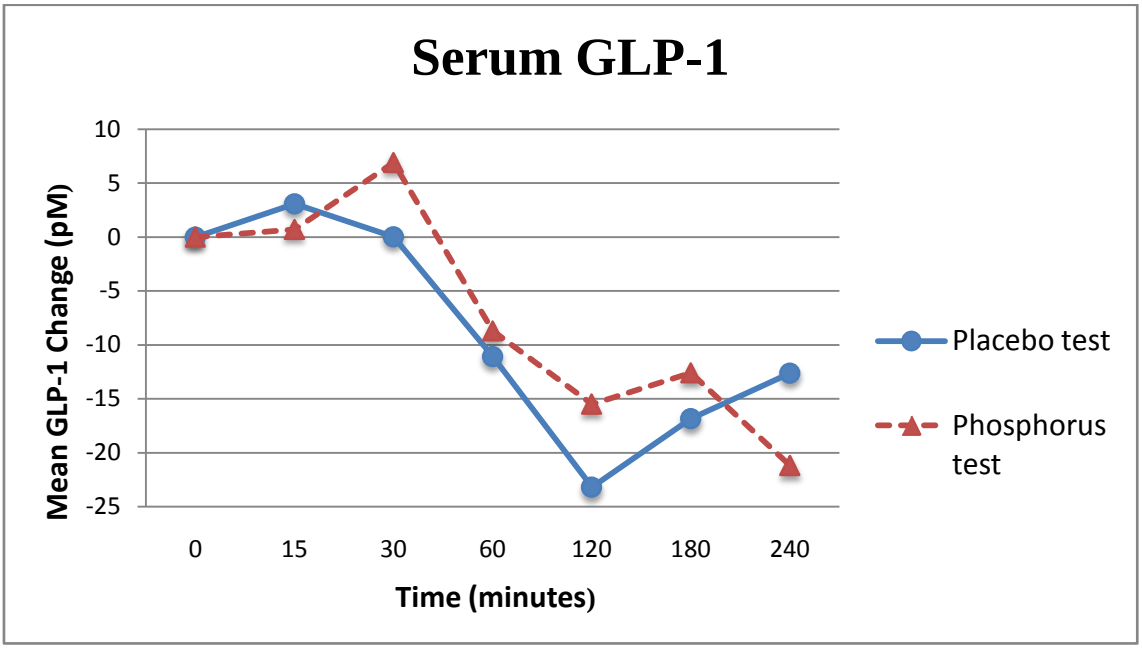

Figure 12: Mean GLP-1 change
* values are significantly different between the phosphorus group and the placebo group at $p<0.05$ using paired t-test.

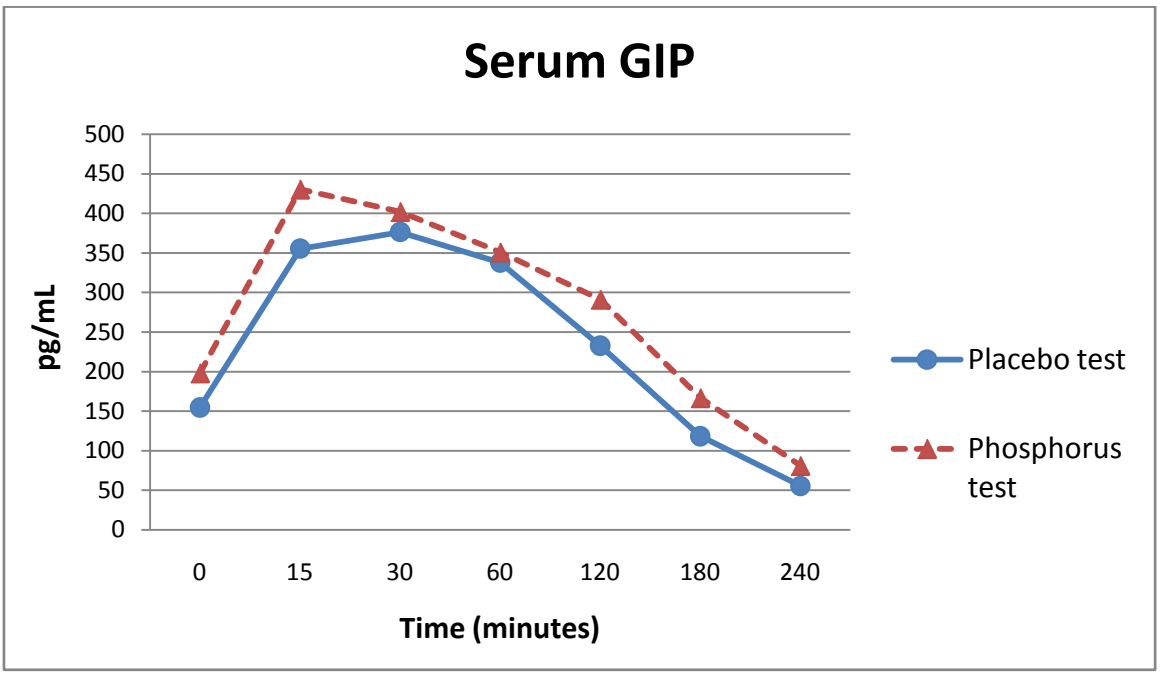

Figure 13: Serum GIP curve
* values are significantly different between the phosphorus group and the placebo group at $p<0.05$ using paired t-test.

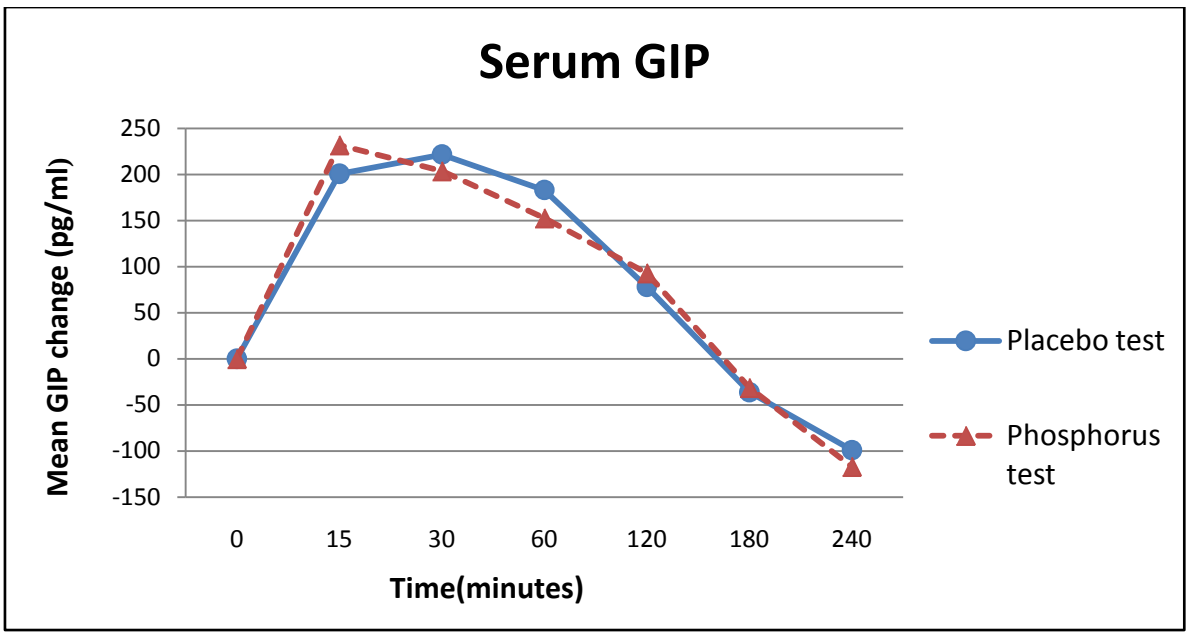

Figure 14: Mean GIP change
* values are significantly different between the phosphorus group and the placebo group at $p<0.05$ using paired t-test.

5.Area under the curve

The AUC represents the total amount over the whole period of the study (240 minutes). The AUC of total phosphate was close to significance (p value = 0.063), but did not reach statistical significance (Table 11). However, the AUC of GLP-1 was statistically significant with a p value of 0.041 (Table 11). All the others parameters did not show any difference between the phosphorus treatment and the placebo treatment.

6.The General Linear Model

GLM was calculated for each of the hormones GLP-1, GIP, and insulin to account for the different variables (such as age, gender, time…). GLP-1 was shown to be significantly different across the different times and across the two groups with a p value less than 0.05. Insulin and GIP were significantly different across time within the same group but not across the different groups.

Table 11: Area under the curve of all parameters

	LDL	**HDL**	**Total cholesterol**	**Total phosphate**	**Glucose**	**Triglyceride**	**Insulin**	**GIP**	**GLP-1**
Placebo test	894± 726	149.3± 79.9	312± 184	0.571± 0.571	5706± 1199	894± 499	14334± 2537	28182± 6425	156.5± 46
Phosphorus test	1552± 747	395± 160	557± 411	25.6± 11.1	5194± 2032	972 ± 580	13976± 2654	23821± 5013	383± 124
p value	0.243	0.224	0.441	0.063	0.690	0.915	0.768	0.241	0.041*

Values are means ± standard error of the mean
*p value <0.05 is considered significant when comparing the placebo treatment versus the phosphorus treatment analyzed by paired t-test.

CHAPTER V

DISCUSSION

This study is investigating the effect of 500 mg of phosphorus together with a glucose solution on phosphate levels, glucose levels, incretins, and lipid profile in the serum.

Serum phosphate levels were significantly different at time 120, 180, and 240. The mean phosphate change showed that the drop in phosphate levels after the glucose solution was greater in the placebo treatment as compared to the phosphorus treatment. The serum phosphate levels decreased after glucose solution ingestion in both the placebo and phosphate treatments. This drop was also evident in previous studies whereby serum phosphate levels dropped following glucose infusions or consumption due to an intracellular shift of phosphate (Berthelay et al., 1984; Pollack et al., 1934; Harrop and Benedict, 1923; Amanzadeh and Reilly, 2006). Similarly serum phosphate levels decreased with sodium chloride and glucose infusion whereas it increased with sodium phosphate and glucose infusion (Nowicki et al., 1996). Likewise, Khattab et al. (2011) observed a drop in serum phosphate levels starting 30 minutes till 240 minutes after the glucose solution was ingested without phosphorus, but this reduction was significantly prevented when glucose was ingested with phosphorus. The glucose load causes the release of insulin which leads to the uptake of glucose together with the serum phosphate into the cells (Fuentebella and Kerner, 2009). In this study, serum phosphate started to pick up after 60 minutes in the phosphorus treatment as compared to the placebo group where the serum phosphate started increasing after time 120.

Serum insulin and glucose increased after glucose solution ingestion. Serum glucose reached its peak at 30 minutes post glucose solution ingestion. Insulin followed the same trend as glucose of increasing post glucose ingestions and then decreasing after around 60 minutes. This post meal rise of insulin and glucose is a previous physiological finding whereby glucose reaches its peak 30 minutes after a meal with insulin following a similar trend as glucose (Rask et al., 2001).

Serum glucose and insulin were not significantly different between the different treatments even when different methods were used for comparison (mean change, GLM, and AUC). These results are not in line with a previous study done on non-diabetic individuals with a BMI range of 16.2 to 52.6kg/m^2 by Haap et al. (2006) that found a negative correlation between serum phosphate and 2 hour OGTT and a positive correlation between serum phosphate and insulin sensitivity with no change in insulin concentration. Similarly other studies showed that hypophosphatemia leads to increased plasma glucose and decreased insulin sensitivity (DeFronzo and Lang, 1980; Xie et al., 2000). Another study done on eleven healthy men (within 10% of their ideal body weight) showed no change in serum glucose or insulin levels with phosphate infusion as compared to controls, but the glucose uptake into the cell was higher with phosphate infusion which was demonstrated by euglycemic clamp technique and was evidenced by a greater decrease in potassium levels (Nowicki et al., 1996). A different experiment performed on eight healthy male subjects showed that insulin decreased, insulin sensitivity improved when subjects were given glucose solution (75g) with phosphorus (500mg) versus a glucose solution without phosphorus (Khattab et al., 2011). However, glucose levels were not different across the two treatments (treatment with glucose and phosphorus versus treatment with glucose solution alone). The results of this experiment were not in line with the results of previous studies in the literature,

but those previous two studies were done on normal weight healthy subjects. The participants in this study are all overweight or obese subjects with a BMI ranging between 26.5 and 36.1 kg/m^2 and 5 subjects out of 7 subjects had elevated fasting insulin levels greater than 15μIU/ml. Insulin levels ≤15 μIU/ml are considered normal (Kapur et al., 2010). Therefore, one can hypothesize that the glucose and insulin levels were not different between the treatments due to insulin resistance that is already developed in those recruited patients but not yet detected. In line with this, a study performed on healthy young insulin resistant offsprings of patients with type 2 diabetes showed that insulin did not increase intramyocellular phosphate concentration or ATP production (Peterson et al., 2005). Thus, insulin resistance affects phosphorus transport and ATP production. This could be the reason behind the results obtained whereby the rise in insulin post a glucose solution did not cause significant uptake of phosphate into the cells and therefore the phosphate did not affect insulin secretion and sensitivity as observed in previous studies done on lean healthy participants. Nevertheless, insulin and glucose curves started to decrease after 60 minutes of the glucose infusion, but serum phosphate increased thereafter at time 120.

Similar to insulin and glucose, the lipid profile was not affected by phosphorus ingestion. In a cross sectional study, serum phosphate has been found to be positively correlated with HDL, LDL, and total cholesterol and correlated negatively with triglyceride levels (Park et al., 2009).An aortic atheroma was formed when apolipoprotein E knockout mice were fed an atherogenic diet with different phosphorus contents for 20 weeks, but no alteration in lipid profile was observed (Ellam et al., 2011). Atheroma was not evident at 12 weeks which indicates that phosphorus does not exert an effect on the lipid profile from one single dose, but has to be taken over a longer period of time to show a change in results.

Triglyceride levels were not significantly different across the two treatments. It was suspected to increase with phosphorus deficiency since low serum phosphate causes an impaired insulin production and thus low clearance of triglycerides from the circulation (Mattar et al., 2010). However, triglycerides did not vary.

The incretins, GIP and GLP-1, were also studied in response to phosphorus loading. This is the first study to assess the effect of phosphorus intake together with a glucose solution on GLP-1 and GIP. GIP was not significantly different between treatments, but it was significantly different within the same treatment group whereby it increased at 15 minutes post glucose ingestion and decreased thereafter. As for GLP-1, it approached significance at time 30 with a p value of 0.051 using a paired t-test analysis with GLP-1 concentrations being higher in the phosphorus test group. Nevertheless, further analysis using AUC showed significant results for GLP-1 with higher concentrations in the phosphorus treatment group. Similarly, the data was analyzed using GLM which also showed significance for GLP-1 across treatments.

Both GLP-1 and GIP usually increase after meal ingestion (Drucker, 2003). GLP-1 and GIP were found to peak after 15 to 20 minutes of glucose ingestion (Schirra et al., 1996) which is in line with our findings whereby GLP-1 peaked at time 15 and GIP peaked at time 30 minutes. GLP-1 is secreted by the L-cells which are located in the ileum and colon, however, the GLP-1 rise was rapid post glucose ingestion (Lim and Brubaker, 2006).GLP-1 has been found to have a biphasic pattern: the first is the initial rapid phase which occurs 15 to 30 minutes after a meal due to an indirect neuro/endocrine stimulation from the proximal intestine by the vagus nerve and a second minor peak that occurs at around 120 minutes due to the direct contact of nutrients with the L-cells (Lim and Brubaker, 2006). In this study, GLP-1 also had a

second peaked at around 180 minutes in phosphorus group and started increasing again after 120 minutes in the placebo group.

GLP-1 levels at baseline were higher than baseline levels reported in the literature. In this study, mean GLP-1 in placebo and phosphorus group were 47.18 pM and 54.22 pM respectively. Usually, basal GLP-1 levels should range between 5 and 15 pM increasing post glucose ingestion to a range between 20 and 60 pM (Nauch et al., 2011). A study performed on insulin resistant mice with similar glucose levels showed a 1.7 fold higher basal GLP-1 levels (after an overnight fast) as compared to controls and this was attributed to hyperinsulinemia (Lim et al., 2009). Therefore, this could explain the higher baseline levels observed in this study. However, another study done by Rask et al. (2001) on insulin resistant non-diabetic men showed no significant difference in fasting serum GLP-1 levels between the different insulin sensitivity tertiles. Nevertheless, interracial differences alter GLP-1 levels. GLP-1 was found to be higher in African American obese subjects as compared to obese Caucasians (Velasquez-Mieyer et al., 2003).

The increase in GLP-1 in response to phosphorus load is desirable in overweight and obese individuals since research has shown that GLP-1 stimulates insulin secretion and insulin sensitivity, improves glucose disposal, reduces food intake, protects pancreatic ß cells, and improves pancreatic ß cell function (Drucker, 2003; Seino et al., 2010). However, the incretin secretion is impaired after a mixed meal ingestion in insulin resistant male subjects (Rask et al., 2001). The GIP levels in insulin resistant men was 34% less than the insulin sensitive group and the GLP-1 levels were 56% less in the insulin resistant group as compared to the insulin sensitive group (Rask et al., 2001). Although GLP-1 secretion postprandially is reduced in diabetic subjects, but its response is still preserved (Drucker, 2003). Subcutaneous GLP-1 infusion for 48

hours in diabetic subjects lowered fasting plasma glucose level and decreased appetite (Toft-Nielsen et al., 1999). Similarly in healthy subjects GLP-1 infusion at a lower dose (1.2 pmol/kg/ min versus 2.4pmol/kg/min which was infused in the previous study) caused a reduction in glucose levels (Meier et al., 2006). GLP-1 infusion at different concentrations showed that GLP-1 increase in the blood and GLP-1clearance were not significantly different between healthy and diabetic subjects (Vilsboll et al., 2003). Therefore, only GLP-1 secretion is affected in type 2 diabetics as reported by some studies (Nauck et al., 2011; Toft-Nielsen et al., 2001). The reason for this decrease is hypothesized to be related to the increased BMI levels, increased glucagon levels observed in diabetics (Nauck et al., 2011), and insulin resistance (Lim et al., 2009). Despite the decreased GLP-1 secretion observed postprandially in diabetics, phosphorus caused an increase in GLP-1 secretion.

The relationship between phosphate and GLP-1 secretion is cAMP production which is needed for the synthesis of GLP-1 in the L-cells. Increased cAMP production, in response to food intake, activates the PKA pathway upregulating proglucagon gene expression in the L-cells (Baggio and Drucker, 2007). Proglucagon is then cleaved by convertases to produce GLP-1 (Baggio and Drucker, 2007). This could further elaborate on the reason why GLP-1 increased post phosphorus loading. Consequently, the increase in GLP-1 levels observed with the phosphorus treatment group could be promising.

A high protein diet followed for 4 days by twelve healthy female subjects has been shown to increase satiety, decrease hunger, increase thermogenesis, and increase GLP-1 levels after mixed meals (Lejeune et al., 2006) all of which are previously observed with phosphorus loading (discussed in details in the literature review). High protein foods are usually high in phosphorus (Kalantar-Zadeh et al., 2010). Similarly, a

high protein diet followed for 2 weeks caused an increase in satiety, a decrease in hunger scores and an increase in insulin secretion and when the same subjects followed an ad libitum high protein diet for 12 weeks, their net caloric intake decreased (Weigle et al., 2005). Another study by Smeets et al (2008) observed an increase in satiety and thermogenesis after only one single meal high in protein, but no change was observed in GLP-1 levels which could also possibly show that one protein load or one phosphorus load was not able to cause any hormonal changes.

Phosphorus in the literature showed similar effect as GLP-1 in terms of improved appetite, improved glycemia and insulin sensitivity, but in this study the results did not show any change in glucose and insulin concentration. This could be the result of three possibilities: 1) GLP-1 is rapidly cleaved by DPP-4 (Song and Wolfe, 2007; Seino et al., 2010); thus GLP-1 did not affect insulin and glucose levels. 2) GLP-1 secretion is decreased in those overweight and obese insulin resistant subjects which is evident in the literature (Nauck et al., 2011) and thus the increase in GLP-1 did not reach physiological significance to impact insulin and glucose levels. 3) The phosphorus given as a single dose and the single rise in GLP-1 are not enough to exert an effect on insulin production, on the pancreatic ß cells, and on insulin sensitivity. However, this study has several limitations:

- The study examined the effect of one phosphorus load which may not be enough to exert a change in the postprandial metabolites.
- The study sample size was small which increases the margin of errors.
- There were many variables available in the sample studied: subjects were both males and females, having a wide range of BMI, with some participants being insulin resistance (4 out of 7) while others were not.

- The pill containing phosphorus also contained potassium; however, the placebo pill did not contain neither phosphorus nor potassium. Therefore, the presence of potassium in the phosphorus pill could have exerted an effect on the results since potassium is transported intracellularly together with glucose in response to insulin (Fuentebella and Kerner, 2009), although previous studies did show an effect of phosphorus on glucose and insulin (Kattab et al., 2011)

More research is needed to study the effect of a phosphorus load on overweight and obese subjects since this area of research is lacking in the literature. Moreover, studies with larger sample sizes and less subject variability are needed. The next step could be studying only male subjects and screening for insulin resistance before participating in the study. Future research should also try to compare the postprandial response in lean subjects as compared to overweight and obese subjects. Additionally, the long term effect of phosphorus administration or ingestion in overweight and obese subjects should be studied since one high phosphorus load might not have caused any change in glucose and insulin when insulin resistance was present.

CHAPTER VI

CONCLUSION

This study is the first to examine the effect of a phosphorus load on the incretin hormones GLP-1 and GIP in overweight and obese subjects. In our study, ingesting 500 mg of phosphorus with a glucose solution caused an increase in serum phosphate after its consumption and an increase in serum GLP-1. Blood glucose, insulin, triglycerides, cholesterol levels, and GIP were not significantly different between the two test groups.

Both phosphorus and GLP-1 have similar effects such as decreasing blood glucose levels, improving insulin secretion, and decreasing appetite. Therefore, the positive effect of phosphorus ingestion examined in previous studies could be the result of a rise in GLP-1 levels. However, insulin and glucose levels were not affected by phosphorus ingestion, which we hypothesized to be due to insulin resistance observed in the subjects enrolled in this study.

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