

The treatment effect of Rooibos on vascular function and signalling pathways of aortas from diet induced obese rats.

By

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DECLARATION:

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ABSTRACT

Introduction:

Obesity is becoming a worldwide health problem and is a well-documented independent risk factor for the development of endothelial dysfunction (decreased nitric oxide (NO) bioavailability) and cardiovascular disease (CVD). It is also associated with chronic inflammation and decreased insulin sensitivity. As a highly active endocrine organ, perivascular adipose tissue (PVAT) releases a variety of adipokines and inflammatory cytokines that activate several signalling pathways. Recently, observational studies have indicated a role for oral supplementation of Rooibos (RB) in lowering the risk for cardiovascular disease by improving endothelial dysfunction by means of RB's anti-inflammatory and anti-oxidant properties. The acute effect of RB in an *ex vivo* aortic model has not been investigated yet.

Aims:

Therefore, this study aimed to investigate the effect of direct RB administration to aortas from obese and lean Wistar rats, by measuring the aortic function as well as changes in the signalling pathways and the inflammatory markers. .

Methods:

Aortas were obtained from three groups of euthanized male Wistar rats: young control (aged 16 weeks), aged-matched lean group (aged 22 weeks) on a normal rat chow and diet group (aged 22 weeks) on a 16 week high fat diet, HFD (high in sucrose and cooking fat). Aortic segments, with or without PVAT, were placed into an organ bath containing Krebs buffer at 37⁰ C. Aortic ring function was determined by cumulative phenylephrine (PE) induced contraction (PE: 100nM-1µM), followed by cumulative acetylcholine (Ach) induced relaxation (Ach: 30 nM-10µM). RB (0.02%) was administered for 30 min during stabilisation before initiation of contraction and relaxation. Intracellular signalling pathways: eNOS, and the upstream activators PKB and AMPK, MAPKs (ERK1/2, JNK1/2, P38), as well as Nitrotyrosine and CREB were evaluated using Western blot analysis. Inflammatory markers were also analysed using the multiplex kit. Data was analysed using two-way ANOVA with Bonferroni's post-test.

Results:

The HFD caused an increase in body weight, intraperitoneal fat, fasting glucose level, as well as HOMA index. Aortas with PVAT of the older lean group showed a significant increase in contractility compared to the young control ($p < 0.05$). However aortas with PVAT of the obese diet group showed a reduced contractile response and enhanced relaxation response compared to the lean group ($p < 0.05$). The addition of RB decreased the contractile response in lean group with PVAT. However, RB enhanced the contractility of the diet group (with and without) PVAT, as well as reduction in relaxation on the diet group without PVAT.

The diet group had increased phosphorylation and phospho/total ratio of eNOS and ERK1/2 in aortas with and without PVAT, compared to the lean group. However, RB decreased this effect. Inflammatory markers showed variable results.

Conclusion:

Sixteen weeks on a HFD was effective to induce insulin resistance. The anti-contractile effect of the HFD on aortic function and the enhancement in the relaxation might be due to the elevation of phosphorylated eNOS that was associated with increased ERK1/2 phosphorylation.

This study demonstrated that RB may reverse the contractile inhibition of obese rat aorta with PVAT, with no effect on PVAT relaxation. Furthermore, RB has shown a pro-contractile and anti-relaxant effect in aorta of the diet group without PVAT. This demonstrates that RB is not deleterious when PVAT is present and may be beneficial to endothelial function in obese individuals.

OPSOMMING

Inleiding:

Vetsug is besig om 'n wêreldwye gesondheidsprobleem te word en is 'n goed-gedokumenteerde onafhanklike risikofaktor vir die ontwikkeling van endoteel disfunksie (afgeneem in stikstofoksied, NO, biobeskikbaarheid) en kardiovaskulêre siekte (KVS). Dit word ook geassosieer met chroniese inflammasie en verminderde insulien sensitiwiteit. As 'n hoogs aktiewe endokriene orgaan, stel perivaskulêre vetweefsel (PVAT) 'n verskeidenheid van adipokines en inflammatoriese sitokiene vry wat verskeie seintransduksiebane aktiveer. Onlangs het waarnemingstudies 'n rol vir orale aanvulling van Rooibos (RB) 'n verlaging van die risiko vir KVS aangedui deur die verbetering van endoteel disfunksie deur middel van RB se anti-inflammatoriese en anti-oksidadant eienskappe. Die akute effek van RB in 'n *ex vivo* aorta model is nog nie ondersoek nie.

Doelwitte:

Hierdie studie is daarop gemik om die effek van direkte RB administrasie op aortas van oorgewig en maer Wistar rotte te ondersoek, deur die meting van die aorta funksie asook veranderinge in die sein paaie en die inflammatoriese merkers.

Metodes:

Aortas is verkry van drie groepe van kant gemaakte manlike Wistar rotte: 'n jong kontrole (16 weke oud), 'n maer groep (22 weke oud) op 'n normale rottdieet, en 'n dieet groep (22 weke oud) op 'n hoë vet dieet, HVD (hoog in sukrose en kookvet) vir 16 weke. Aorta segmente, met of sonder PVAT, is in 'n orgaan bad met Krebs buffer teen 37⁰ C geplaas. Aorta ring funksie is bepaal deur kumulatiewe fenielefrien, PE, geïnduseerde kontraksie (PE: 100nM-1µM), gevolg deur kumulatiewe asetielcholien, Ach, geïnduseerde verslapping (Ach: 30 nM-10µM). Die RB (0,02%) is toegedien vir 30 min tydens stabilisering voor aanvang van kontraksie en verslapping. Intracellulêre sein paaie: eNOS, en die aktiveerders PKB en AMPK, asook MAPKs (ERK1/2, JNK1/2, p38), Nitrotyrosine en CREB is geëvalueer met behulp van Western-klad analise. Inflammatoriese merkers is ook geanaliseer met behulp van die multiplex kit.

Data is geanaliseer deur van twee-rigting ANOVA met Bonferroni se post-toets gebruik te maak.

Resultate:

Die HVD veroorsaak 'n toename in die liggaamsgewig, intraperitoniale vet, vastende glukose vlak, asook HOMA indeks. Aortas met PVAT van die ouer maer groep het 'n beduidende toename in kontraktiliteit vergeleke met die jong kontroles getoon ($p < 0.05$). Die aortas met PVAT van die vetsugtige dieet groep het 'n afname kontraktiele respons getoon en verbeterde verslapping in vergelyking met die maer groep ($p < 0.05$). Die toediening van RB het die kontraktiele respons in die maer groep met PVAT verlaag. RB het die kontraktiliteit van die dieet groep (met en sonder PVAT) verhoog, asook die verslappings effek in die dieet groep sonder PVAT verlaag.

Die dieet groep het toename in fosforilering en fosfo / totale verhouding van eNOS en ERK1/2 in aortas met en sonder PVAT getoon, in vergelyking met die maer groep. Maar RB hierdie effek laat afneem. Inflammatoriese merkers toon veranderlike resultate.

Gevolgtrekking:

Sestien weke op 'n hoë vet dieet was effektief om insulienweerstandigheid te veroorsaak. Die anti-kontraktiele effek van die HVD op aorta funksie en die pro-verslappings effek kan verband hou met die toename van gefosforileerde eNOS en 'n verhoogde ERK1/2 fosforilering.

Hierdie studie bewys dat RB die kontraktiele inhibisie van oorgewig rot aortas met PVAT kan omkeer, met geen effek op PVAT ontspanning nie. Verder het RB 'n pro-kontraktiele en anti-verslappings effek in aorta van die dieet groep sonder PVAT getoon. Dit dui aan dat RB nie nadelige is wanneer PVAT teenwoordig is nie en dat dit tot voordeel van endoteel funksie in vetsugtig individue kan wees.

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Abbreviation

Ad 36 = adeno virus 36.

AFR = Africa region.

AMPK = adenosine monophosphate-activated protein kinase.

AMPK =5' Adenosine Monophosphate-activated Protein Kinase

AMR = Americas region.

AngII = angiotensin II.

ANOVA = Analysis of Variance

APS = Ammonium Persulfate

ATP: Adenosine Triphosphate.

BH4= tetrahydrobiopterin.

BMI = body mass index.

BSA = Bovine Serum Albumin

Ca²⁺= calcium.

CAD = coronary artery disease.

DIO = diet induced obesity

ECL = Enhanced Chemiluminescence

ED = Endothelial Dysfunction

EDHF = Endothelial hyperpolarization factor.

EDTA = Ethylenediaminetetraacetic

EMR = Eastern Mediterranean region.

eNOS = Nitric oxide synthase.

EUR = Europe region.

FAD= flavin adenine dinucleotide.

FFA= free fatty acid.

FMN= flavin mononucleotide.

H₂O₂= hydrogen peroxide.

H₂S = hydrogen sulphide.

HDL-C= high density lipoprotein cholesterol.

HFD= high fat diet.

HOMA = homeostasis model assessment.
IL= Interleukin.
iNOS = inducible nitric oxide synthase.
IP = intra peritoneal.
IR = insulin resistance.
K⁺ = potassium channels.
KHB = Krebs Henseleit buffer.
LDL-C = low density lipoprotein cholesterol.
MHO = metabolically healthy obese.
NADPH = Nicotinamide adenine dinucleotide phosphate.
nNOS = neural nitric oxide synthase.
NO = nitric oxide.
OS = oxidative stress.
(OONO⁻) = peroxy nitrite anion.
PAI-1=plasminogen activator inhibitor.
PKB/Akt = Protein Kinase B.
PKG = protein kinase G.
PVAT= perivascular adipose tissue.
RB = rooibos.
ROS = reactive oxygen species.
SAT = subcutaneous adipose tissue.
SDS = Sodium dodecyl sulfate.
SEAR = South-East Asia region.
SEM = Standard Error of the Mean
TEMED = 1,2-Bis(dimethylamino)ethane
TG = triglyceride.
TNF- α = Tumor necrosis factor alpha.
VAT = visceral adipose tissue.
WB = western blot.
WHO = world health organization.

WPR = Western Pacific region.

1. LITETAURE REVIEW

1.1 Obesity

1.1.1 Obesity and contributing factors

Obesity contributes to a wide field of research, since it has increased in prevalence throughout the world over the last few decades (Kim et al. 2008). Research is now more focused on the association between body weight, cardiovascular risk factors and cardiovascular disease (Britton, Fox 2011), as well as insulin resistance, type 2 diabetes and coronary heart disease (Molica et al. 2015) .

Despite the fact that obesity is due to dietary intake exceeding energy loss, etiological factors such as; genetics, physical activity, food type, drugs, etc., that are associated with the development of obesity (fig.1.1) are highly complex.

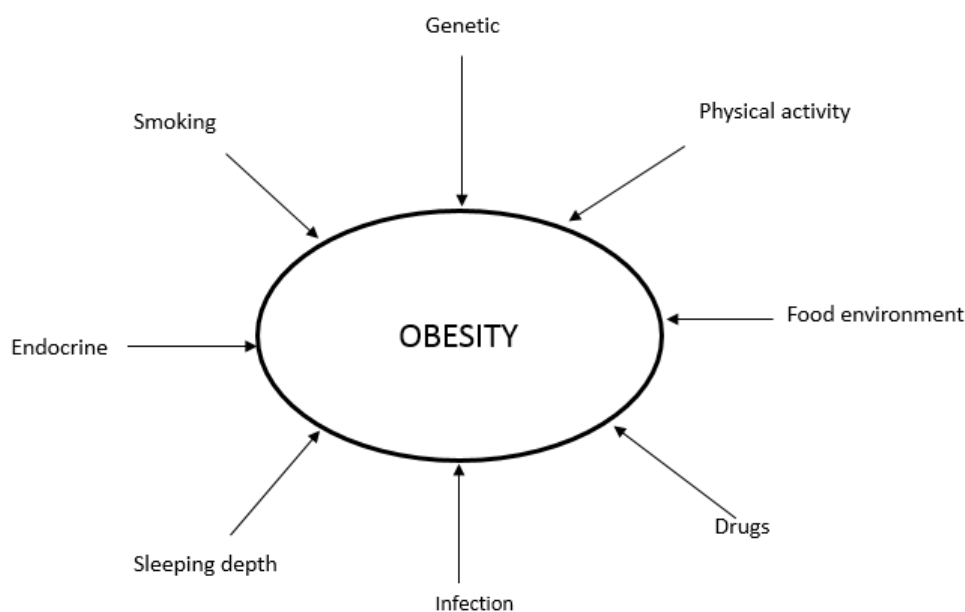


Figure 1.1: A representation of factors contributing to obesity.

The increased access to highly palatable foods which are rich in sugar, fat, and sodium, is increasing globally and contributes to a high risk lifestyle. Physical exercise has been decreasing in the last few years (Rolls 2003). Moreover, using certain types of drugs such as; antihypertensive, anti- psychotic, steroid hormones, contraceptives, anti-histamine, and protease inhibitors, all play a critical role in weight gain (Aronne,

Segal 2003). There is a negative relationship between the amount of sleeping hours per night and Body mass index (BMI), as a reduction in sleeping hours increase hunger and stimulates appetite (Gangwisch et al. 2005, Spiegel et al. 2004). Even Adenovirus (Ad-36) has been shown to cause obesity in humans (Atkinson et al. 2005). Research has shown that rare mutations in genes may contribute to genetic obesity (Swinburn et al. 2011). Chemical substances such as dichlorodiphenyltrichloroethane, some polychlorinated biphenols and some alkylphenols may cause endocrine disturbance leading to increase body weight (Keith et al. 2006). Additionally, cigarette smokers have shown to have an increase in body weight as soon as they stop smoking, due to increased appetite (Wright, Aronne 2012).

1.1.2 Obesity Statistics

According to the World Health Organization (WHO) website, obesity is defined as “abnormal or excessive fat accumulation that may impair health”. A person with BMI of 25-kg/m² or more is considered to be overweight, while BMI equal or more than 30kg/m² is considered to be obese.

Globally according to the WHO website in 2014, 39% of adults aged 18+ were overweight (39% men and 40% of women), of which 13% were obese (11% of men and 15% of women). Thus, nearly 2 billion adults worldwide are overweight and, of these, more than half a billion are obese figure (1.2).

The prevalence of overweight and obesity were highest in the WHO Regions of the Americas (61% of individuals including both sexes, were overweight, 27% were obese) and lowest in the WHO Region of South East Asia (22% overweight in both sexes with 5% being obese). In the WHO Region of the Americas and European and Eastern Mediterranean Regions over 50% of women were overweight. In all three of these regions, roughly half of overweight women were obese (25% in Europe, 24% in the Eastern Mediterranean, 30% in the Americas). In all WHO regions women were more likely to be obese than men. In the WHO African, Eastern Mediterranean and South-East Asia Regions, women had roughly double the obesity prevalence of men.

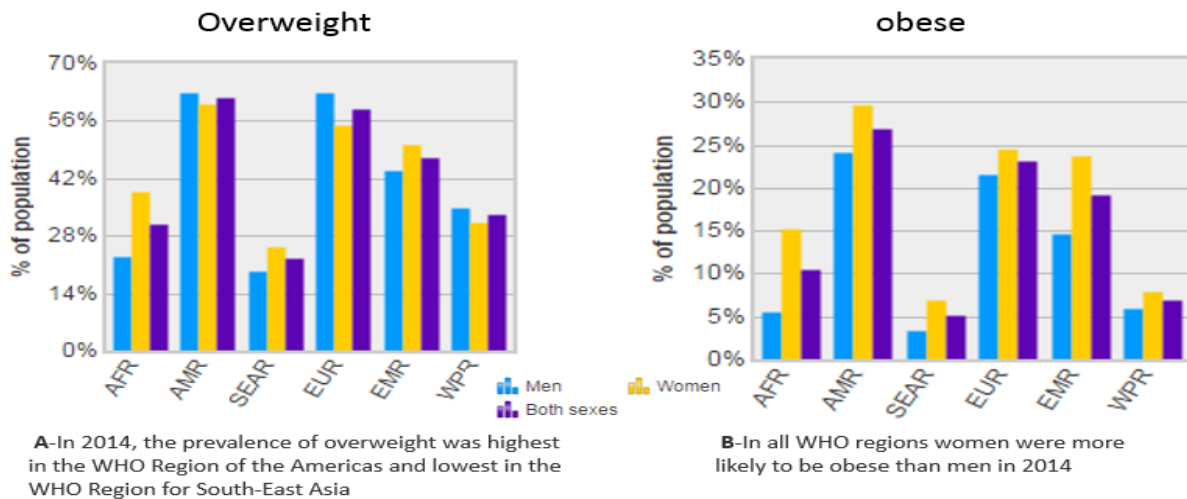


Figure 1.2 The trend of obesity and overweight individuals in 2014 (AFR = Africa, AMR = Americas, SEAR = South-East Asia, EUR = Europe, EMR = Eastern Mediterranean, WPR = Western Pacific) image from http://www.who.int/gho/ncd/risk_factors/overweight/.

1.1.3 Obesity and Cardiovascular Disease

Comorbidities, like cardiovascular disease may depend more strongly on the distribution of the body fat than the amount of fat, when it is compared to the generalized measuring of adiposity such as BMI (Britton, Fox 2011). Furthermore, due to the fact that BMI cannot be used as an accurate measurement to distinguish between fat mass and muscle mass, waist circumference is used as a good predictor of coronary artery disease (CAD) (Alberti, et al 2006).

Abdominal body fat can be classified into; subcutaneous adipose tissue (SAT) or visceral adipose tissue (VAT) (Molica et al. 2015). The chance of developing a high risk of hypertension, insulin resistance and coronary heart disease is linked to the VAT which represent only around 10% of the total body fat (Thomas et al. 1998), while the SAT has a mild effect.

A strong relationship exists between obesity and cardiovascular disease as obesity is often associated with the well-known disorder called Metabolic Syndrome. The mechanism of this disorder is thought to be due to excessive visceral fat accumulation and insulin resistance (Kihara, Matsuzawa 2015). Insulin has a critical impact on the

endothelium by elevation of nitric oxide (NO) bioavailability, which enhances vasodilation and acts in an anti-atherogenic manner (as reviewed by Ritchie and Connell in 2007). Insulin resistance is a condition which affects the response of vasculature, leading to endothelial dysfunction. Moreover obesity contributes to insulin resistance which will increase the risk of cardiovascular diseases (Ritchie, Connell 2007).

Despite the above mentioned, approximately 6-40% of obese people are metabolically healthy with a BMI ≥ 30 kg/m² as reviewed by (Phillips 2013). This observation could be explained by the fact that metabolically healthy obese individuals have a lower fat amount in their VAT compared to other obese individuals and was unlikely to suffer from a cardiovascular event (Phillips 2013). Research is on-going with regards to the mechanism responsible for metabolically healthy obese, but it is postulated to be due to factors which are involved in lipogenesis and lipid metabolism (Lozzo 2011).

1.1.4 Vascular wall structure

There are three layers as illustrated in figure1.3 that surround the blood vessel, namely: the endothelial cell layer (tunica intima), the vascular smooth muscle layer (tunica media), and the adventitial layer (tunica externa) (Zhao, Vanhoutte & Leung 2015).

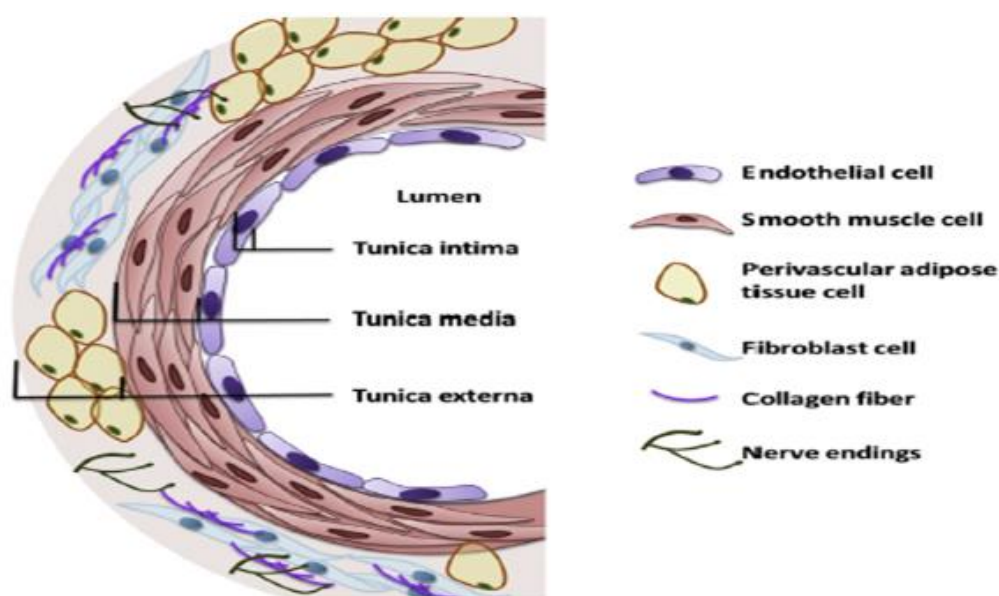


Figure1. 3: The composition of the vascular wall (Zhao, Vanhoutte & Leung 2015)

The endothelial cell layer, is the layer that covers the internal surface of the vasculature, starting from the heart to the smallest capillary, it acts as a barrier between the surrounding tissue and the blood. The endothelial layer plays a crucial role in modulating the diameter of the blood vessels by secreting NO which is derived from the endothelium. In addition it secretes contracting substances such as prostanoids and peptides, as well as stimulating endothelium dependent hyperpolarization.

The vascular smooth muscle layer, is responsible for the vascular tone of the blood vessels. Contraction of the smooth muscle can be stimulated either mechanically (pressure, stretch), or by pharmacological stimulation (by linking the ligands to receptors). This will result in intracellular calcium influx through activation of calcium channels in the plasma membrane or through calcium release from the sarcoplasmic reticulum. Furthermore, the released calcium will bind to calmodulin and will form a calcium – calmodulin complex. Thereafter, actin-myosin will be activated and phosphorylated and lead to the smooth muscle cell contraction.

The adventitial layer, is the main focus of our study, as it contains nerve ending and most importantly the perivascular adipose tissue (PVAT) as well as fibroblasts and collagen. This layer plays a critical role in the vascular development and remoulding. In addition it plays a role in immunity, and inflammatory cell processes, as well as providing an environment for signal exchange.

Vascular endothelium is considered to be a paracrine, autocrine, and endocrine organ that plays a major role in vascular haemostasis, as well as secreting various mediators, which in turn, play roles in platelet aggregation, coagulation, fibrinolysis and vessel tone. Moreover, endothelial cells have the ability to regulate vascular tone either by secreting vasoconstriction substances, including endothelin-1, thromboxane A₂ and reactive oxygen species (ROS), or vasodilatory substances, such as NO, prostacyclin and endothelium-derived hyperpolarizing factor (EDHF) (Avogaro, de Kreutzenberg 2005). Furthermore, endothelial cells are thought to be the main target for cardiovascular diseases including: atherosclerosis, myocardial infarction, and stroke (Barton, Baretella & Meyer 2012).

1.1.5 Perivascular adipose tissue (PVAT)

PVAT is defined as the fat which is localized around blood vessels (Schlett et al. 2009). The amount of fat differ from one site to another, for example the aorta has more fat situated on the outside in comparison with the cerebral or microcirculation where the fat is often absent (Szasz, Bomfim & Webb 2013).

It had been shown that adipose tissue acts as a massive endocrine organ in our body and PVAT is thought to play a role in supporting blood vessels. Recent studies have focused on the secretory role of PVAT as endocrine organ which is metabolically active (Eringa et al. 2007), and have its affect in the vasculature function (Table 1.1).

The active metabolites secreted termed Adipokines, include; cytokines, chemokines, and hormones that act in a paracrine, autocrine or endocrine way (Karastergiou, Mohamed-Ali 2010).

1.1.6 The mechanism of PVAT on the vasculature

Table 1.1: The effect of PVAT on the vascular contraction (adapted from Szasz and Webb, 2012).

Mechanism	Effect	Condition	Vascular bed and species	Reference(s)
K ⁺ -channels: K ⁺ ATP K ⁺ Ca	Anti-contractile Anti-contractile	Physiological Physiological	Rat aorta Rat aorta	(Dubrovskaya et al. 2004, Gao et al. 2007).
Adiponectin	Anti-contractile	Physiological	Rat aorta	(Fesus et al. 2007)
Superoxide	contractile	Physiological	Rat aorta	(Gao et al. 2007)
H ₂ O ₂	Anti-contractile	Physiological	Rat aorta	(Gao et al. 2007)
Nitric oxide	Anti-contractile	Early (DIO)	Mouse mesenteric	(Gil-Ortega et al. 2010, Greenstein et al. 2009)
Oxidative stress	Blocks anti-contractile effect	(DIO)	Mouse mesenteric	(Greenstein et al. 2009)

Inflammation	Blocks anti-contractile effect	Obesity	Human subcutaneous fat arterioles	(Greenstein et al. 2009)
H ₂ S ₂	Anti-contractile	Physiological hypertension	Rat aorta	(Fang et al. 2009, Schleifenbaum et al. 2010)
Angiotensin II	Contractile	Physiological	Rat mesenteric	(Lee et al. 2009)
AMPK	Endothelial dysfunction	Obesity	Rat mesenteric	(Ma et al. 2010)
Infiltration macrophages +hypoxia	Blocks anti-contractile effect	Obesity and metabolic syndrome	Mouse mesenteric +rat aorta	(Withers et al. 2011)
Changes in fatty acid composition	Blocks anti-contractile effect	Metabolic syndrome	Rat aorta	(Rebolledo et al. 2010)
K⁺ : potassium channels, ATP: Adenosine Triphosphate, Ca: calcium, H₂ O₂ : hydrogen peroxide, DIO: diet induced obesity H₂ S₂ : hydrogen sulphide, AngII: angiotensin II, AMPK: adenosine monophosphate-activated protein kinase.				

1.1.7 The Pathophysiology of obesity

Mechanisms responsible for the pathophysiology of obesity include: inflammation, oxidative stress, vascular dysfunction, dyslipidaemia and insulin resistance (Prieto, Contreras & Sánchez 2014a).

1.1.7.1 Inflammation

Once over supply of nutrients occur it will lead to hypertrophy and hyperplasia of adipocytes, which in turn alter the normal metabolism in adipose tissue which to an environment of mild inflammation. Adipocytes secrete well-known inflammatory markers such as interleukins (IL1-B –IL-6) and tumour necrosis factor-alpha (TNF- α) (Fernández-Sánchez et al. 2011). These pro-inflammatory markers have been found to be higher in obese subjects than their lean counterparts. Moreover there are other adipokines such as: adiponectin, leptin, plasminogen activator inhibitor (PAI-1), angiotensinogen, resistin, visfatin, and glucocorticoids (Ritchie et al. 2004b), all of which play an important role in inflammation. Additionally, obesity is associated with

the occurrence of insulin resistance, which will in turn lead to enhanced lipolysis and free fatty acid (FFA) release from triglycerides, which further enhances inflammation through production of inflammatory cytokines (Sethi, Vidal-Puig 2007). This elevation in FFA will also lead to alternation of endothelial function, proliferation of vascular smooth muscle cells, as well as changes in high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C) levels (Wyne 2003).

There is a wide range of other different cytokines that modulate inflammation and can be classified into either pro-inflammatory or anti-inflammatory cytokines (Table 1.2).

Table 1.2: The classification of cytokines:

Pro-inflammatory	IL-1 α , IL-1 β , IL-12, IFN-g, TNF- α .
Anti-inflammatory	IL-2, IL-4, IL5, IL-10, IL-13.

1.1.7.2 Oxidative stress (OS)

OS is the result of imbalance between the reactive oxygen species (**ROS**) production and anti-oxidant. In addition to the role of adipokines in secreting inflammatory markers, it also contributes to the production of reactive oxygen species (**ROS**), which gives rise to OS. The latter may occur under physiological or pathological conditions and may cause direct or indirect damage to surrounding tissues. This can in turn lead to development of additional pathological conditions such as obesity, diabetes, and cardiovascular disease (Fernández-Sánchez et al. 2011).

As mentioned by Fernández-Sánchez, et al. (2011) the mechanisms that are responsible for formation of ROS due to obesity is summarized as (figure 1.4):

- i) Production of inflammatory cytokines that are secreted from the adipose tissue.
- ii) Oxidation of free fatty acids by mitochondrial action.
- iii) Increases in the mechanical load and myocardial metabolism.
- iv) Excessive accumulation of fat with the associated increase in cellular damage.
- v) Diets rich in fat may affect oxygen metabolism.

Although there is a strong link between oxidative stress and ageing, there is insufficient evidence that the increased level of oxidative stress due to obesity can be responsible for stimulation of the aging process (Zhang et al. 2015). However, it has been shown

that obese mice, either genetic or hyperphagic models, had a shorter life span in comparison with lean mice (Baur et al. 2006).

Moreover, the secretion of adipokines by adipose tissue as well as the production of ROS can alter Nitric Oxide (NO), bioavailability which leads to endothelial dysfunction (Viridis et al. 2011).

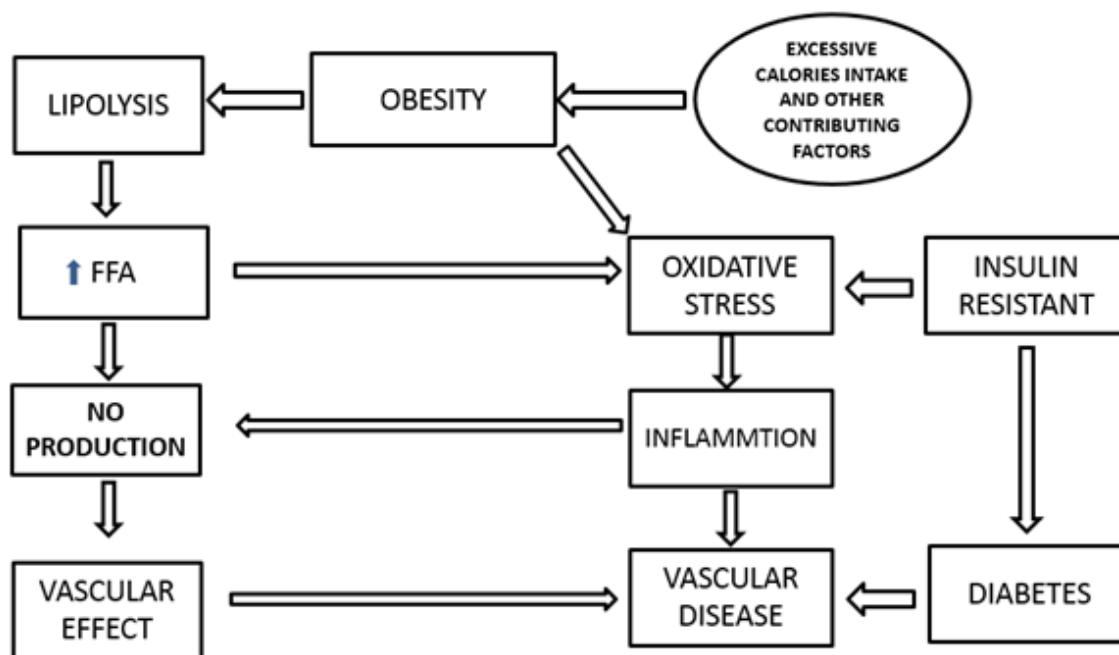


Figure 1.4: Summary of the connection between obesity, oxidative stress, insulin resistance, inflammation and atherosclerosis (modified from Tangvarasittichai 2015).

1.1.7.2.1 Nitric oxide synthase (NOS)

The main source of nitric oxide in the blood vessels is from L-Arginine conversion by the enzyme endothelial Nitric oxide synthase (eNOS) (Chen, Pittman & Popel 2008). There are three NOS isoforms, which are all responsible for the production of nitric oxide from L-arginine, namely neuronal NOS (nNOS or NOS-1), cytokine-inducible NOS (iNOS or NOS-2) and endothelial NOS (eNOS or NOS-3) (Andrew, Mayer 1999).

Nitric oxide was reported in 1986 by Robert Furchgott and Louis Ignarro to be endogenously produced from the endothelium and several studies showed a role for NO in physiological processes and signaling (Furchgott 1988).

1.1.7.2.2 Nitric oxide in physiological processes

In addition to the vasodilatory effect of NO, it plays a vital role in the regulation of the cardiovascular system, including smooth muscle proliferation and migration, platelet function, oxidation of LDL, as well as inhibition of leukocyte adhesion to endothelial cells (Yetik-Anacak, Catravas 2006). Furthermore, NO also exhibit a neurological function through parasympathetic innervations including regulating the behaviors, intestinal motility, as well as protection against infection and tumors (Toda, Ayajiki & Okamura 2012)

In order to produce nitric oxide from L-arginine NOS requires several co-factors including Nicotinamide adenine dinucleotide phosphate (NADPH), tetrahydrobiopterin (BH₄), flavin adenine dinucleotide (FAD) , flavin mononucleotide (FMN), calmodulin and iron protoporphyrin IX (haem) (Forstermann, Munzel 2006a). Moreover, the catalytic and regulatory activity of the three isoform of NOS are unique. (Bredt et al. 1991).

The chemical structure of the NOS enzyme consists of a reductase and oxygenase domain which are linked together by Calcium-calmodulin binding sites. Although the haem and BH₄ binding sites are located on the oxygenase domain, and the FAD, FMN, and NADPH are located at the reductase binding domain. The NOS enzyme functions as the NADPH cytochrome p-450 which transfers electrons from its reductase group to a haem group (Panda, Ghosh & Stuehr 2001). Moreover, the isoforms of NOS are produced as monomers and in order for full enzymatic activity to catalyse NO production, it must be in dimer form (Rafikov et al. 2011) (figure 1.5). In conditions such as: hypercholestremia, diabetes, hypertension, and CAD, the monomer will produce ROS instead of NO which will lead to NOS uncoupling (Forstermann, Munzel 2006a). The latter is a PEnomena which correlates with endothelial dysfunction and its related diseases, such as atherosclerosis (Heitzer et al. 2001).

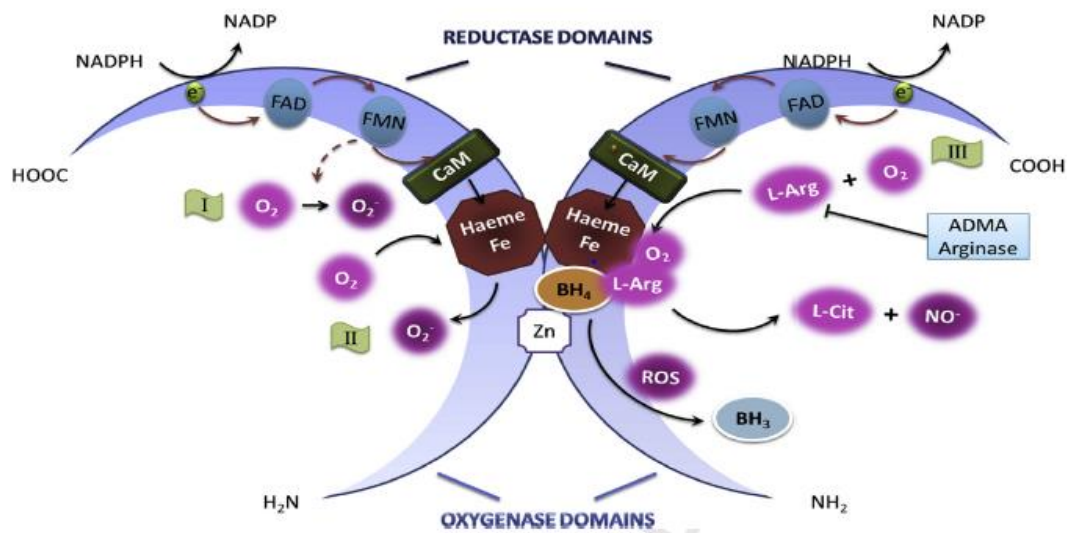


Figure 1.5: Homodimeric structure of nitric oxide synthase (NOS) (Zhao, Vanhoutte & Leung 2015).

Endothelial cells express both arginine I and II (Arg-I and Arg-II). Arg-II is a NOS substrate and plays a vital role in eNOS uncoupling. In an angiotensin II-induced hypertension mouse model, endothelial dysfunction was accelerated through activation of mitogen activated protein kinase p38 MAPK which acts on Arg-II (Huang 2009).

1.1.7.2.3 Nitric oxide signaling pathway

The formation of nitric oxide (NO) is triggered either by vasodilators such as: acetylcholine or bradykinin (through Ca²⁺ calmodulin-dependent), or through phosphorylation of PKB/Akt and AMP-activated kinase (AMPK) by (protein kinase A (PKA) and protein kinase G) (PKG) (Huang 2009). In addition, stimulation of the Akt kinases is enhanced by insulin and other factors namely: beta-adrenoceptors, and vascular endothelial growth factor (VEGF) (Huang 2009). eNOS generates NO which, under physiological conditions, signals through the guanylyl cyclase-cGMP-PKG pathway to stimulate vasodilation, However, NO produced by eNOS might be rapidly inactivated during oxidative stress. Superoxide (O₂⁻) if produced in sufficient volume can react with NO to form the peroxynitrite anion (OONO⁻) (Pacher, Beckman & Liaudet 2007). Thus, O₂⁻ scavenges NO and renders it unavailable to mediate its physiologic functions, including binding to soluble guanylate cyclase to stimulate vasodilation. In addition, peroxynitrite can cause direct oxidative damage (Beckman,

Koppenol 1996). Therefore, the decrease in the bioavailability of NO normally oppose atherosclerosis.

1.1.7.2.4 Endothelial dysfunction

Endothelial dysfunction is defined as decrease the bioavailability of nitric oxide production, which in turn decreases the vasodilation and enhance the activation of smooth muscle contraction (Hadi, Carr & Suwaidi 2005).

The correlation between obesity and endothelial dysfunction can be either by direct or indirect mechanisms, direct mechanism includes the production of inflammatory cytokines that activates ROS and in turn decrease the bioavailability of nitric oxide (Vigili de Kreutzenberg et al. 2000). Whereas indirect mechanisms, such as insulin resistance and its related complications like hypertension, dyslipidaemia, and diabetes mellitus (Bakker et al. 2009).

1.1.7.3 Dyslipidaemia

Obesity is linked to dyslipidaemia which is one of the risk factors of cardiovascular disease, and it is define as an elevation of triglyceride (TG) levels, reduction in high-density lipoprotein (HDL) levels, and increased low density lipoprotein (LDL) levels (Howard, Ruotolo & Robbins 2003). These lipid changes are similar to what is associated with type 2 diabetes mellitus and insulin resistance (which is considered to be the main contributor to metabolic syndrome) (Olefsky 2000, Taskinen 2003).

Nitric oxide formation can be affected by lipid levels. LDL cholesterol levels can influence the production of nitric oxide by decreasing eNOS activity, this may be more relevant when LDL is taken up by macrophages and converted to oxidize LDL (Steinberg, Witztum 2002). Conversely, HDL can play a role in decreasing the blood pressure by elevation of the nitric oxide bioavailability (Mineo et al. 2006)

1.1.7.4 Insulin Resistance

Insulin binds to insulin receptors (IR) (figure1.6) and stimulates the recruitment of insulin receptors substrate (IRS) proteins. This in turn leads to phosphoinositide 3-kinase (PI3K) activation, resulting in phosphorylation of PKB which has been shown to have a metabolic effect as well. Insulin induced PKB phosphorylation will result in

phosphorylation of eNOS at serine 1177 (Michell et al. 1999) and stimulation of NO production.

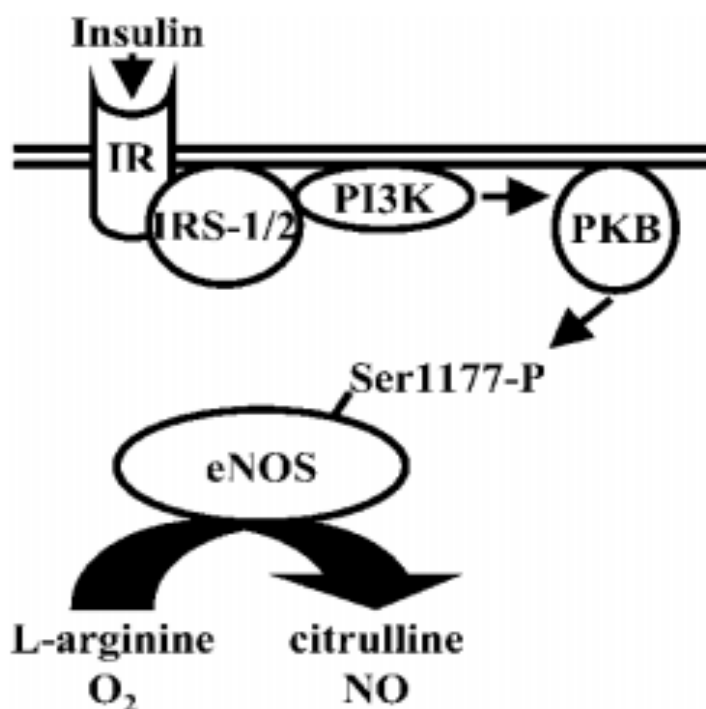


Figure 1.6 : Mechanism of insulin mediated NO production. Insulin binds to the IR receptor which in turn recruits IRS leading to activation of PI3K. PI3K activates PKB that leads to phosphorylation of eNOS at Ser-1177. NOS utilizes L-arginine as substrate and the co-substrate, molecular oxygen to produce NO and L-citrulline as end- products (Copied from Ritchie et al. 2004a).

Insulin resistance is a condition in which the three main metabolic tissues in the body (liver, skeletal muscle, white adipose tissue) become sensitive to insulin. Insulin up-regulates eNOS activation, which is mediated by the mitogen activated protein kinases (MAPK) consisting of three subfamilies, P38 kinase, JNK1/2, and ERK1/2. The main role of these kinases are to regulate basic metabolism. Due to the anabolic metabolic effects of insulin, it activates the MAPK pathway which plays a role in development of insulin resistance (Gehart et al. 2010).

The correlation between insulin and endothelial dysfunction is highly complicated as (reviewed by Huang 2009). As shown in figure (1.4) the metabolic changes seen in obesity, diabetes and metabolic syndrome involve insulin resistance and visceral

adiposity, potentially resulting in endothelial dysfunction which leads to atherosclerosis.

1.1.8 Adipokines and signalling

Studies have shown that insulin sensitivity is influenced by adipokine secretion (Ritchie et al. 2004c). Adipokines include adiponectin, resistin, leptin, TNF- α , IL-1 β and IL-6. Moreover, adiponectin enhances the production of NO by phosphorylation of AMPK, while resistin, IL-6, and TNF- α suppress NO production through decreased phosphorylation of eNOS (Huang 2009) (figure. 1.7).

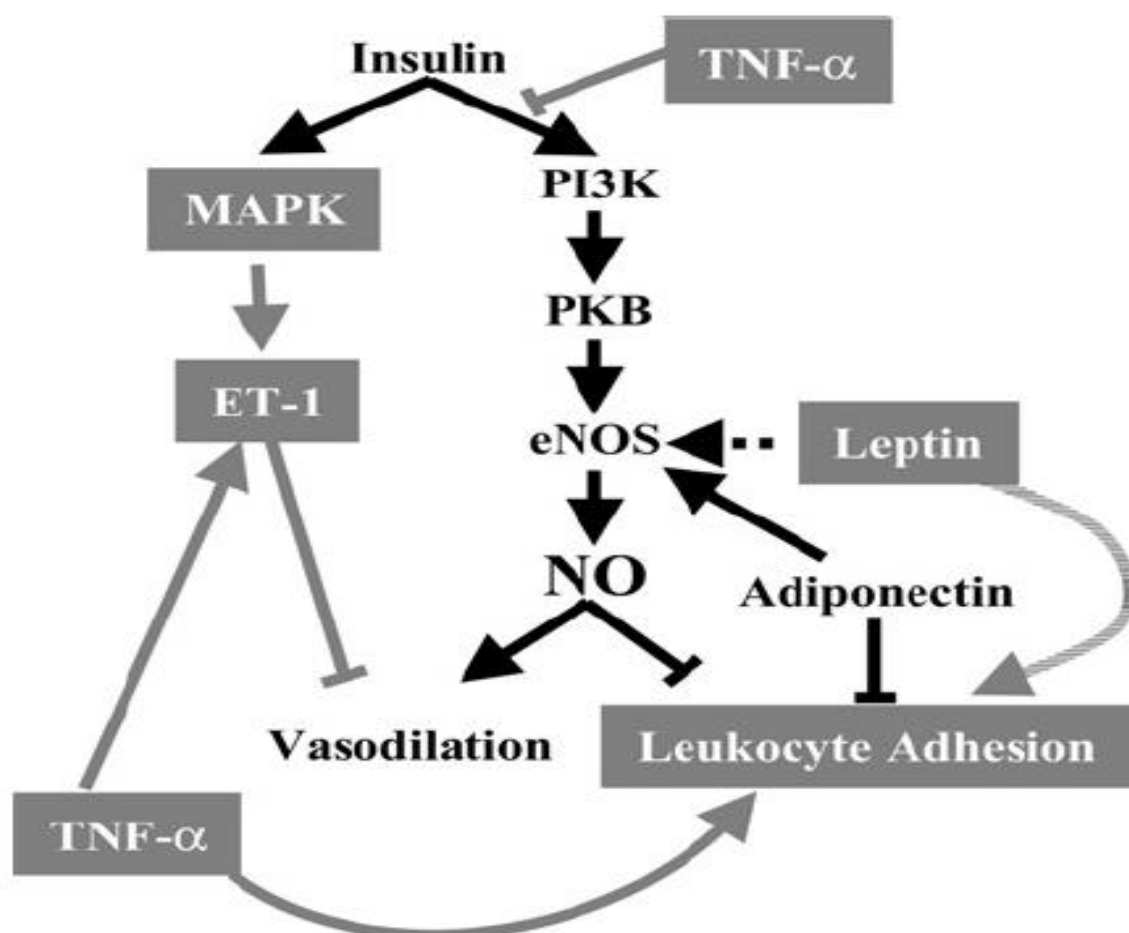


Figure 1.7 Summary of adipokine mediated effects on insulin signalling. Insulin acts within the endothelium to stimulate both NO synthesis, by utilizing the PI3K/PKB/eNOS pathway, and ET1 production via direct MAPK activation. Leptin, adiponectin and TNF- α have been proposed to directly interact with these pathways (copied from Ritchie et al. 2004).

A study by Chen et al (2008) has shown that resistin decreased the expression of eNOS and increased ROS production in endothelial cells of human coronary artery via activation of P38 MAPK, and JNK (Molica et al. 2015). Another study has shown that resistin causes proliferation of cells through activation of ERK1/2, as well as activation of PKB/AKT (Calabro et al. 2004).

Leptin is one of the adipokines that plays a role in inflammation and oxidative stress. A study by Li et al (2014) has shown that leptin can influence vascular smooth muscle cell (VSMC) proliferation, through activation of the P38 MAPK signalling pathway (Li et al. 2014).

As reviewed by Chen et al (2015), TNF- α is a pro-inflammatory cytokine that plays a critical role in insulin resistance by stimulation of lipolysis. Glucose uptake is increased in the visceral and subcutaneous adipocyte by stimulation of AMPK signalling pathway, which in turn stimulate insulin resistance by activation of JNK1/2 (Chen et al. 2015).

Interleukin IL-1 β is another pro-inflammatory cytokine which may enhance insulin resistance by alteration of the insulin signalling pathway that leads to a decrease in insulin sensitivity of pancreatic β -cell, and impair the secretion thereof in the peripheral tissue. This pro-inflammatory marker is thought to be highly expressed in different types of cells, such as endothelial cells and monocytes which increase during elevation of the blood sugar levels (Su et al. 2009). Moreover, IL-1 β decreases insulin stimulation of adipocytes which lead to activation of the ERK pathway (Jager et al. 2007a).

According to a review by McArdle et al. (2013), white adipose tissue secretes large amounts of IL-6 that has been found to be more prevalent in obesity, and is usually associated with insulin resistance and type 2 diabetes. IL-6 is mainly found in the skeletal muscle after exercise and plays a role in muscle contraction. Skeletal muscle metabolism is influenced by IL-6, and it plays a role in AMPK α 2 activity, oxidation of free fatty acids, as well as glucose uptake (McArdle et al. 2013) .

These are the adipokines which are best characterised in literature. Other adipokines and cytokines are still under investigation with little evidence about their role in signalling pathways.

1.1.9 Adipokines and atherosclerosis

Narrowing in the blood vessels due to formation of plaque is known as atherosclerosis. Molica et.al 2015 has reviewed the role of adipokines in cardiovascular disease. As mentioned above the adipokines play an essential role in inflammation, which is linked to cardiovascular disease. Adipokines diffuse into blood vessels via the *vasa vasorum*, the main mechanism of this process is still unclear. It has been hypothesised that adipokines affects the migration and proliferation of smooth muscle activity, which is likely to be the underlying cause of hypertension, atherosclerosis, as well as formation of thrombosis (Molica et al. 2015). The effect of the adipokines on obesity and cardiovascular disease is summarized in table 1.3.

The association between obesity and atherosclerosis has been hypothesised to be the result of prolonged inflammation. The early atheroma formation is caused by the interaction between the immune cells and endothelium, in addition decreased nitric oxide, as well as an increase in ROS and oxidative stress that will lead to endothelial dysfunction (decreased in NO bioavailability) and pro-atheromatic conditions (Molica et al. 2015).

The complication of obesity and the elevation of blood pressure have also been indicated as a precursor of endothelial dysfunction and is associated with a reduction in nitric oxide. Moreover, the adipokines that are secreted from the perivascular adipose tissue plays an important role in developing atherosclerosis via its involvement in the process of endothelial dysfunction (Dorresteijn, Visseren & Spiering 2012).

Table 1.3: The role of adipokines in obesity and cardiovascular disease adapted from (Molica et al. 2015).

Adipokines	Function in obesity	Function in cardiovascular disease
TNF-α	↑ IR, hyperinsulinemia and Inflammation (Cawthorn, Sethi 2008, Skoog et al. 2002).	↑Endothelial dysfunction (Münzel et al. 2008). ↑Hypertension (Steinberg et al. 2000). ↑VSMC proliferation and migration (Wang, Castresana & Newman 2001).

		<p>↑Atheroma (Skoet et al. 2002).</p> <p>↑Thrombosis (Kruithof 2008).</p>
IL-6	<p>↑CRP release, inflammation. (Popko et al. 2010).</p>	<p>↑Endothelial dysfunction, hypertension (Schrader et al. 2007).</p> <p>↑ Adhesion molecules and inflammation (Libby, Ridker 2006).</p> <p>↑Thrombosis (Niessen et al. 1997).</p>
Leptin	<p>Regulate appetite (Mantzoros et al. 2011)</p> <p>↑Inflammation (Mantzoros et al. 2011, Kiguchi et al. 2009).</p>	<p>↑ Endothelial dysfunction (Korda et al. 2008).</p> <p>↑Hypertension (Aizawa-Abe et al. 2000).</p> <p>↑VSMC proliferation and migration (Bohlen et al. 2007). ↑Foam cell Atheroma formation (Shanker et al. 2012). ↑Thrombosis (Bodary et al. 2005).</p>
Resistin	<p>↓Glucose homeostasis and insulin sensitivity, ↑T2DM (Singhal et al. 2008, McTernan, Kusminski & Kumar 2006).</p>	<p>↑ Endothelial dysfunction, hypertension. (Chen et al. 2010).</p> <p>↑VSMC proliferation and migration (Miao, Li 2012).</p> <p>↑Foam cell Atheroma formation (Lee et al. 2009).</p> <p>↑Thrombosis (Calabro et al. 2011).</p>
Adiponectin	<p>↓T2DM and IR. (Waki et al. 2003, Lamers et al. 2011)</p> <p>↓ Inflammation. (Ohashi et al. 2010).</p>	<p>↓ Endothelial dysfunction (Hui et al. 2012).</p> <p>↓Hypertension (Tan et al. 2004).</p> <p>↓VSMC proliferation (Takaoka et al. 2009) .</p> <p>↓Migration (Lamers et al. 2011).</p> <p>↓Inflammation (Wang et al. 2013).</p> <p>↓Thrombosis (Chen et al. 2008).</p>

TNF- α : tumour necrosis factor, IR: insulin resistance. VSMC: vascular smooth muscle cell, IL-6: interleukin 6, ROS: reactive oxygen species, T2DM: type 2 diabetes mellitus.

TNF- α , IL-6, Leptin and resistin (as mentioned in table 1.3) have been shown to play a vital role in the cardiovascular disease through causing endothelial dysfunction, hypertension, VSMC proliferation and migration. In contrast to, adiponectin that has a protective effect on the cardiovascular by decreasing the endothelial dysfunction, lowering the blood pressure, as well as reducing the incidence of thrombosis.

1.1.10 Obesity and Aging

The rate of obesity in elderly people is increasing, which contribute to cardiovascular risk factors. A study done by Barandier et al. (2005) showed that the proliferation of adipocytes was significantly elevated in old and high fat diet (HFD) mice when compared to young mice. Furthermore, aging was shown to enhance vascular inflammation and endothelial dysfunction due to elevation of oxidative stress and infiltration of peri-aortic tissue with macrophages in old age model (Bailey-Downs et al. 2013)

1.1.11 Management of Obesity

Obesity is a reversible condition that can be managed by reversing the causes. Diet intervention, physical exercise, improving the quality of life style, as well as treatment of the underlying pathology is necessary. However, using chemical drugs, which are frequently prescribed to decrease body weight may lead to undesirable side effects, besides being costly.

Observational studies have repeatedly shown that diets high in plant-based foods and beverages are associated with a lower risk of chronic diseases such as cardiovascular disease (Hertog et al. 1993; Hollman et al. 1999; Hu 2003). There is a growing body of literature that recognises the importance of herbal treatments in obesity.

According to the World Health Organization (WHO), 80% of the world's population uses medicinal plants in the treatment of diseases and, in African countries, this rate

is much higher. Furthermore, 25% of modern medicines are made from plants that were first used traditionally. For instance, in China, traditional herbal preparations account for 30-50% of the total medicinal consumption, whereas in Ghana, Mali, Nigeria, and Zambia, the first line of treatment for 60% of children with high fever resulting from malaria is the use of herbal medicines at home. Herbal teas also became a popular research topic due to their beneficial effect on health and lack of side effects. Examples of such herbal teas are green tea, black tea, and Rooibos.

1.2 Rooibos (RB)

Rooibos originates from the Cedarberg mountain region in the Western Cape province of South Africa, (Beltrán-Debón et al. 2011a). It is harvested from a plant called *Aspalathus linearis*. It is a very popular and commonly used beverage not only among the South African population, but also among other countries such as: China, Japan, Germany, England, Poland, Malaysia, South Korea, and USA (Khan, Gilani 2006a).

RB gained its popularity due to its health effects. RB has a fruity, sweet taste in addition to its low tannin content, lack of caffeine and rich mineral content, including iron, potassium, sodium, calcium, copper, zinc, magnesium, fluoride, and manganese. RB also has high levels of polyphenols and flavonoids (Marnewick et al. 2011a).

RB is used in an unfermented and fermented form. *Aspalathin* and *Nothofagin* are the two major flavonoids found in unfermented (green) RB, while phenylpyruvic acid-2-O- β -D-glucoside (PPAG) and *Aspalathin* are the major flavonoids present in fermented RB (Dludla et al. 2014a). *Aspalathin* is the most abundant compound present in the aqueous extract of unfermented RB (Joubert et al. 2005). In contrast, the fermented form has lower levels of flavonoids that makes the anti-oxidant capacity lower than the unfermented form (Standley et al. 2001). Flavonoids have been proven to play a role in anti-oxidant, anti-inflammatory and vasorelaxant activity (Chan, Pannangpetch & Woodman 2000)

Several studies have been conducted on the low bioavailability of RB (Kreuz et al. 2008) in pigs and (Courts, Williamson 2009) humans. Those studies confirmed that RB has a low extent of absorption, and the flavonoids are detected in blood t after 24 hours of excretion in urine

1.2.1 Health benefits of rooibos

RB have been shown to have a variety of health benefits in the cardiovascular system, respiratory system, gastrointestinal system, along with its anti-inflammatory, anti-oxidant, and anti-cancer properties.

A strong correlation has been reported between the consumption of RB and the decrease in mortality from cardiovascular disease, through vascular tone regulation, reduction of oxidative stress, and improved function of the platelets (Bøhn et al. 2012).

A study done by Dłudla and co-workers, indicated that fermented rooibos has a cardio protective effect on cardiomyocytes in diabetic rats (Dłudla et al. 2014b). Another study also showed that *in vivo* supplementation of Rooibos has the ability to inhibit angiotensin- converting enzyme ii (ACE ii) activity, which is the key enzyme for renin –angiotensin aldosterone system. Activation of this enzyme will lead to endothelial dysfunction and inhibition of NO production. ACE inhibition is used for treatment of cardiovascular diseases such as hypertension. Ingestion of a single dose (400ml) of RB within 30-60 min will inhibit the activity of this enzyme (Persson et al. 2010a). The summative effect being improvement of endothelial function.

Pantsi and colleagues also showed that *in vivo* RB can protect against myocardial ischemia / reperfusion damage in a rat model (Pantsi et al. 2011).

RB could enhance smooth muscle relaxation by the activation of K⁺ channels and antagonising Ca⁺⁺(Khan, Gilani 2006b). Therefore RB could be used as treatment to lower blood pressure, gastrointestinal disorders, as well as bronchial asthma.

A study by Baba (2009) investigated the relationship between the anti-inflammatory effect and the anti-oxidant activity of RB. Colitis was induced in a rat with dextran sodium sulphate (DSS), and the anti-oxidant activity was measured by serum superoxide dismutase (SOD). The results showed an elevation of SOD levels and lower levels of DSS in the Rooibos group when compared to the control. This proved that rooibos has an anti-oxidant capacity *in vivo* (Baba et al. 2009a).

The anti-oxidant effect of rooibos has also been seen to protect the brain from CNS age deterioration by scavenging free radicals and decreasing lipid peroxidation (Inanami et al. 1995)

Rooibos tea exerted an anti-diabetic effect, this was proven by a study done by Kawano and co-workers who examined the effect of Aspalathin on glucose uptake in L6 myotubes and on insulin secretion by cultured pancreatic cells. The results showed that Aspalathin elevate insulin secretion by pancreatic cells, as well as enhanced glucose uptake from the muscle cells (Kawano et al. 2009)

Since NO is the main product of eNOS, a variety of studies have been done to see the effect of rooibos on eNOS. A study by Persson showed an increase in NO production in human umbilical veins endothelial cell culture (HUVEC). In contrast, in another study they found that the tea did not show any effect on eNOS after supplementary dose was given to humans (Persson et al. 2010a).

Table 1.4: Summary of the studies on RB from 2008-2014 adapted from (Joubert, de Beer 2011)

Infusion/extract	Model	Bioactivity	References
Fermented (aq. extract)	CCl4- induced liver damage in rats	Regeneration of liver damage	(Ulicna et al. 2008)
Unfermented (aq. extract)	Wistar rat DSS-induced colitis	Anti-inflammatory activity	(Baba et al. 2009b)
Unfermented and Fermented (aq. extract.)	Oral administration to Fischer rats: cancer promotion and initiation by FB and DEN, respectively	Chemo protection	(Marnewick et al. 2009)
Fermented (EtOH extract)	Murine neuroblastoma cell line derived from spontaneous malignant tumour	Tumouricidal activity	(Mazzio, Soliman 2009)
Fermented (DMSO extract)	Polar screen PPAR γ ligand binding competitive assay Lantha screen TR-FRET PPAR γ coactivator assay	PPAR γ ligand binding activity	(Mueller, Jungbauer 2009)

Unfermented and Fermented (aq. extract. and MeOH extracts)	Inhibition of substrate binding assay to CYP17 and CYP21 in adrenal microsome	Inhibition of adrenal steroidogenic p 450 enzyme	(Perold 2009)
Fermented and unfermented (EtOH extract)	Two stage skin carcinogenesis mouse models:UVB and DMBA as promotor and initiator, respectively	Anti-carcinogen and phot protective effect	(Petrova 2009)
Fermented (Aq. Infusion)	Single oral dose to healthy humans	Oxidative stress reduction	(Wanjiku 2009)
Fermented (70% aq. MeOH extracts)	Chinese hamster lung fibroblast V79-4 cells	Cytoprotective activity	(Yoo, Hwang & Moon 2009)
Commercial extract blend with Camellia sinensis extract	Topical application in female humans	Anti-wrinkle activity	(Chuarienthong, Lourith & Leelapornpisid 2010)
Fermented (aq. Infusion)	Single oral dose containing sugar with a high fat meal to healthy humans	Inhibition of post-prandial oxidative effect	(Francisco 2010)
Blend of bontical extract containing unfermented rooibos	Topical application to human (male) with androgenic alopecia (male pattern baldness)	Promotion of hair growth	(Glynn 2009)
Fermented (aq, infusion)	Whole blood culture assay : unstimulated, endotoxin stimulated, PHA stimulated	Anti-inflammatory effect	(Hendricks, Pool 2010)

Fermented (aq. extracts and Aspalathin-enriched)	STZ induced hyperglycaemia in wistar rats	Hypoglycaemic activity	(Joubert et al. 2010)
Unfermented (aq. extract)	Diet induced type ii diabetes vervet monkey		
Fermented (DMSO extract)	LPS stimulated macrophages	Anti-inflammatory activity	(Mueller, Jungbauer 2009)
Fermented (aq, infusion)	Single oral dose to healthy humans	Angiotensinogen converting enzyme inhibition	(Persson et al. 2010b)
Fermented (aq, infusion)	Oral administration to human after 2 hours wrestling practice	rehydration	(Utter et al. 2010)
Fermented and unfermented (aq. extract in ready to drink beverage)	Single oral dose to healthy humans	Oxidative stress reduction and lipid profile modulation	(Villaño et al. 2010)
Fermented rooibos extract	Hyperlipidaemic male LDL rat/mice	Anti-hyperlipidemic activity	(Beltrán-Debón et al. 2011b)
Fermented rooibos extract	3T3-L1 mouse adipocytes	Anti-adipogenesis activity	(Beltrán-Debón et al. 2011b)
Fermented (aq. Infusion)	Oral administration to human with cardiovascular risk factors over a 6 week period	Oxidative stress reduction and lipid profile	(Marnewick et al. 2011b)

Fermented and unfermented (aq. extraction)	Male Wistar rats consumed aqueous rooibos and green tea (Camellia sinensis) extracts	cardio protective effect against ischaemia/reperfusion injury	(Pantsi et al. 2011)
Fermented (Aspalathin linears) aq. extract	Wistar rats were injected with 40 mg/kg of STZ.	protects cardiomyocytes from oxidative stress and ischemia induced from diabetic rats	(Dludla et al. 2014b)
Aq: aqueous, DSS: Dextran Sodium sulphate, EtOH: Ethanol, DMSO: Dimethylsulphoxide, PPAR: Peroxisome proliferators –activated receptors, MeOH: methanol, UVB: ultraviolet b, DMBA: 7,12 dimethylbenza(α)anthracence, PHA: phytochemagglutinin, STZ: streptozocin, LPS: lipopolysaccharide, LDL: Low density lipoprotein, FB: Fumonisin B1, DEN: diethylnitrosamine.			

1.3 Rationale and Aims

The international diabetes federation estimated that by 2030 the incidence of diabetes mellitus will increase up to 552 million. Moreover, the changes that occur in the vasculature as an underlying mechanism of obesity has become a high priority in research.

The linked between obesity and aging had contributed to a lot of studies, the deterioration and pathology of organs that occurs with chronic obesity is in many ways similar to that which occurs in normal aging leading some to suggest that chronic obesity accelerates the aging process. Therefore using three models of rat aortas, including the young control rats (control), aged-matched lean rats (lean) and diet-induced obese rats (diet), will allow investigate the effect of aging on aortic function and clarify the differences between them.

Changes in composition of the layer of the endothelial cells and the surrounding layers of the vasculature (i.e. PVAT) have been implicated in the pathophysiology of cardiovascular disease, making it a prominent focus area for research. The presence of PVAT and the secretion of the adipokines is influencing the vascular function, therefore it is important to elucidate the responsiveness of vascular activity in the presence of PVAT, and after the PVAT was removed.

There is a lot of variation regarding the management of obesity and diabetes, including in the use of herbal treatment, including RB. Most of the literature referred to used RB as a supplement in different models including *in vivo* and *ex vivo* models. The lack of literature using RB as a future medicine or treatment through direct infusion suggested that this could be a novel study. Moreover, since the bioavailability of RB is very low, and no significant side effects, the direct administration of RB could provide valuable insight about its effect on vascular function, and possibly even improve obesity-induced endothelial dysfunction.

According to literature RB exerts anti-inflammatory and anti-oxidant effects. Therefore, we aimed to investigate these claims through measuring the inflammatory and oxidative stress markers in aortas when RB was added directly into an organ bath.

We hypothesise that adding RB directly into the organ bath can treat endothelial dysfunction caused by obesity, through its anti-inflammatory and anti-oxidant properties. Furthermore, by investigating the underlying signalling pathways, we may be able elucidate the effect of RB on cellular mechanisms that is involved in the pathophysiology of the disease.

1.3.1 Aims of the study

In view of the above, the aims of this study were to explore adding RB directly into an aortic-organ bath. Therefore, the following aspects were investigated:

The first aim: To investigate the effect of diet and age on aortic responsiveness in aged-matched lean rats (lean) and diet-induced obese rats (diet) compared to young control rats (control).

The second aim: to investigate the effect of RB after 30 minutes of adding it into the organ bath on vascular function (contraction-relaxation) of aortas (with or without PVAT) in a lean and high fat diet rat model.

The third aim: To investigate the effect of RB on the underlying cellular signalling mechanisms using Western blot (WB) analysis, by determining the phosphorylation and expression status the following proteins:

- eNOS and upstream activators: PKB/Akt and AMPK.
- MAPK which include: p38 MAPK, ERK and JNK.
- Cyclic AMP response element binding protein (CREB).
- Oxidative stress markers: NITROTYROSINE.

The fourth aim: To investigate whether rooibos has an effect on inflammation caused by obesity by measuring:

Pro-inflammatory markers: IL-1 α , IL-1B, IL-12, IFN- γ , TNF- α . IL-6,

Anti-inflammatory markers: IL-2, IL-4, IL5, IL-10, IL-13.

2. MATERIALS AND METHODS

2.1 Animals

One of the principles of animal ethics, which is upheld at the University of Stellenbosch, is to reduce the amount of animals in research, and in order to meet this principle we used tissue from animals that have been used by researchers in other studies. These studies complied to the Revised South African National Standard for the Care and Use of Animals for Scientific Purposes (South African Bureau of Standards, SANS 10386, 2008), and was approved by the Research Ethics Committee for Animal Care and Use, Stellenbosch University (protocol numbers: SU-ACUM12-00040, SU-ACUD14-00020). Rats were housed in an animal care facility and had *ad libitum* access to food and water.

A total of 158 animals were used in different groups to investigate the aims of our study, the protocol is summarized in figure 2.1.

In order to investigate the effect of rooibos tea on the vasculature, we added RB to the organ bath, measuring the function of the aorta (contraction and relaxation) by recording the aortic ring tension with an isometric force transducer in a tissue-organ bath system. We further assessed the influence of the tea and obesity on the vasculature by analysing the underlying signalling mechanism, using Western blots. Inflammatory bio-markers were also analysed using the Luminex Multiplex immune assay.

Aortas were obtained from three groups of adult male Wistar rats, after euthanasia by lethal intraperitoneal injection of sodium pentobarbital (160 mg/kg). Young rats (CONTROL) were fed with normal diet before termination at an age of 16 weeks. The fat or diet-induced obese group (DIET) was fed normal chow for 6 weeks (170-180g) and then started on the high fat diet for 16 weeks, reaching the age of 22 weeks at termination. The third group was the aged matched lean group (LEAN) which was on normal diet for 22 weeks. Control animals were fed normal rat chow while the high fat diet (HFD) was prepared as follows: normal rat chow (2.4Kg) sugar (520 g) condensed milk (full cream) 8 cans; Holsum cooking fat (550g); water (2L). The composition of the control rat chow and the HFD is summarized in table 2.1. Chemical analysis of the composition of the diets was performed by MicroChem specialized Laboratory Services, Cape Town.

Table 2.1 Composition of control and high fat diets (Salie, R et al, 2014).

Diet composition	Fat (g/100g)	Cholesterol (mg/100g)	% protein	% CHO	Sugar (g/100)	KJ/100g
control	4.8	3	17.1	34.6%	6.6	1272
Diet+Holsum	11.5	13	8.3	42	24.4	1354

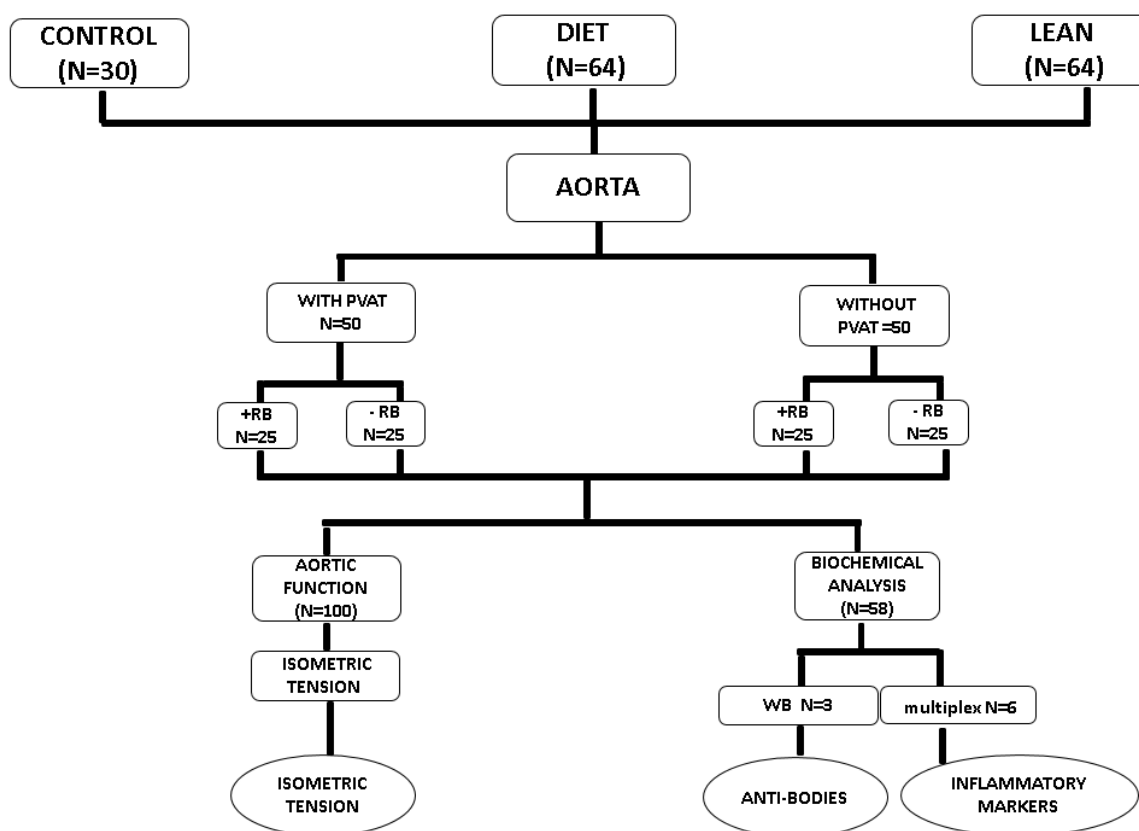


Figure 2.1: Summary of the protocol used in this experimental study. Two types of analysis were done, the aortic ring function in which we measured the vascular reactivity (contraction and relaxation) and biochemical analysis to investigate the underlying cellular mechanism, using western blot analysis and the inflammatory biomarkers using multiplex kit analysis.

2.2 Material

Acetylcholine and phenylephrine were purchased from Sigma-Aldrich (St Louis, Mo, USA), all other chemicals and buffer reagents were purchased from Merck (Darmstadt, Germany).

RB extract (2%) was prepared using 100ml boiled double distilled water and 2 grams of leaves and stems of fermented RB plants, RB was allowed to steep for 30 minutes. By using this protocol for preparation of tea, we mimicked the way in which RB is brewed at home, and subsequently filtered very carefully. Finally, 250µl Rooibos tea was administered to the organ bath of the aortic tension system containing 25 ml Krebs Henseleit buffer (KHB), resulting in a 100 times dilution (0.02% Rooibos). The RB extract was analysed with high performance liquid chromatography, HPLC (Addendum Figure 5.1).

2.3 Aortic function determination

The isometric tension system is used to monitor the aortic activity and assess the endothelial function (Fig. 2.2). It includes an organ bath (AD Instruments, Bella Vista, New South Wales, Australia) for insertion of the aorta, force transducer for activity measurement, a Power Lab 4/35 data acquisition system, a bridge amp and Lab Chart Pro 7 data capturing software. A stainless steel stationary hook was fashioned along with a stainless steel hook that was attached to a silk suture and in turn attached to the isometric force transducer (TRI202PAD, Pan lab, Cornella, BCN, Spain) to record the aortic activity (contraction and relaxation).

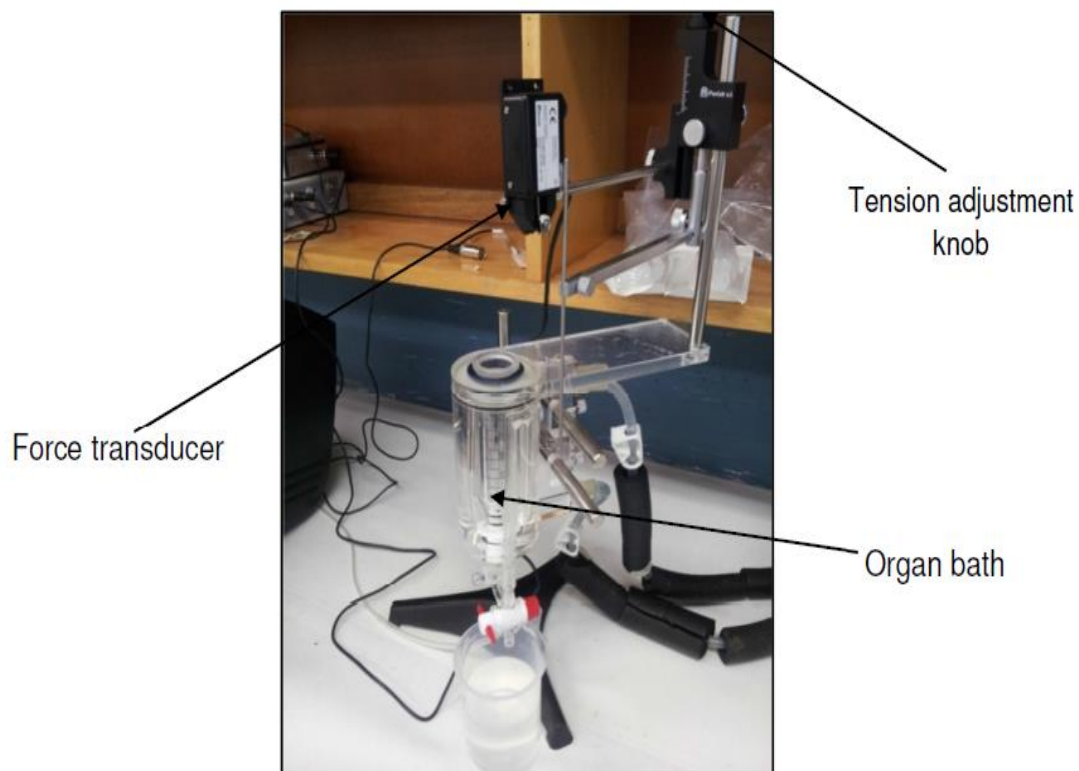


Figure 2.2: The tissue-organ bath with the force transducer (copied from Loubser 2014).

Before starting the experiment, the Power Lab, computer, and the organ bath was switched on. The organ bath was rinsed three times with distilled water before commencing the experiment and then filled with oxygenated (KH) buffer. Time was allowed to ensure that the buffer in the organ bath reach optimal temperature. Krebs-Henseleit bicarbonate buffer (KHB) at 37.5 °C , pH 7.4 contained (in mM); NaCl 119, NaHCO₃ 25, KCl 4.75, KH₂ PO₄ 1.2, MgSO₄ 0.6, NaSO₄ 0.6, CaCl₂ 1.25 and glucose 10, and was oxygenated with 95% O₂ and 5% CO₂ . Additional KH buffer was pre-warmed for buffer exchange every 10 minutes.

The system was calibrated in Lab Chart 7.0 by setting the tension of the system between 0g and 2g by suspending paper clips of 2 g on the free hanging steel hooks.

2.3.1 Drugs for aortic function experiments

A concentrated stock of the drugs, used during the aortic function experiments, were prepared fresh before the experiments. Phenylephrine (PE, light sensitive), is an α -adrenergic receptor agonist that causes contraction via smooth muscle cells, while Acetylcholine (Ach) cause relaxation and NO release through binding to endothelial cell receptors.

Preparation of the PE and Ach stocks:

1 mM (PE stock) = 0.002 g PE in 10 ml 0.9% saline

10 mM (Ach stock 1) = 0.0182 g of Ach in 10 ml 0.9% saline

1 mM (Ach stock 2) = dilute 1 ml of Ach stock 1 in 9 ml 0.9% saline

0.1 mM (Ach stock 3) = dilute 1 ml of Ach stock 2 in 9 ml of 0.9% saline

The final concentration of the drugs were as follow:

Cumulative concentrations of PE were obtained by adding different volumes of PE stock (1mM) to 25 ml KHB in the organ bath:

- 100 nM PE = 2.5 μ L of PE stock (1mM)
- 300 nM PE = 5 μ L of PE stock into 25ml KHB containing the 100nM PE
- 500 nM PE = 5 μ l of PE stock into 25ml KHB containing the 300nM PE
- 800 nM PE = 7.5 μ l of PE stock into 25ml KHB containing the 500nM PE
- 1 μ M PE = 5 μ l of PE stock into 25ml KHB containing the 800nM PE

Cumulative concentrations of Ach were obtained by adding different volumes of Ach stocks to 25 ml KHB in the organ bath:

30 nM Ach = 7.5 μ L of Ach stock 3

100 nM Ach = 17.5 μ L of Ach stock 3

300 nM Ach = 42.5 μ L of Ach stock 3

1 μ M Ach = 14.3 μ L of Ach stock 2

10 μ M Ach = 220 μ L of Ach stock 2

2.3.2 Excision and mounting of the aorta

After intraperitoneal injection of the rat with phenobarbital 160mg/kg, and once the rat stopped reacting to the pedal pinch test, body weight and fasting blood glucose level was measured, an incision was made through skin and muscle layers across the ventral side of the rat, just below the thoracic region. The diaphragm was cut and then the ribcage cut in a cranial direction as to expose the thoracic cavity. Heart, lungs, trachea and oesophagus were removed. The thoracic aorta (above the diaphragm to distal end of the aortic arch) was excised and immediately placed in ice cold buffer to arrest metabolic activity. The intraperitoneal fat (IP) was removed and weighed. Aortas were quickly cleaned from any clotted blood and then the surrounding PVAT was either removed (without PVAT) or the PVAT was not cleaned from the aorta (with PVAT), as illustrated in figure 2.3. The aorta was cut into a 3-4 mm ring segment that was subsequently mounted onto two stainless steel hooks and placed into an organ bath containing 25 ml KHB.



Figure 2.3 Two pieces of aorta with and without PVAT (Loubser 2014)

2.3.3 Cumulative aortic contraction and relaxation

The isometric tension measurement protocol (representative graph in Figure 2.4) was based on a modification of a previously described technique (Privett et al. 2004) and standardized in our laboratory (Loubser 2014). Aortic tension was stabilized for 30 minutes by increasing the resting tension gradually till 1.5g. Once the tension has been stabilized, testing the activity was done with a single dose of 100 nM phenylephrine (PE) for contraction followed by a single dose of 10 μ M (ACH) for relaxation. This test was performed to make sure that the smooth muscle is still intact and the endothelium was not damaged. The buffer was changed every 10 minutes for 30 minutes, after

which the tension was stabilized again to 1.5 g for 30 minutes. Rooibos tea (0.02%) was added and remained in the organ bath for 30 minutes without adjusting with the tension or changing KH buffer. Cumulative concentrations of PE 100 nM, 300 nM, 500 nM, 800 nM and 1 μ M was added followed by cumulative concentrations of ACH 30 nM, 100 nM, 300 nM, 1 μ M and 10 μ M. The data was analysed using Lab Chart 7.0.

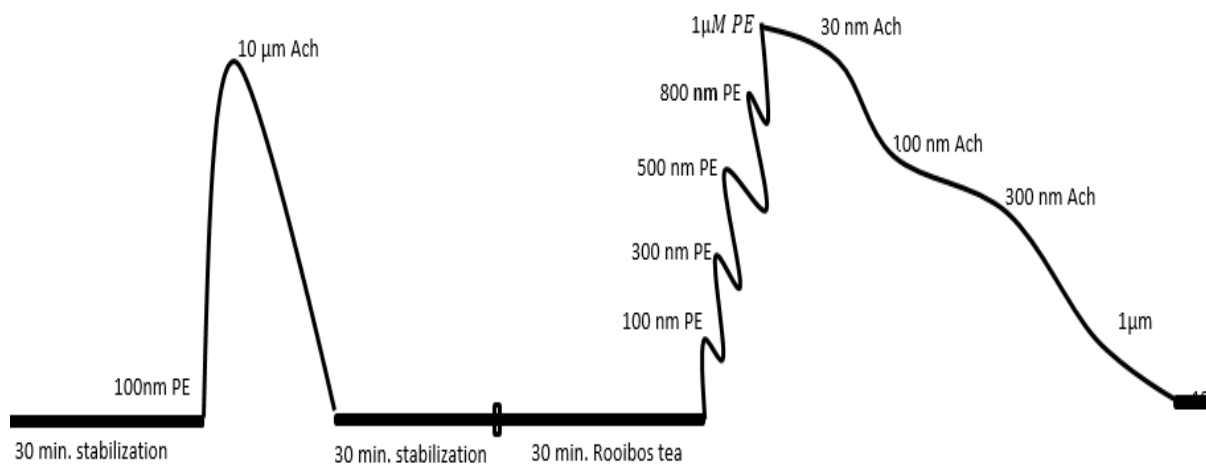


Figure 2.4: Representation of the protocol used in aortic ring function study.

We used aortas from the lean control young rats to confirm that the protocol used above had achieved optimal contraction (when contraction response to final PE concentration of 1 μ M plateaued) followed by a relaxation of more than 60 %. In Figure 2.5 the contraction and relaxation response in the young control groups indicated that our system was producing valid results.

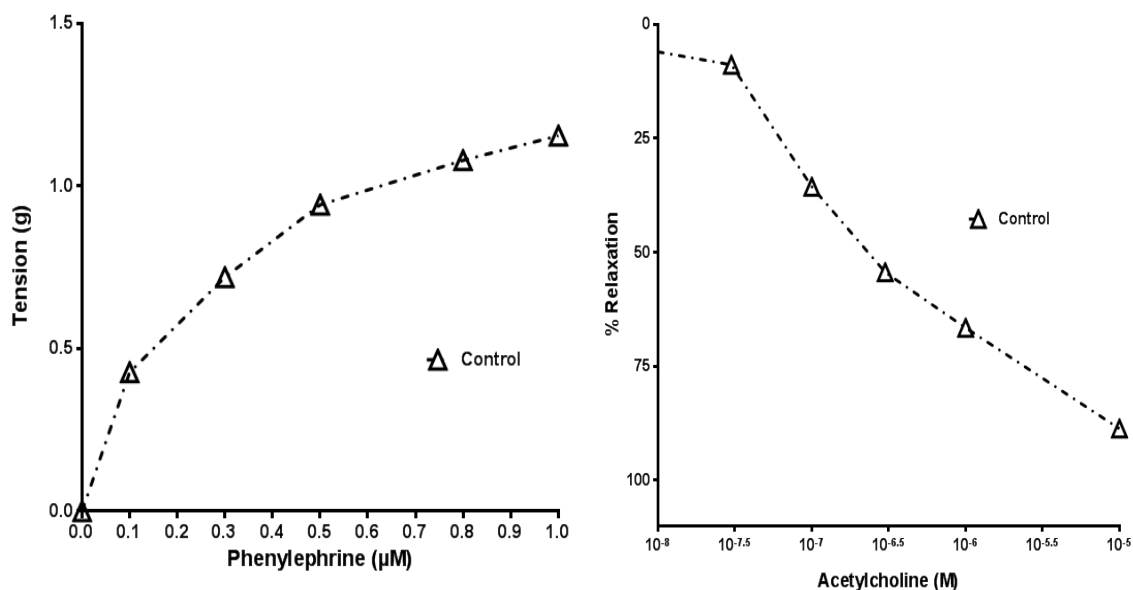


Figure 2.5: Representation of contraction and relaxation dose response curves in the control group

2.4 Biochemical analysis of the aorta

The second aim of this project was to determine the mechanism by which RB influences aortic signalling pathways. Aortic tissue was collected from three different groups: control, lean group (aged matched), and diet (obese) rats. Each group was subdivided into two groups: (i) the first group consisted of 50 aortas without PVAT, which was further divided into a group where RB was added (0.02% final concentration) to the aortas, while the other group was without RB. (ii) The second group, consisted of 50 aortas with PVAT, also divided into two groups: one with RB and one without RB, as demonstrated in figure 2.1.

The excised aortic tissue with or without PVAT (as described above) was attached to the hook and submerged into oxygenated KH buffer at 37⁰ C in an organ bath. In one group the buffer was changed every 10 minutes for a maximum duration of 60 minutes without adding RB, while in the other group the buffer was changed for the first 30 minutes and stabilizing it for another 30 minutes when rooibos tea was added (Figure 2.6). Liquid nitrogen was used to freeze the sample at the end of the experiment and the samples were stored in -80⁰ C for western blot and multiplex analysis.

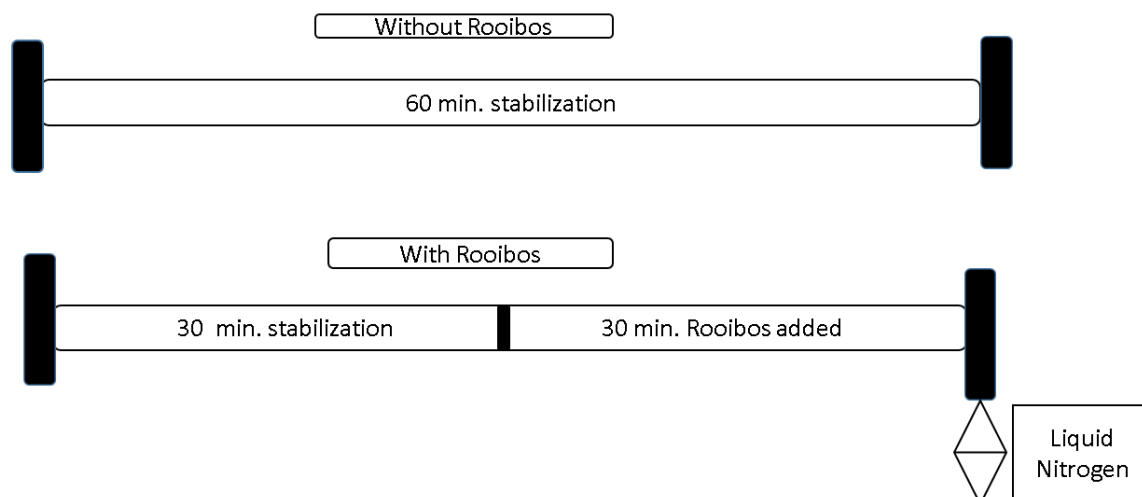


Figure 2.6: Protocol used for preparation the samples to perform biochemical analysis.

2.4.1 Western blot analysis

WB analysis was done on aortic tissue after proteins were extracted with a lysis buffer. This technique allows for the detection of proteins involved in underlying signalling pathways. Specific primary antibodies were used to detect the total as well as phosphorylated status of 9 different proteins including; eNOS, PKB/AKT, ERK p44/p42 MAP Kinase (p38 MAP kinase AMPK/ JNK / CREB), Nitrotyrosine and P22PHOX. A secondary antibody (horseradish peroxidase-conjugated anti-rabbit immunoglobulin G) was used to bind to the primary antibodies to enhance the detection signal. All antibodies were obtained from Cell signalling Technology.

2.4.2 Protein extraction

Lysis buffer was made for the aortic tissue from the following:

Table 2. 2: The composition of the lysis buffer for aortic tissue.

Reagent	Stock	FINAL Content	Amount for 10ml
Triton X	10%	1%	1 ml
Tris-Hydro chloride with EGTA (pH 7.5)	200 mM	20.0 mM	1 ml
Ethylenediaminetetraacetic acid (EDTA)	100 mM	1 mM	100µl
Sodium chloride (NaCl)	1 M	150 mM	1.5 ml
Beta-glycerophosphate	-	1 mM	0.002g
Tetra-sodium-pyrophosphate	-	2.5 mM	0.01g
NaF	-	50nM	0.0213g
Na ₃ VO ₄ [0.018g/10ml]	10 mM	1.0 mM	1 ml
Leupeptin	10 µg/µl	10 µg/µl	10 µl
Aprotinin	10 µg/µl	10 µg/µl	10 µl
Sodium dodecyl sulphate (SDS)	10 %	0.1 %	10 µl
PEnylmethylsulfony fluoride (PMSF)	100 mM	50 µg/ml	30 µl
Distilled water			Fill up to 10ml

The frozen aortic tissue (30-50 mg) was pulverized with mortar and pestle which were pre-cooled in liquid nitrogen, and placed in Eppendorf tubes filled with 600 μ l from the above lysis buffer. About 7-10 stainless steel beads (1.6 mm) were added to the Eppendorf's, then homogenised at 4 ° C in the bullet blender at speed 8 for three times one minute periods, and with five minute intervals in between. Samples were kept on ice for 15 minutes and then centrifuged for 20 minutes at 4 ° C at 15000 rpm (12074 x g). The centrifuged supernatant was transferred into new Eppendorf tube and placed on ice to be used for Bradford determination before WB and Multiplex analysis.

2.4.3 Bradford protein analysis

The protein concentration in the sample supernatant was determined using the Bradford technique (Bradford, 1976). 20 μ l of the sample was added to 80 μ l of distilled water to prepare the first set of sample dilution (5x), while the second set was prepared in duplicate using 20 μ l of the first set of sample dilution adding 80 μ l of distilled water. Standards were made from 100 μ l bovine serum albumin (BSA) with a known concentration of 5 mg/ml that was diluted in 400 μ l of distilled water and pipette into tubes for the standard curve as, indicated in Table 2.3.

Table 2. 3: The content of the BSA standards for the Bradford protein analysis

BSA concentration (ug/ml)	BSA volume (μ l)	Distilled H ₂ O (μ l)	Bradford reagent (μ l)
0	0 (blank)	100	900
1.25	5	95	900
2.5	10	90	900
5	20	80	900
10	40	60	900
15	60	40	900
20	80	20	900

Two pieces of Wathman filter papers (pore size 0.4 μ m) were used to filter the diluted (5x) Bradford reagent. From the latter 900 μ l was added to the Standards as well as to the second set of sample dilutions. The tubes were vortex and left standing for 15

minutes using the spectrophotometer. The absorbance was measured at 595 nm and was carried out using a 1 ml cuvette, reading the prepared dilution against the blank.

The protein concentration that the samples yielded was 2-4µg/µl for the aorta without PVAT, and 6-13µg/µl for the aorta with PVAT. These were then diluted with lysis buffer and Laemmli sample buffer (content: 62.5 mM Tris-HCl (pH 6.8), 4% SDS, 10% Glycerol, 0.03% BromoPEnol Blue and 5% β-mercaptoethanol) was in a 1:2 ratio. This yielded 15 µg/ 15 µl for the aorta without PVAT and 40µg/ 10µl for the aorta with PVAT (to be loaded into the gel). Samples boiled for 5 minutes and stored in -80 ° C freezer overnight.

2.4.4 Protein Gel separation and transfer to membrane

Samples were pre-boiled again for 4 minutes, before loading them into the wells of a 4% stack (Table 2.5). This stack was on top of a 7.5% or 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel that was prepared according to Bio –RAD mini gel system (Table 2.4). We added 2, 2, 2 Trichloroethanol which is a new method to enable visualization of the proteins in the gel. This system is based on UV modification by trichloro compounds of the naturally occurring tryptophan remnants within the proteins. After separation of the proteins in the gel they can be visualized in a Chemi-Doc MP imager (Bio-Rad) system without staining the gel.

Table 2.4: The composition of the 7.5% or 10% SDS gels

Reagent	Stock	7.5%	10%
H₂O (MILLIPORE)		5.475ml	4.85ml
2, 2, 2 Trichloroethanol	99%	50 µL	50 µL
Tris –HCl (pH8.8)	1.5 M	2.5 ml	2.5ml
Sodium dodecyl sulphate (SDS)	10%	100 µL	100 µl
Acrylamide	40%	1.875 ml	2.5 ml
Ammonium persulfate (APS)	10%	50 µL	50 µL
Tetramethylethylenediamine (TEMED)	99%	20 µL	20 µL

After the gel had been set for half an hour the stack was added, which consisted of the following;

Table 2.5: The composition of 4% stack

<u>Reagent</u>	<u>Stock</u>	<u>4%</u>
H₂O (MILLIPORE)		3.05 ml
Tris –HCl (pH6.8)	0.5 M	1.25 ml
Sodium dodecyl sulphate (SDS)	10%	50 µL
Acrylamides	40%	500 µL
Ammonium persulfate(APS)	10%	50 µL
Tetramethylethyldiamine (TEMED)	99%	10 µL

The loading of the samples was done using the Hamilton syringe (Purchased from Sigma–Aldrich, St. Louis .MO). The 15-well gel was loaded as follows: lane 1: 5 µl of the marker (Page Ruler pre stained proteins ladder obtained from Thermo Scientific); lanes 2-4 LEAN samples, lanes 5-7: LEAN with RB samples, lanes 8-10: DIET samples, lanes 11-13: DIET with RB samples, lanes 14-15: normal young CONTROL samples.

Then the polyacrylamide gel was placed in a tank filled with running buffer composed of 50 mM Tris, 384 mM glycine and 1% SDS. The polyacrylamide gel was subjected to sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE) for 10 minutes at 100 V, and 200 mA followed by 50 minutes at 200V and 200 mA. Then the separated proteins were activated using the Chemi-Doc MP imager (Bio-Rad) system.

The transfer of separated proteins on the gel to immobilon membrane (Millipore, Billerica, MA, USA; polyvinylidene difluoride,PVDF) was done using the Trans blot Turbo transfer system for 10 minutes at mixed molecular weight setting. Then the proteins on the membrane were visualized in the Chemi-Doc system to confirm that all proteins were transferred to the membrane, and to determine equal loading. The non-specific sites on the membrane was blocked for 1-2 hours at room temperature

by using 5% long life fat free milk diluted in TBST (Tris buffer saline and 0.1 % Tween-20). Washing the membrane with TBST (3 x 5 minutes) for 15 minutes is mandatory before adding the primary antibody. 5 µL of primary antibodies were added to 5 ml TBST incubated for overnight at 4⁰ C.

After incubation, membranes were washed with TBST for 15 minutes before adding the 1.25 µL horseradish peroxidase- labelled secondary anti-body (Amersham life science, Buckinghamshire, UK) in 5 ml TBST, except for the eNOS membranes where Signal Boost™ instead of TBST was added. The membrane was washed for the last time before adding the enhanced chemiluminescence (ECL) detection agent for one minute, then exposed to the Chemi-Doc system and analysed with Lab Image 5.

2.5 Luminex Multi- plex immune assay

The last aim of this project was to determine the inflammatory markers in aorta samples, using the Luminex multiplex system. It was done with the assistance of Dr N. Chegou (from Stellenbosch University Immunology Research Group, Division of Molecular Biology and Human Genetic). The concentrations of 9 cytokines namely: Interleukin; IL-1 α, IL 1β, IL-2, IL-4, IL-6, IL-10, Tumour necrosis factor (TNF-α), Interferon gamma (IFN-γ), and granulocyte macrophage colony stimulating factor (GM-CSF) were investigated in all samples using Bio-plexPro™ magnetic bead-based assays (obtained from Bio –Rad laboratories, Hercules, USA). The preparation of the samples was the same way that we prepared the WB.

The experiment was performed strictly according to the manufacturer's instructions as specified in the package insert. The technique uses magnetic beads which are coated with capture anti - bodies that are specific to the cytokines of interest. After addition of samples, a cocktail of detection anti-bodies and a reporter dye was added as shown in figure 2.7. A bead-based sandwich immune assay is created around each bead, which is similar to immune ELISA assay, the experiment at the result is read using a flow cytometry based analyser which then distinguishes between the different beads. The samples that was analysed by the kit was calculated in pg/ml, and due to the fact that we used aortic tissue we converted the units from pg/ml to pg/mg. Therefore 50 µl of the lysate in duplicate in n=6 was done, 6-12mg/ml per aorta with PVAT, and 2-4 mg/ml of aorta without PVAT.

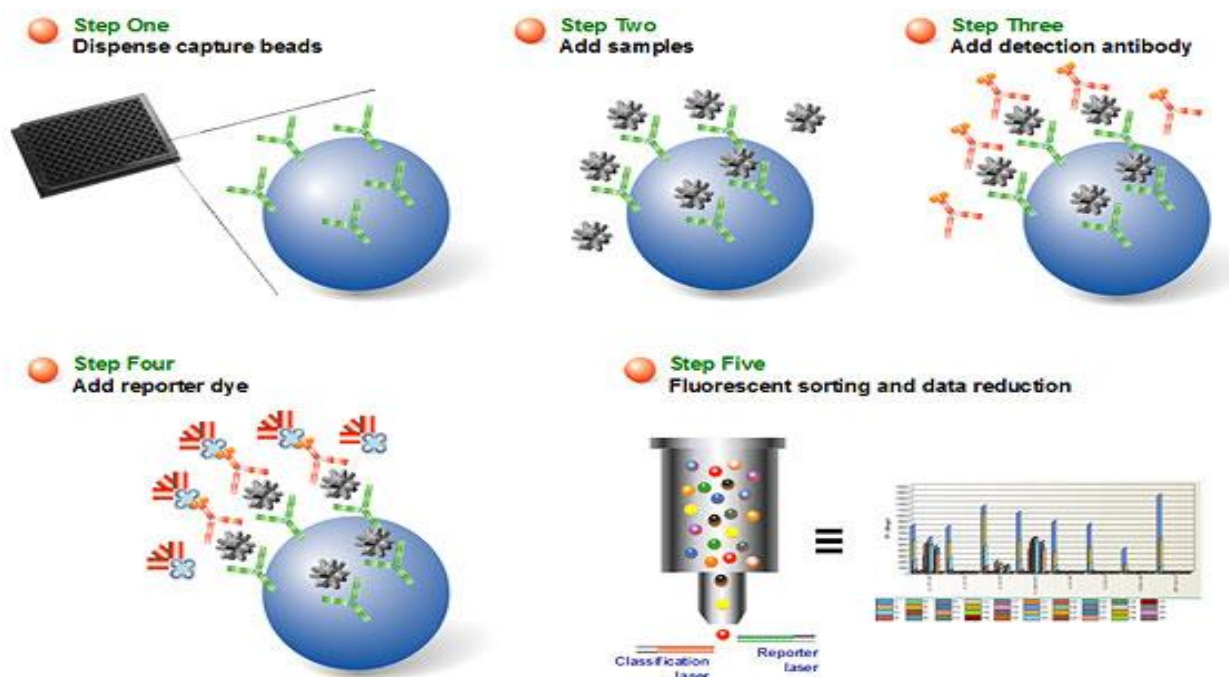


Figure 2. 7: Schematic representation of an immunoassay sandwich-based assay (image from [workflow. www.Bio-Rad.com](http://workflow.www.Bio-Rad.com))

Finally the assay was read on a Bio -Plex 200 machine (Bio - Rad Laboratories). The Bio-plex Manager Software version 6.1 was used for bead acquisition and analysis of median florescent intensity.

2.6 Calculation of data and Statistical analysis

Normalizing the Western blots with the control was done using the Image Lab 5 to correct for unequal loading.

The aortic ring function data was analysed as previously described (Razali et al., 2013). Briefly, concentration-response curves to PE was expressed in grams. The response was calculated as the difference between the absolute tension and the baseline tension. The relaxation with Ach was calculated as a % of the final pre-contraction response to PE (at 1 μ M).

Data was statistically analysed by Graph Pad Prism 6 and values were expressed as mean \pm standard error of the mean (SEM). One way and two way analysis of variance (ANOVA) followed by Bonferroni post-hoc test was done to determine the differences between different groups. Differences with a p-value < 0.05 were considered statistically significant.

The analysis that applied was agreed upon by Biostatistician from the department of biostatistics at University of Stellenbosch, and longitudinal analysis was also done to see the most effective intervention.

3. RESULTS

3.1 Biometric data:

Herewith we determined the Body weight and IP fat weight. The fasting blood glucose level, insulin and homeostasis model assessments (HOMA) IR index was determined by another MSc student.

3.1.1 Body weight:

There was a significant difference in the body weight between the diet group vs the lean group, as the diet group showed an increase in body weight after the feeding period (lean : 394.1 ± 12.29 g vs diet : 443.1 ± 17.08 g, $p < 0.05$).

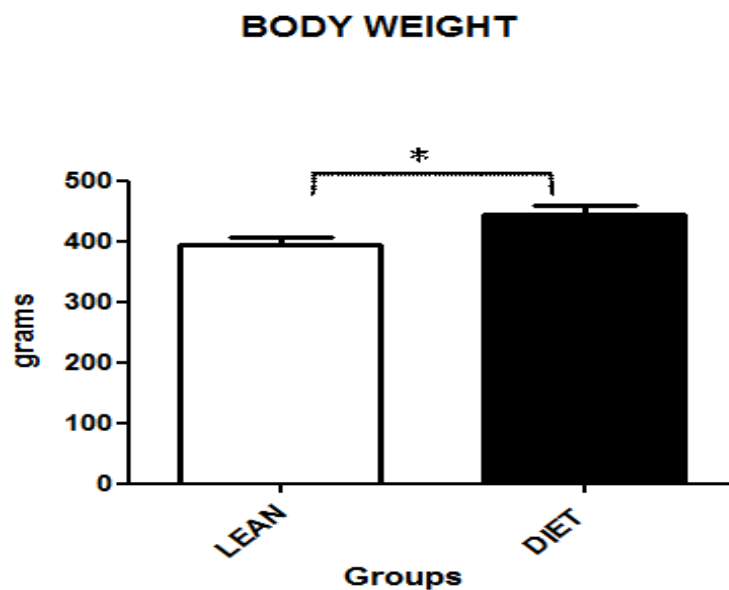


Figure 3. 1.1. The body weight of the diet group vs the lean group. * $P < 0.05$; $n = 17$ per group.

3.1.2 IP fat weight:

There was a significant difference between the lean group vs the diet group , as the diet group showed an increase in the IP fat when compared to the lean group after the feeding period (lean: 19.04 ± 2.64 g vs diet: 36.93 ± 4.71 g, $p < 0.01$).

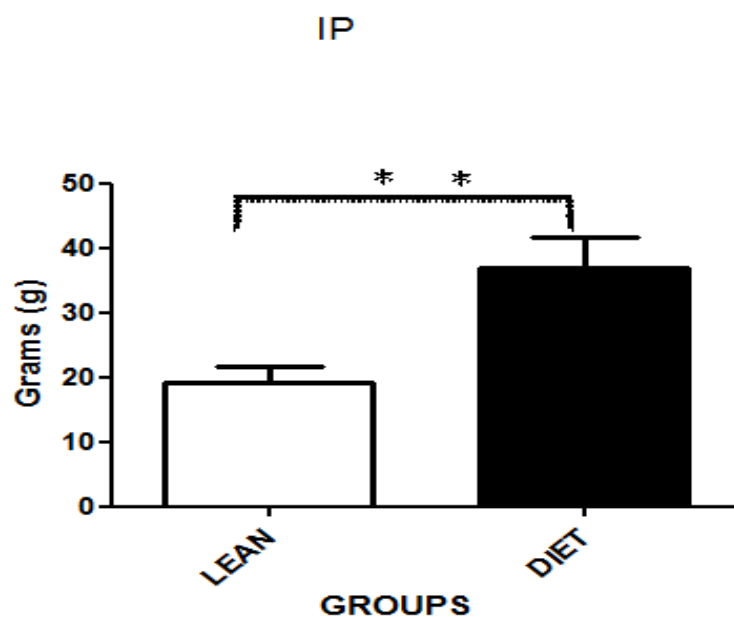


Figure 3.1.2. The IP fat weight between diet group vs lean group. **P < 0.01; n = 17 per group

3.1.3 Insulin level, fasting blood glucose level and HOMA index

The same group of animals used in our experiments were also used by another MSc student. She determined insulin and fasting blood glucose level using appropriate kits and calculated the HOMA index of both lean and diet animals (Addendum Table 5.1).

There was a significant increase in fasting blood glucose levels in diet group when compared to the lean group. Moreover the insulin level was elevated in the diet group when compared to the lean group.

Homeostasis model assessment of insulin resistance is calculated as follows: HOMA IR index = basal blood glucose level (mmol/L) x Fasting insulin level (mIU/ml) / 22.5.

The HOMA IR index was higher in diet group when compared to the lean group.

3.2 Aortic ring function

PE induced concentration-dependent contractions and Ach induced relaxation of aortic rings, with or without PVAT, in young control rats (control), aged-matched lean rats (lean) and diet-induced obese rats (diet) were studied. Furthermore the effect of RB on these contraction and relaxation responses of aortas in the different groups were also determined.

3.2.1 The effect of the diet and lean on aortic ring contraction with and without PVAT

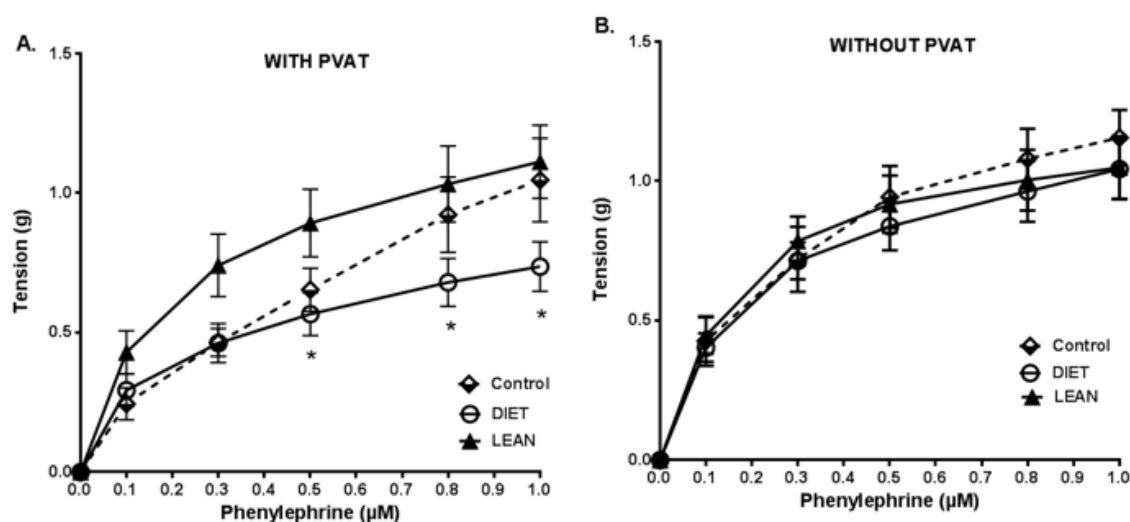


Figure 3.2.1 Diet and lean effect on PE-induced contraction. (A) The effect of the diet in aortic ring contraction induced by PE in aortas with PVAT (* $p < 0.05$, the diet vs lean at concentrations: 500nM, 800nM, 1µM PE; 2-way ANOVA). (B) The effect of the diet in aortic ring contraction induced by PE in aortas without PVAT. (n = 6-11).

In figure 3.2.1 (A) a significant increase in the PE induced aortic contraction in lean group with PVAT was observed compared to control group ($p < 0.05$, 2-way ANOVA). However aortas with PVAT of the diet group contracted significantly less than the lean group in response to PE. This was noticed in the decreased contraction in the diet group compared to the lean at final PE concentration (1µM PE: 0.73 ± 0.09 g vs 1.11 ± 0.13 g, respectively, $p < 0.05$). The aortas from the diet group was also less

responsive than the lean group at the lower PE concentrations: 500 nM (diet with PVAT 0.57 ± 0.08 g vs lean with PVAT 0.89 ± 0.12 g, $p < 0.05$) and 800 nM (0.68 ± 0.09 g vs 0.13 ± 0.13 g, $p < 0.05$).

However in figure 3.2.1 (B) when the PVAT was removed this significant difference disappeared.

3.2.2 The effect of the diet and lean on aortic ring relaxation in aorta with and without PVAT:

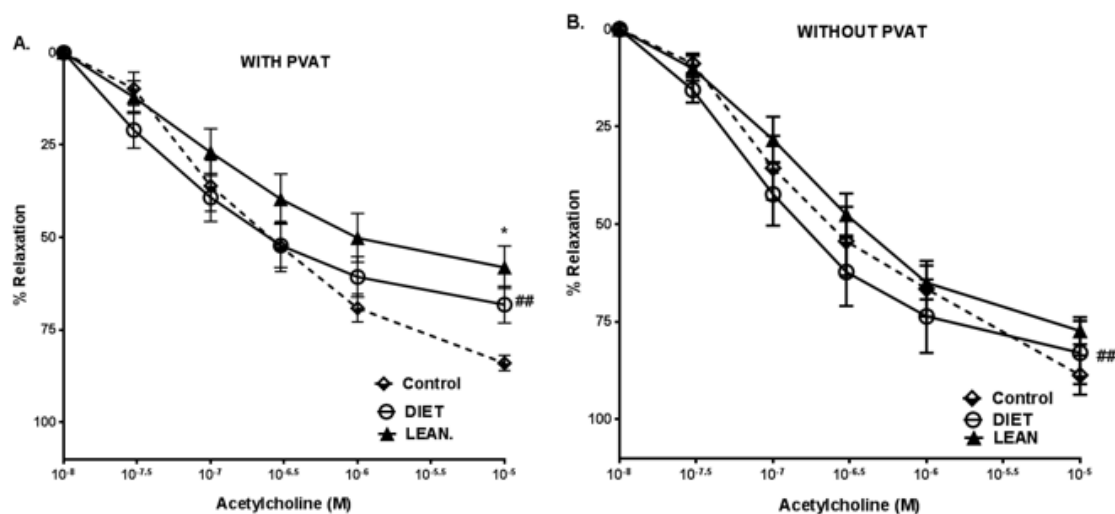


Figure 3.2.2 Diet and lean effect on ACH induced relaxation. (A) The effect of the diet in aortic ring relaxation induced by ACH in aorta with PVAT. (* $P < 0.05$ the control vs lean at $10 \mu\text{M}$, ## $P < 0.01$ between the diet vs lean). (B) The effect of the diet and lean on ACH induced relaxation in aorta without PVAT (## $P < 0.01$ diet vs lean). (2-way ANOVA, $n = 6-11$).

In figure 3.2.2 **A**, the ACH induced relaxation in aortas with PVAT the diet group relaxed significantly more compared to the lean group ($P < 0.05$). In figure 3.2.2 **B**, when the PVAT was removed it did not change this effect, the diet group still relaxed more than the lean group.

Furthermore, in figure 3.2.2 **A**, a significant decrease in the relaxation of lean group in aorta with PVAT compared to the control group ($p < 0.05$, 2-way ANOVA) and this is also notice at final Ach concentration, $10 \mu\text{M}$ (control: 83.69 ± 2.05 % vs lean: $58.10 \pm$

5.75 %, $p < 0.01$). On the other hand, in figure 3.2.2 **B**, when the PVAT was removed this effect disappeared.

3.2.3 The effect of PVAT on aortic ring contraction in the diet and lean groups:

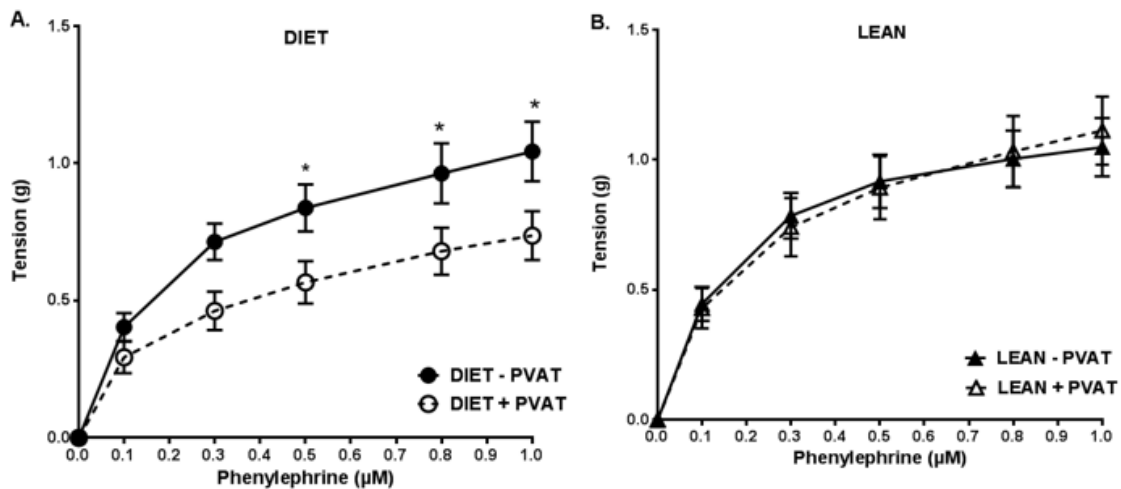


Figure 3.2.3 The effect of PVAT on PE induced contraction in the diet and lean group. (A) The effect of the PVAT on PE induced aortic contraction in the diet group aorta with and without PVAT (* $p < 0.05$, the diet with PVAT vs diet without PVAT at specific concentrations: 500nM, 800nM, 1µM PE; 2-way ANOVA). (B) The effect of PVAT on the lean group with and without PVAT. (n = 10 - 11).

In figure 3.2.3 A, the diet group with the PVAT contracted less significantly than the diet group with PVAT in response to PE. This was noticed in the decreased contraction in the diet group with PVAT compared to the diet group without PVAT at final PE concentration (1µM PE: 0.73 ± 0.09 g vs 1.04 ± 0.10 g, respectively, $p < 0.05$). The aortas from the diet group with PVAT was also less responsive than the aorta of the diet group without PVAT at the lower PE concentrations: 500 nM (diet with PVAT 0.57 ± 0.08 g vs diet without PVAT 0.83 ± 0.08 g, $p < 0.05$), 800 nM (0.68 ± 0.09 g vs 0.69 ± 0.10 g, $p < 0.05$).

3.2.4 The effect of PVAT on aortic ring relaxation in the diet and lean groups:

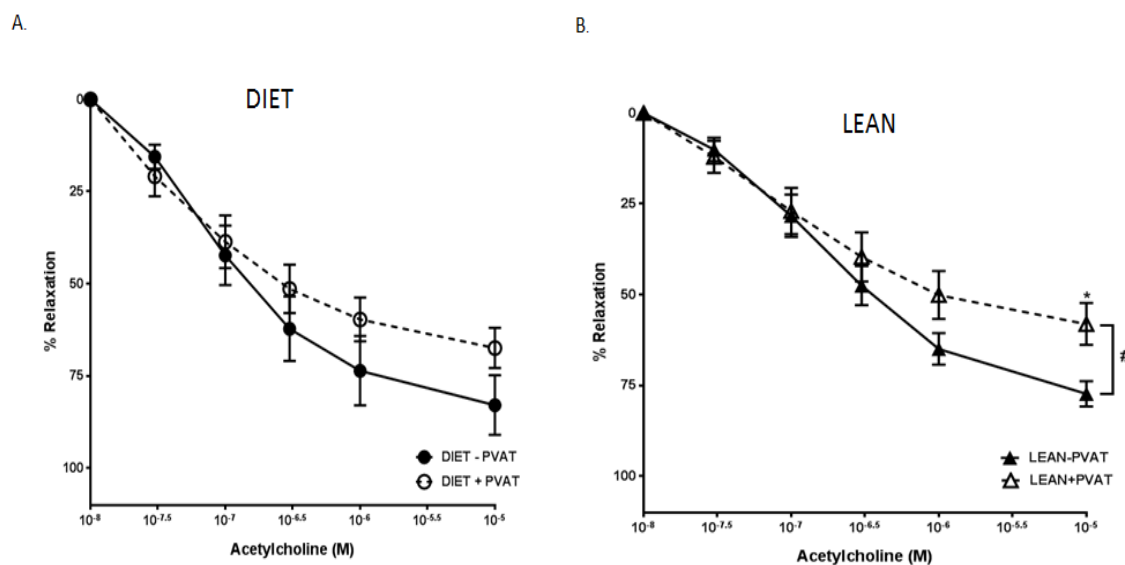


Figure 3.2.4 The effect of PVAT on aortic ring relaxation in the diet and lean group. (A) The effect of the PVAT in the DIET group in aortic ring relaxation induced by ACh in aorta with and without PVAT. (B) The effect of PVAT on aortic ring relaxation in the LEAN group with and without PVAT (# $P < 0.05$ lean without PVAT and lean with PVAT, * $P < 0.05$ at $10 \mu\text{M}$). (2-way ANOVA, $n = 10 - 11$).

In figure 3.2.4 A. When we compared the effect of PVAT on aortic ring relaxation there were no significant differences in diet group with and without PVAT. **In figure 3.2.4 B.** With regards to the lean group a significant decrease in the relaxation of the lean group with PVAT compared to lean group without PVAT at specific cumulative ACh relaxation at $10 \mu\text{M}$ (lean without PVAT $77.3 \pm 3.5 \%$ vs. lean with PVAT $58.1 \pm 5.7 \%$, $p < 0.05$).

3.2.5 The effect of the RB in Lean and Diet groups on aortas with or without PVAT:

3.2.5.1 Contraction of the diet group:

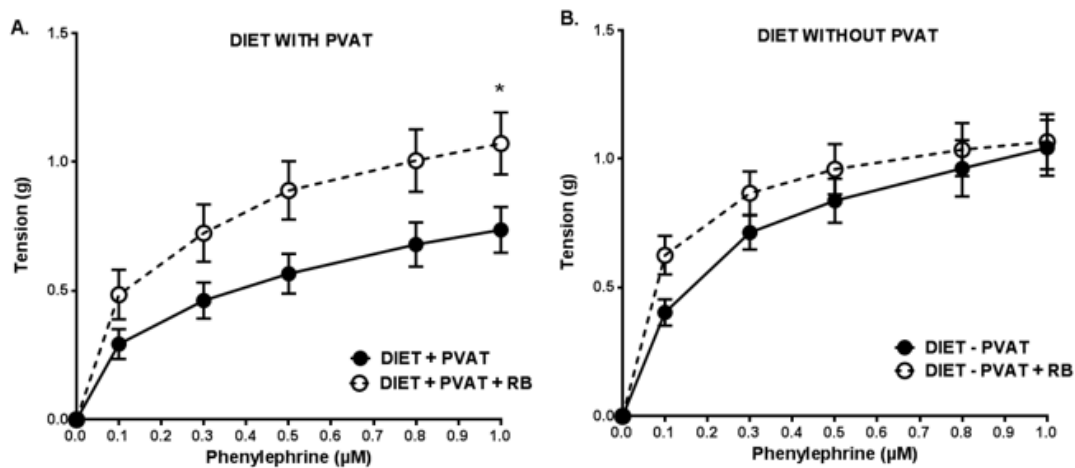


Figure 3.2.5.1 The effect of RB on aortic ring contraction in diet group. (A) The effect of RB on aortic ring contraction in diet group with PVAT (* $p < 0.05$, the diet with PVAT vs diet with PVAT+ RB at 1μM PE; 2-way ANOVA). (B) The effect of RB on aortic ring contraction in the diet group without PVAT. (n = 10 - 11).

In aorta with PVAT, Figure 3.2.5.1 **A**, RB caused increased the contractility of the aorta with PVAT in the diet group with RB when it was compared to the diet group in response at final PE concentration 1μM PE (0.73 ± 0.08 g vs 1.07 ± 0.12 g, $p < 0.05$). In figure 3.2.5.1 **B**, RB did not cause a significant effect on the aortic contraction in the diet group after removing the PVAT.

3.2.5.2 Contraction of the lean group

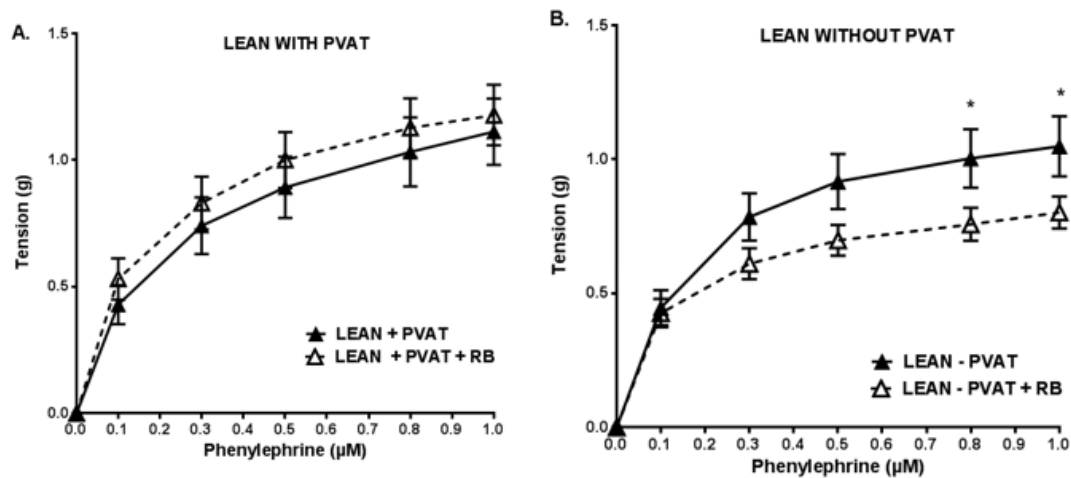


Figure 3.2.5.2 The effect of RB on PE induced contraction in lean group (A)

The effect of RB on PE induced contraction in lean group with PVAT. (B) The effect of RB on aortic ring contraction in lean group without PVAT (* $p < 0.05$ specific effect at 800 nM and 1 μM (2-way ANOVA, $n = 10 - 11$).

In figure 3.2.5.2 **A**, RB did not have any effect on contraction in aorta with PVAT in the lean group with RB when it was compared to the lean. However, **in figure 3.2.5.2 B**, when the PVAT was removed, RB decreased the contractility in the lean group at final PE concentration 1 μM (1.04 ± 0.11 g vs 0.80 ± 0.06 g, $p < 0.05$), the aortas from the lean with RB group without PVAT was also less responsive than the aorta of the without RB group without PVAT at the lower PE concentrations: 800 nM (0.75 ± 0.06 g vs 1.00 ± 0.10 g, $p < 0.05$).

3.2.5.3 Relaxation of the diet group:

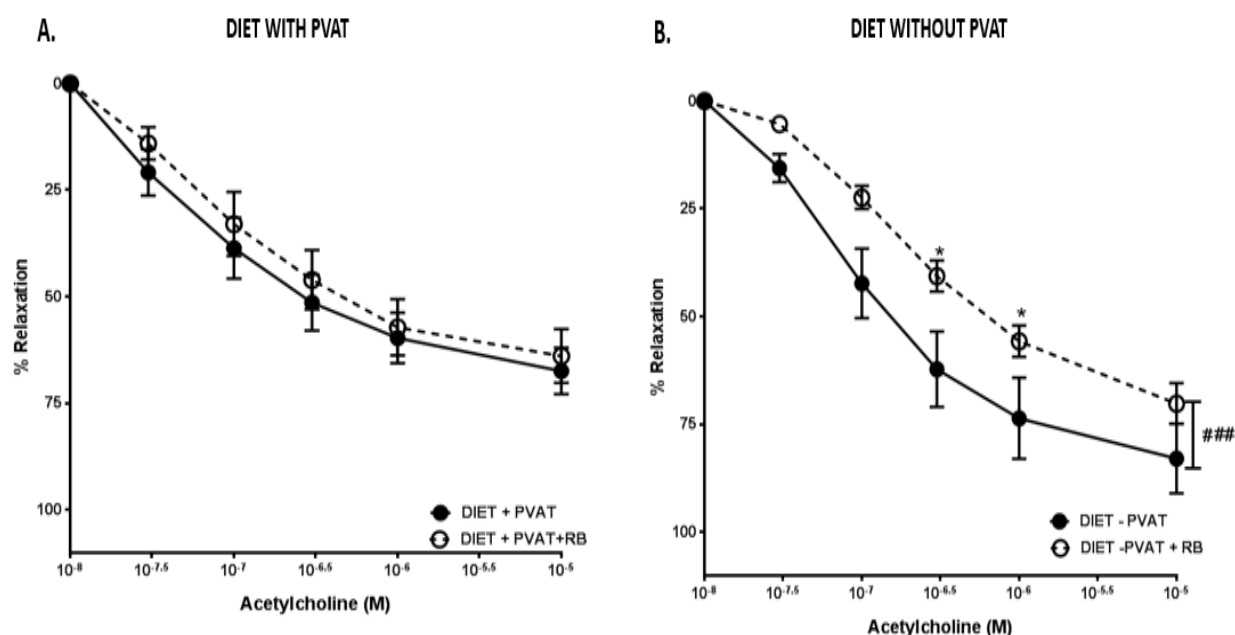


Figure 3.2.5.3 The effect of RB on ACH induced relaxation in aorta in the diet group. (A) The effect of RB on ACH induced relaxation in aorta in the diet group with PVAT. (B) The effect of RB on aortic ring relaxation in aorta in the diet group (### P <0.001 diet without PVAT and diet without PVAT with RB, *P <0.001 at 300 nM and 1 μM. 2-way ANOVA, n = 10 - 11).

In Figure 3.2.5.3 **A**, RB did not show any effect on relaxation in aorta with PVAT in the diet group. However, in figure 3.2.5.9 **B** the diet group after removing the PVAT, RB decreased the relaxation in the aorta $p < 0.001$, as well as specific effect that appeared at 300 nM and 1 μM cumulative ACH concentration: 300 nM (diet without PVAT with RB: 22.36 ± 2.66 % vs diet without PVAT 42.27 ± 8.05 % $p < 0.05$) and 1 μM (diet without PVAT with RB 40.60 ± 3.59 % vs diet without PVAT 62.17 ± 8.74 %, $P < 0.05$).

3.2.5.4 Relaxation of the lean group:

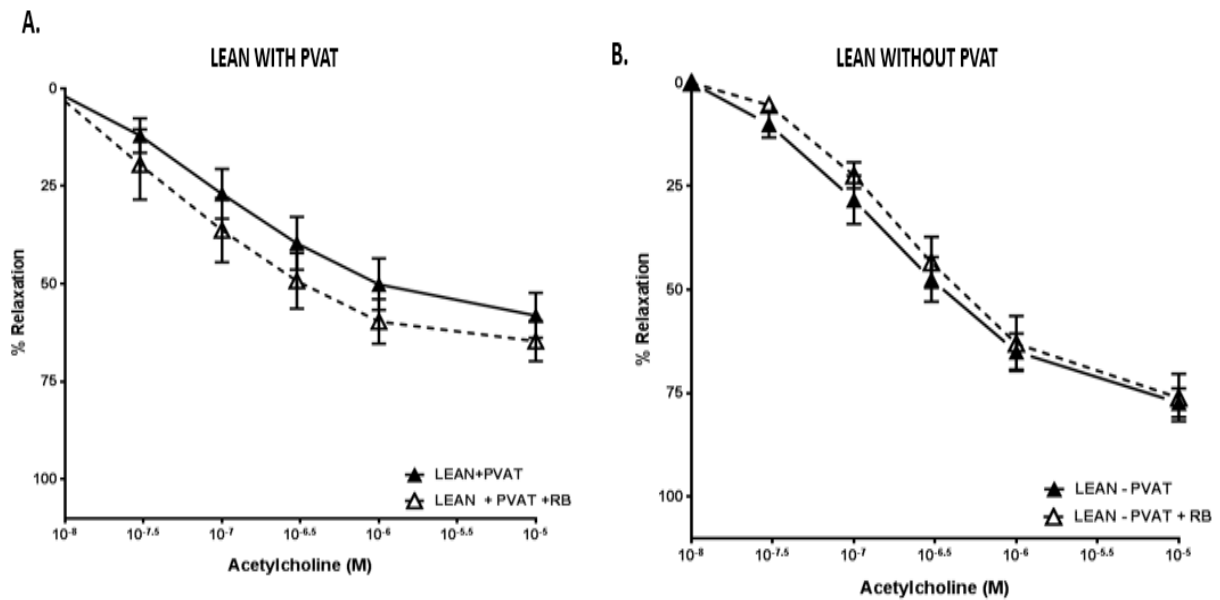


Figure 3.2.5.4 The effect of RB on ACH induced relaxation in aorta in the lean group. (A) The effect of RB on ACH induced relaxation in aorta in the lean group with PVAT. (B) The effect of RB on aortic ring relaxation in aorta in the lean group without PVAT.

The RB had no effect because no difference was observed with regards to the relaxation of aorta in the lean group with or without PVAT.

Table 3.1: Summary of the effect of the diet and RB in aortic ring contraction and relaxation:

	Diet (vs lean)		Diet+RB (vs Diet)		lean+RB (vs lean)	
	with pvat	Without PVAT	With PVAT	Without PVAT	With PVAT	Without PVAT
contraction	↓	-	↑	↑	-	↓
relaxation	↑	↑	-	↓	-	-

- no effect, ↓ decrease, ↑ increase

Table 3.2: Summary of the effect of the PVAT in the diet and lean on aortic ring function:

	Diet+ PVAT (vs DIET - PVAT)	Lean + PVAT (vs Lean – PVAT)
contraction	↓	-
relaxation	-	↓

- no effect, ↓ decrease, ↑ increase.

3.3. Western blot analysis

Western blot analysis was performed on aortas with and without PVAT to determine protein phosphorylation and expression. Blots were done in n=3 aortas per experiment.

3.3.1. The effect of RB on eNOS, PKB and AMPK signalling pathways in lean and diet aortas

Three main effects that will be focused on in this section are the effects of (i) diet induced obesity, (ii) PVAT, and (iii) Rooibos (RB) on the phosphorylation and total protein expression as well as the phosphorylation to total ratio of eNOS, as well as the upstream activators PKB and AMPK.

3.3.1.1 The effect of RB on eNOS signalling in lean and diet aortas with PVAT

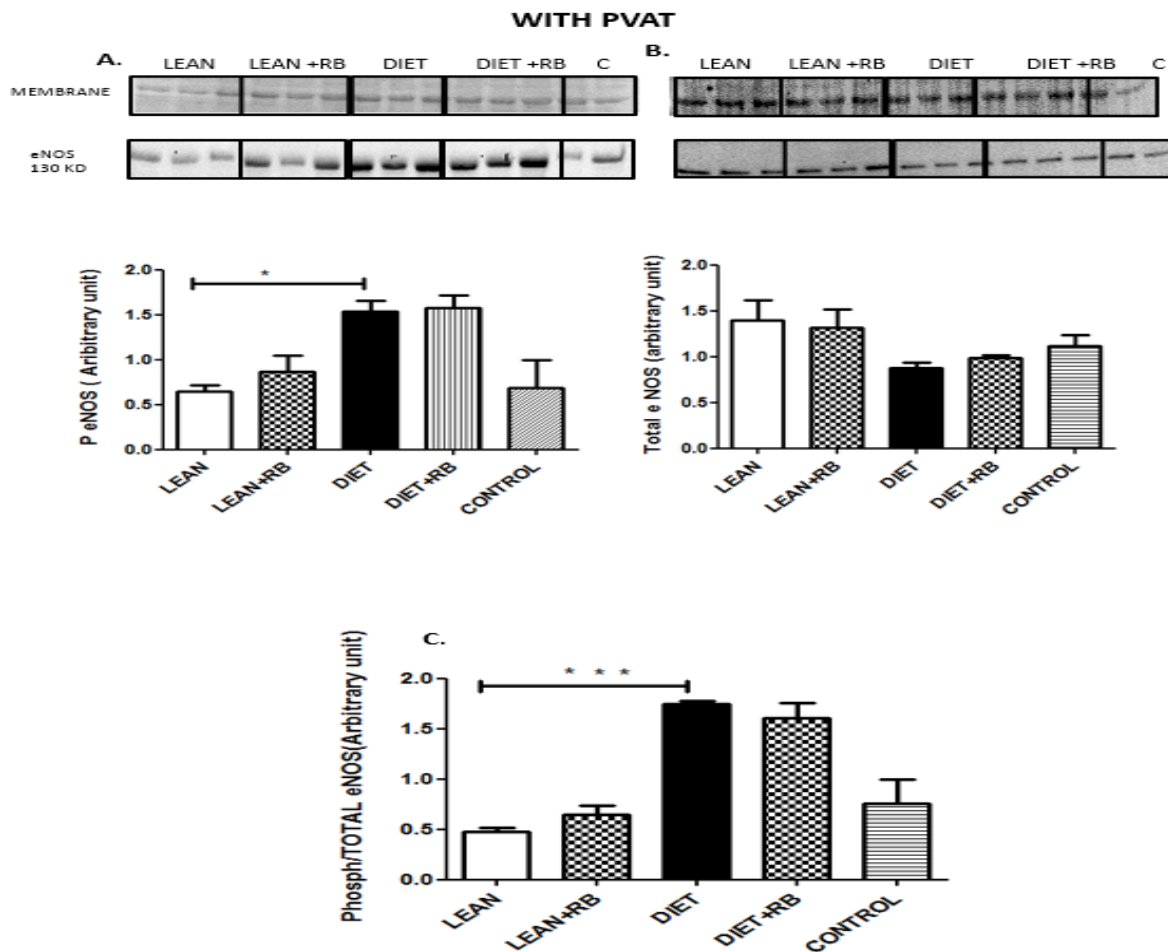


Figure 3.3.1.1: The effect of RB on eNOS in aortic tissue with PVAT. Representative membranes (total proteins) and blots (exposed to antibody) with arbitrary units, A. Phospho-eNOS, B. Total eNOS and C. Phospho/Total ratio of eNOS. Data expressed as mean \pm SEM. *P < 0.05; ***P < 0.001. n = 3.

In aortas with PVAT the only significant effect observed was the effect of the diet (figure 3.3.1.1 **A**) that caused an increase in eNOS phosphorylation in diet group compared to the lean group (Diet: 1.53 ± 0.12 vs. lean: 0.64 ± 0.06 , $p < 0.01$). Furthermore, this increase in phosphorylation was confirmed in the phospho/total eNOS ratio in figure 3.3.1.1 **C**, as this was significantly increased in the diet when compared to lean group.

3.3.1.2 The effect of RB on eNOS signalling in lean and diet aortas without PVAT

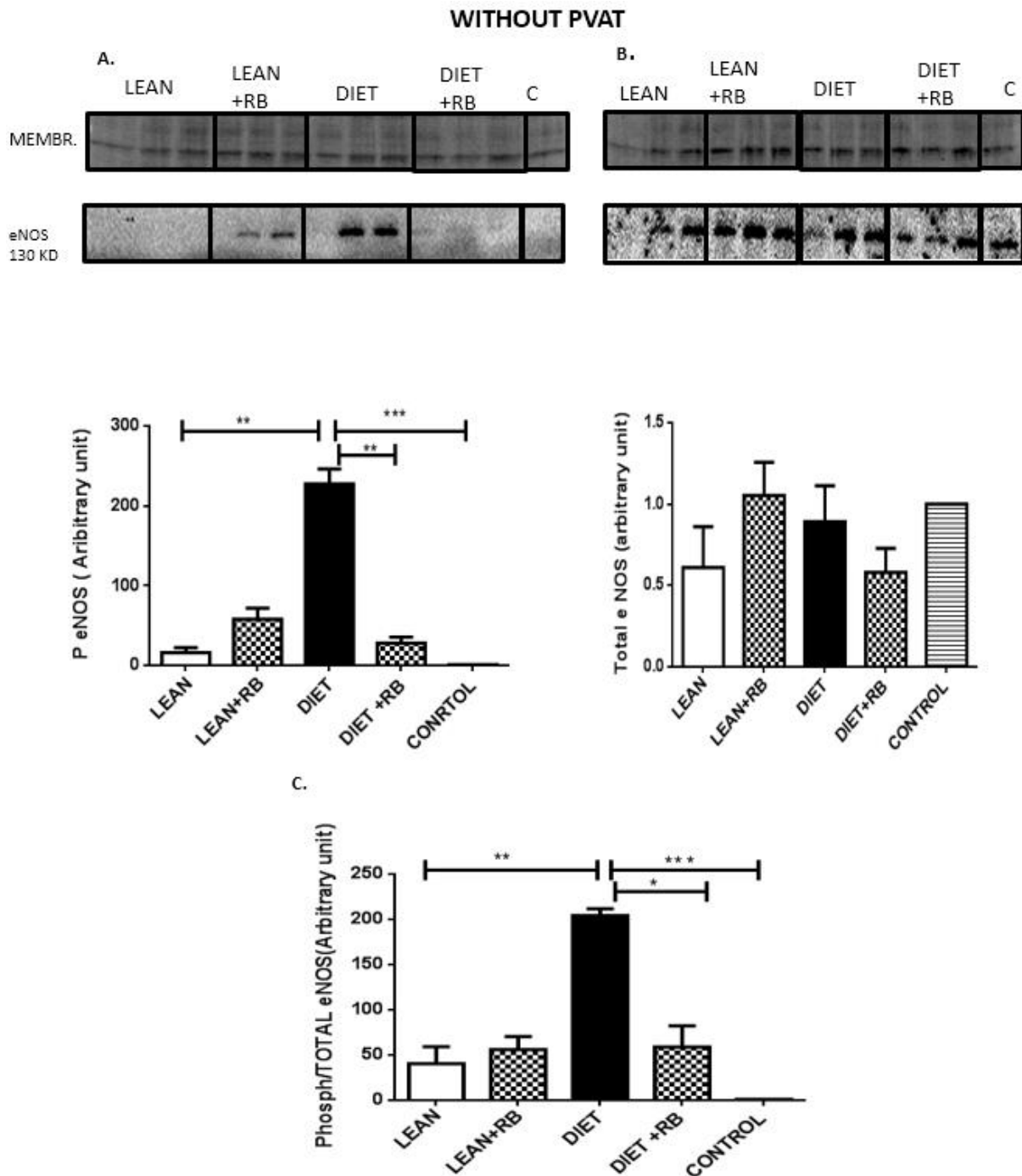


Figure 3.3.1.2: The effect of the RB on eNOS in aortic tissue without PVAT. Representative membranes (total proteins) and blots (exposed to antibody) with arbitrary units, A. Phospho-eNOS, B. Total eNOS and C. Phospho/Total ratio of eNOS. Data expressed as mean \pm SEM. *P < 0.05, **P < 0.01; ***P < 0.001. n = 3.

In the aortas without PVAT (figure 3.3.1.2 A) phosphorylated eNOS was significantly increased in the diet group compared to the control and lean, (diet: 227.0 ± 19.25 vs lean: 15.65 ± 6.43 $p < 0.01$, vs control: 1.00 ± 0.00 , $p < 0.001$). Interestingly, RB decreased the phosphorylation in the diet group (diet: 227.0 ± 19.25 vs diet with RB: 27.47 ± 7.93 , $p < 0.05$). Furthermore in figure 3.3.1.2 C, increase in phosphorylation was confirmed in the phospho/total ratio of eNOS as well.

3.3.1.3 The effect of RB on PKB signalling in lean and diet aortas with PVAT:

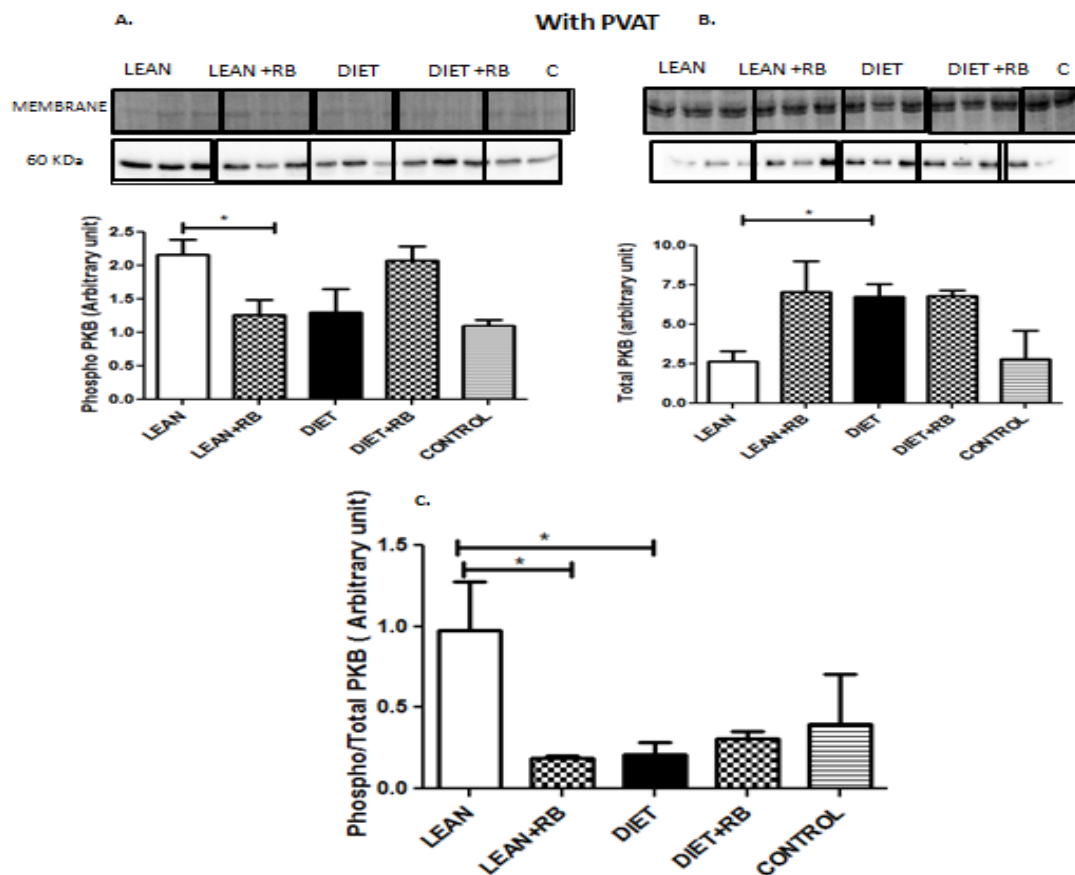


Figure 3.3.1.3: The effect of RB on PKB in aortic tissue with PVAT. Representative membranes (total proteins) and blots (exposed to antibody) with arbitrary units, A. Phospho-PKB, B. Total PKB and c. Phospho /Total ratio of PKB. Data expressed as mean \pm SEM. * $P < 0.05$. $n = 3$.

In the aortas with PVAT, the only significant effect on PKB phosphorylation (figure 3.3.1.3: A) was with RB administration in the lean group, where RB significantly decreased the phosphorylation of PKB compared to the lean (lean: 2.17 ± 0.22 vs lean

with RB: 1.26 ± 0.22 , $p < 0.05$). In figure 3.3.1.3 **B.** the total protein expression of PKB was significantly increased in the diet group compared to lean (diet: 6.76 ± 0.18 vs lean: 2.63 ± 0.67 , $p < 0.01$). Moreover, in figure 3.3.1.3 **C.** RB caused a significant decrease in the phospho/total PKB ratio compared to the lean group (lean with RB: 0.18 ± 0.01 vs lean: 0.97 ± 0.30 , $p < 0.05$), moreover, there was a significant decrease in the phosphor/total ratio of PKB in the diet group compared to the lean (diet: 0.18 ± 0.01 vs lean: 0.97 ± 0.30 , $p < 0.05$).

3.3.1.4 The effect of RB on PKB signalling in lean and diet aortas without PVAT:

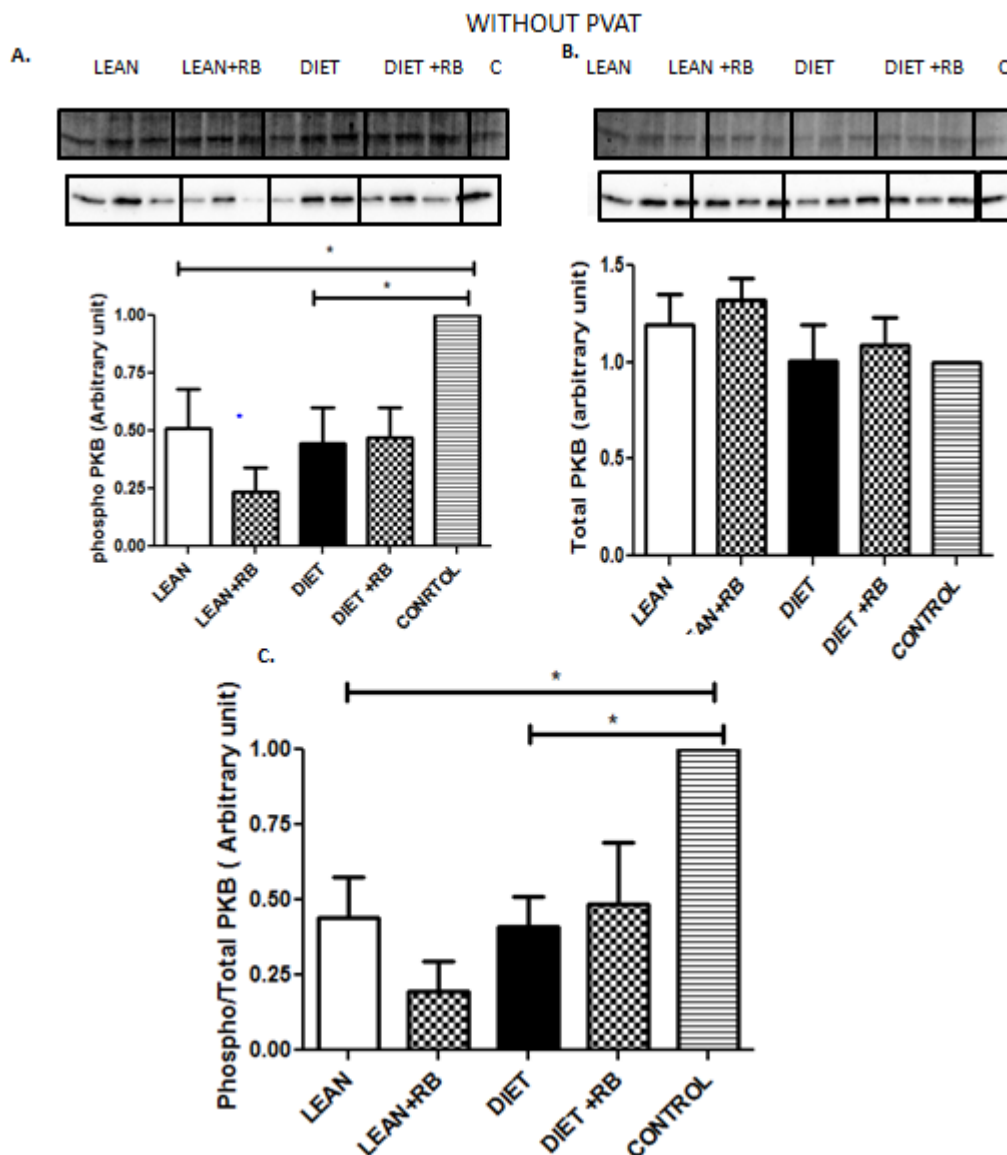


Figure 3.3.1.4: The effect of RB on PKB in aortic tissue without PVAT.

Representative membranes (total proteins) and blots (exposed to antibody) with arbitrary units, of A. Phospho-PKB, B. Total PKB and C. Phospho /Total ratio of PKB. Data expressed as mean \pm SEM. * $p < 0.05$. $n = 3$.

In aortas without PVAT the phosphorylation of PKB (figure 3.3.1.4 **A**) was significantly decreased in the diet group as well as in the lean group compared to the control group (diet: 0.44 ± 0.15 vs control: 1.00 ± 0.00 , $p < 0.05$ lean: 0.51 ± 0.17 vs control 1.00 ± 0.00 , $p < 0.05$). These changes were also reflected in the phospho/total PKB ratio (figure 3.3.1.4 **C**).

3.3.1.5 The effect of RB on AMPK signalling in lean and diet aortas with PVAT:

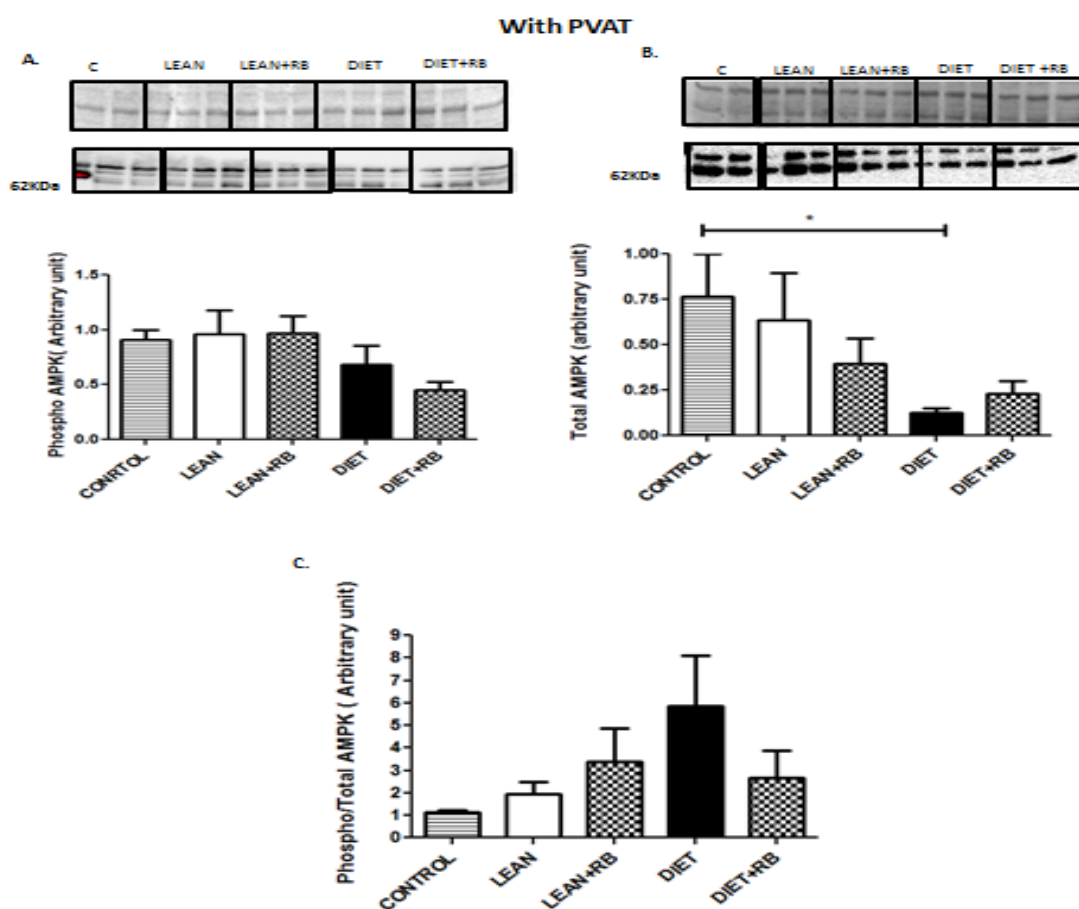


Figure 3.3.1.5: The effect of RB on AMPK in aortic tissue with PVAT.

Representative membranes (total proteins) and blots (exposed to antibody) with arbitrary units, of A. Phospho-AMPK, B. Total AMPK and C. Phospho/Total ratio of AMPK. Data expressed as mean \pm SEM. *P < 0.05. n = 3.

In the aortas with PVAT in figure 3.3.1.5 **B**, the only significant effect was observed in the total protein expression of AMPK which was significantly decreased in the diet group compared to control (diet: 0.23 ± 0.07 vs control: 0.76 ± 0.23 , p < 0.05).

3.3.1.6 The effect of RB on AMPK signalling in lean and diet aortas without PVAT:

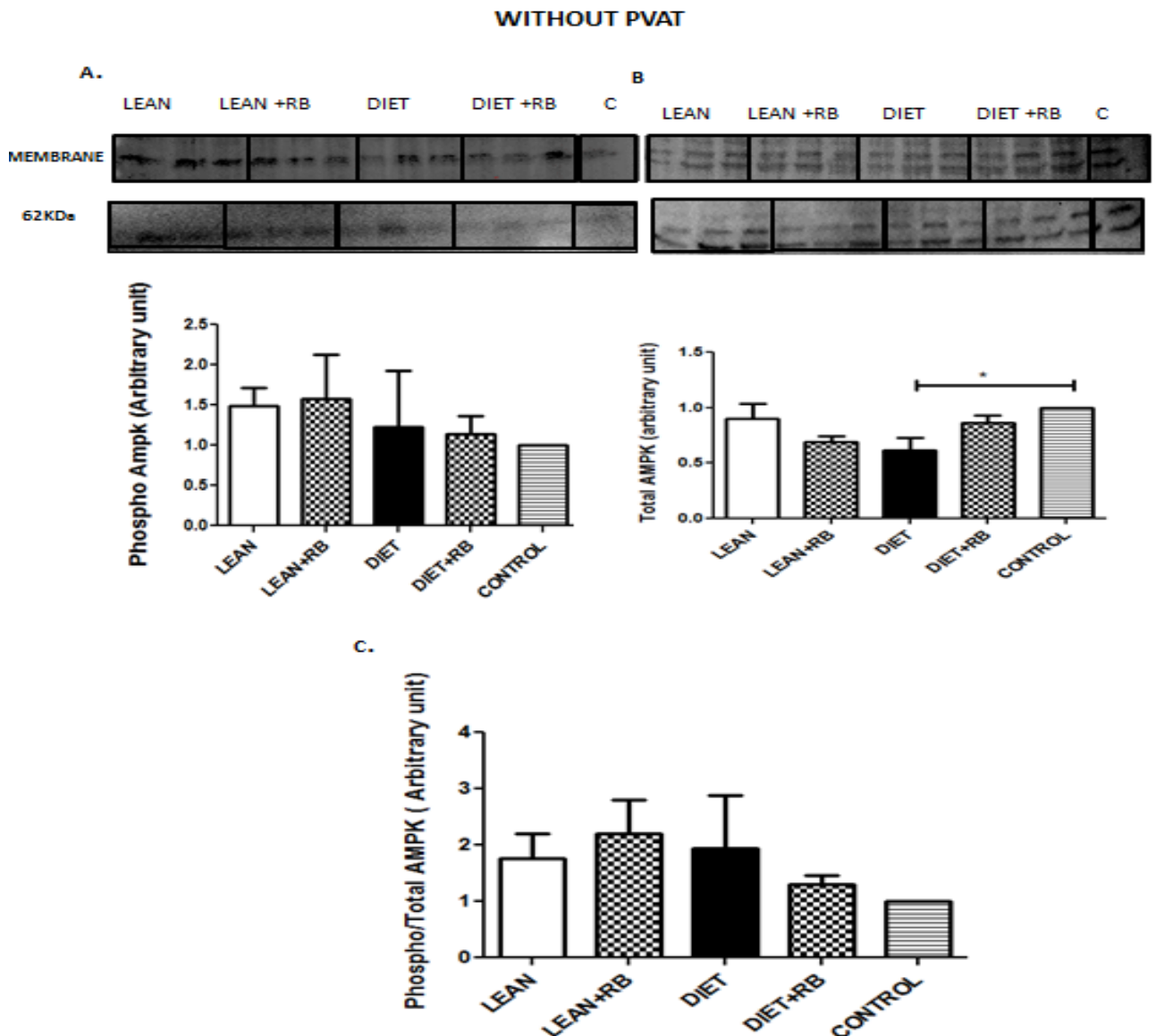


Figure 3.3.1.6 The effect of RB on AMPK in aortic tissue without PVAT.

Representative membranes (total proteins) and blots (exposed to antibody) with arbitrary units, of A. Phospho-AMPK, B. Total AMPK and C. Phospho /Total ratio of AMPK. Data expressed as mean \pm SEM. *P < 0.05. n = 3.

In the aortas without PVAT, figure 3.3.1.6 **B**, the diet significantly decreased the total protein expression of AMPK compared to control (diet: 0.61 ± 0.11 vs. control: 1.00 ± 0.00 , p < 0.05).

Table 3. 1 : Summary of the effect of diet induced obesity, PVAT, and RB on eNOS, PKB and AMPK signalling:

		Diet (vs. Lean)		Diet + RB (vs. Diet)		Lean + RB (vs. Lean)	
		with PVAT	without PVAT	with PVAT	without PVAT	with PVAT	without PVAT
eNOS:	Phospho	↑	↑	↓	-	-	-
	Total	-	-	-	-	-	-
	Phospho/ Total Ratio:	↑	↑	↓	↓	-	-
PKB	Phospho	-	-	-	-	↓	-
	Total:	↑	-	-	-	-	-
	Phospho/ Total Ratio	↓	-	-	-	↓	-
AMPK	Phospho:	-	-	-	-	-	-
	Total:	-	-	-	-	-	-
	Phospho/ Total Ratio	-	-	-	-	-	-

↑ increase, ↓ decrease, - no effect

3.3.2 The effect of RB on mitogen activated kinase (MAPK) and oxidative stress signalling pathways

In the next section the phosphorylation and total protein expression, as well as the phosphorylation to total ratio of the MAPKs, namely **ERK p44/p42**, **JNK p54/p46**, **p38 MAPK** with downstream **CREB**, as well as oxidative stress signalling, namely **Nitrotyrosin**.

3.3.2.1 The effect of RB on ERK P44/P42 signalling in lean and diet aortas with PVAT:

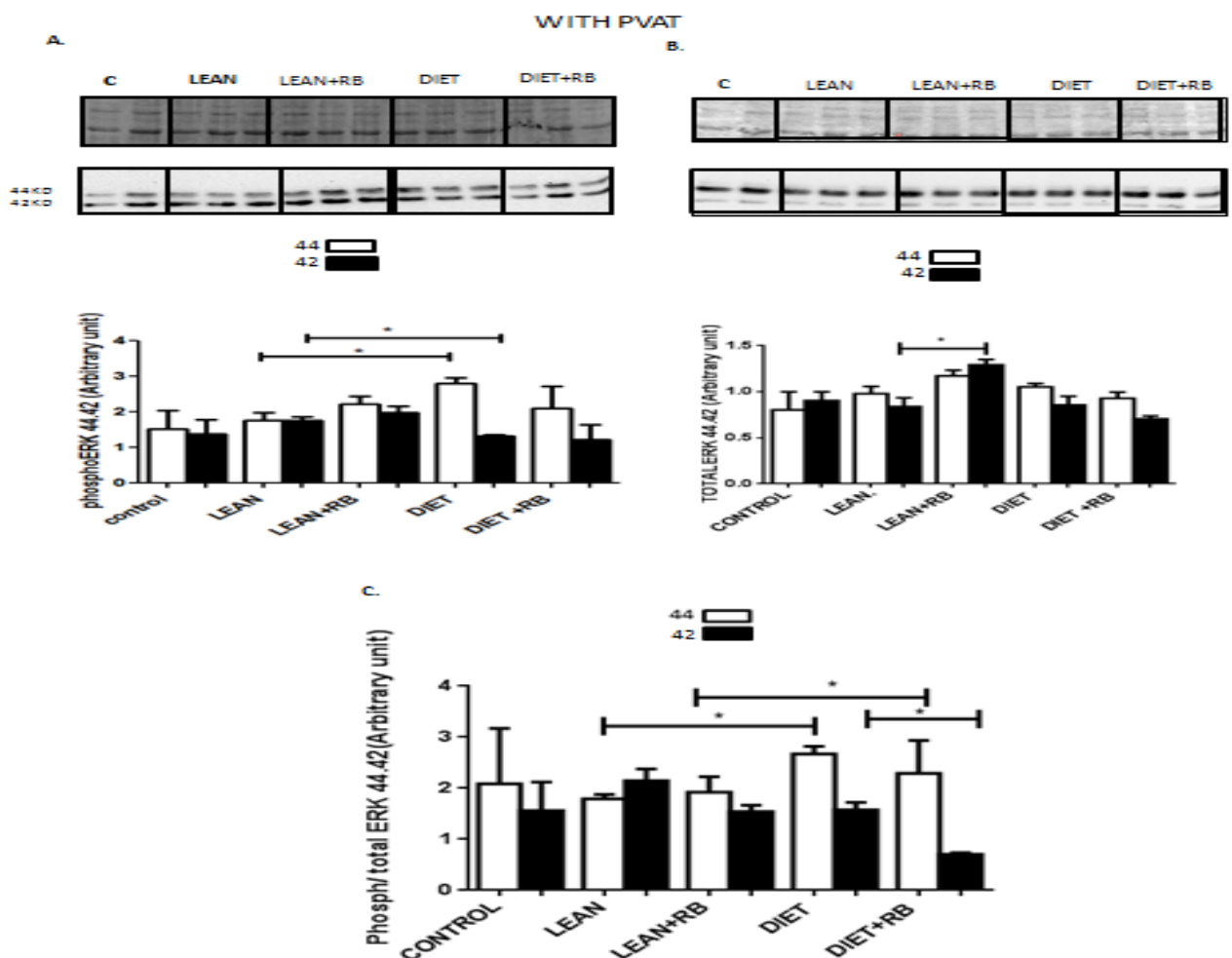


Figure 3.3.2 1: The effect of RB on ERK P44/P42 in aortic tissue with PVAT. Representative membranes (total proteins) and blots (exposed to antibody) with arbitrary units of A. Phospho ERK P44/P42 B. Total ERK P44/P42, and C.

Phospho /Total ratio of ERK P44/P42. Data expressed as mean \pm SEM. *P <0.05. n = 3.

In the aortas with PVAT, the diet significantly (figure 3.2.1: **A**) increased in the phosphorylation of ERK P44 compared to the lean (diet ERK P44: 2.81 ± 0.15 vs lean ERK P44: 1.76 ± 0.21 , P < 0.05). Interestingly the diet significantly decreased ERK P42 phosphorylation compared to lean p44 (diet ERK P42: 1.32 ± 0.02 vs lean ERK P42: 1.39 ± 0.39 , P < 0.05). In figure 3.3.2.1 **B** the total protein expression of ERK /P42 was significantly increased in the lean group after administration of RB compared to the lean alone (lean with RB: 1.29 ± 0.05 vs lean: 0.83 ± 0.09 , p < 0.05). In figure C, the phospho/total ratio of ERK P44 was significantly increased in the diet compared to the lean group, similar to the effect seen in phosphorylation. Furthermore, administration of RB to diet aortas decreased the phospho/total ratio of ERK P42 compared to the diet alone, as well as compared to the lean with RB (diet with RB: 0.70 ± 0.03 vs diet: 2.67 ± 0.15 , p < 0.05).

3.2.2.2 The effect of RB on ERK44/P42 signalling in lean and diet aortas without PVAT:

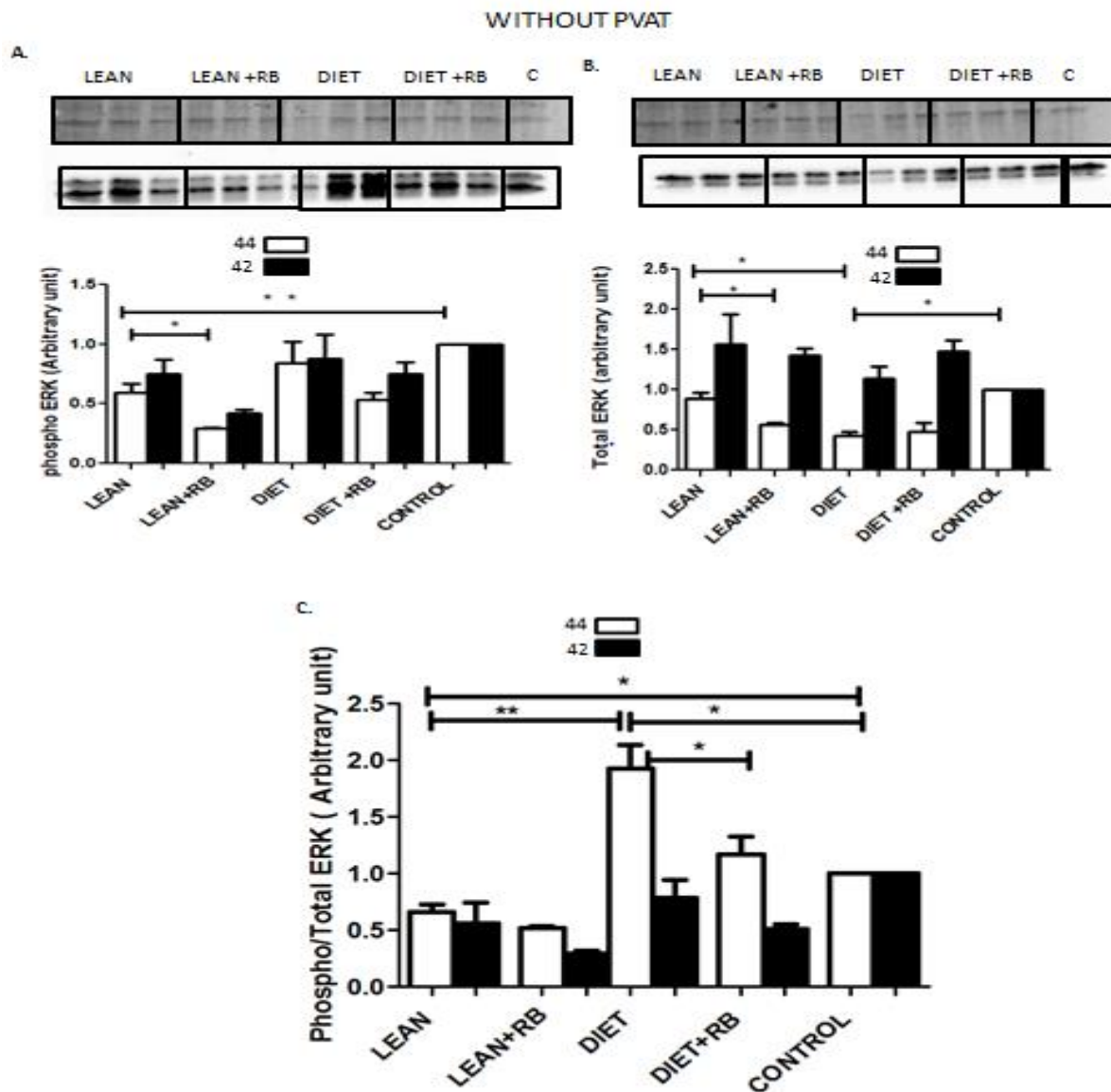


Figure 3.3.2 2: The effect of RB on ERK44/P42 in aortic tissue without PVAT. Representative membranes (total proteins) and blots (exposed to antibody) with arbitrary units of A. Phospho-ERK44/P42, B. TotalERK44/P42, and C. Phospho/Total ratio of ERK44/P42. Data expressed as mean \pm SEM. *P < 0.05. **p < 0.01 n = 3.

In aorta without PVAT in figure 3.3.2.2 **A**, the phosphorylation of ERK P44 decreased significantly in lean group compared to control (lean: 0.59 ± 0.07 vs control: $1.00 \pm$

0.00, $p < 0.01$). In addition, after administration of RB to lean aortas the phosphorylation of ERK P44 decreased significantly compared to lean group (lean with RB: 0.29 ± 0.008 vs. lean: 0.59 ± 0.07 , $p < 0.05$). In figure 3.2.2.2 **B**, the expression of total ERK p 44 was decreased in the diet group compared control (diet: 0.44 ± 0.05 vs control 1.00 ± 0.00 , $p < 0.001$), as well as decreased in diet compared to the lean, (diet: 0.44 ± 0.05 vs lean 0.89 ± 0.07 , $p < 0.01$). Interestingly, after administration of RB to the lean aortas the expression of ERK P 44 decreased compared to the lean group (lean with RB: 0.56 ± 0.02 vs lean 0.89 ± 0.07 , $p < 0.05$). In figure 3.2.2.2 **C**, the ERK P44 phospho/total ratio increased in the diet group compared to the control (diet: 1.93 ± 0.20 vs control: 1.00 ± 0.00 , $p < 0.05$) as well as compared to the lean, (diet: 1.93 ± 0.20 vs lean: 0.66 ± 0.11 , $p < 0.01$). On the other hand, the ERK P44 phospho/total ratio was decreased in the lean group compared to the control (lean: 0.66 ± 0.11 vs control: 1.00 ± 0.00 , $p < 0.01$). Interestingly, the administration of RB to the diet aortas significantly decreased the ERK P44 phospho/total ratio when compared to the diet alone (diet with RB: 1.17 ± 0.15 vs diet: 1.93 ± 0.20 , $p < 0.05$).

3.3.2.3 The effect of RB on JNK p54/p46 signalling in lean and diet aorta with PVAT

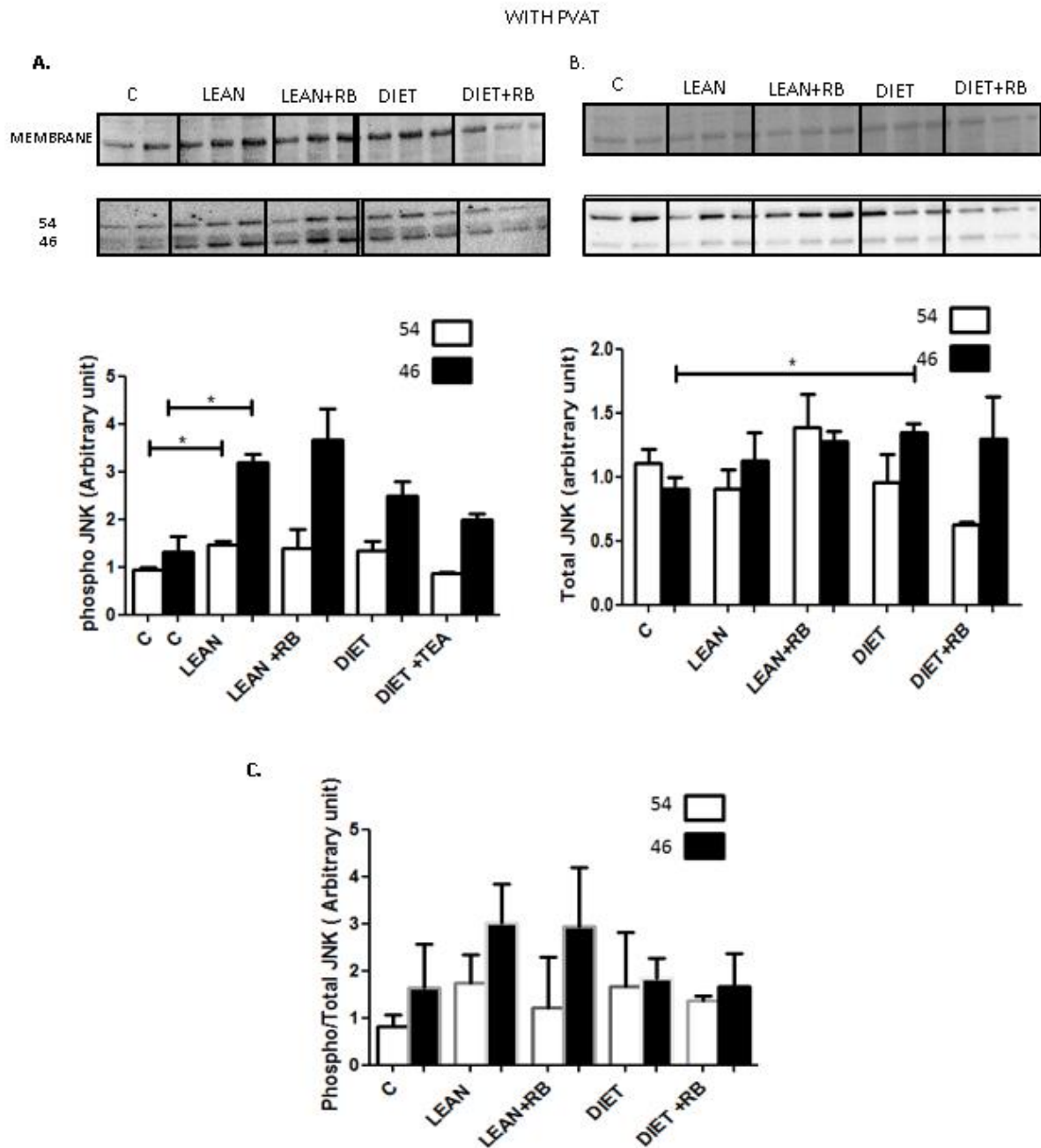


Figure 3.3.2.3 the effect of RB on JNK p54/p46 in aortic tissue with PVAT. Representative membranes (total proteins) and blots (exposed to antibody) with arbitrary units, of A. Phospho- JNK p54/p46 B. Total JNK p54/p46, and C. Phospho /Total ratio of JNK p54/p46. Data expressed as mean \pm SEM. *P < 0.05

In aorta with PVAT, figure 3.3.2.3 **A**, the phosphorylation of JNK 46/54 increased significantly in the lean group compared to the control, (JNK p54: lean 1.47 ± 0.06 vs

control 0.93 ± 0.06 , $p > 0.05$), (JNK p46: lean 3.19 ± 0.16 vs control: 1.31 ± 0.31 $p < 0.01$). In figure 3.3.2.3 B, a significant effect appeared in the total protein expression of JNK 46 which was significantly increased in the diet group compared to control (diet: 1.34 ± 0.07 vs control: 0.90 ± 0.09 vs $p < 0.05$). In figure 3.3.2.3 C, there were no significant differences appeared in the phospho/total ratio of JNK 46/54.

3.3.2.4 The effect of RB on JNK p54/p46 signalling in lean and diet aorta without PVAT:

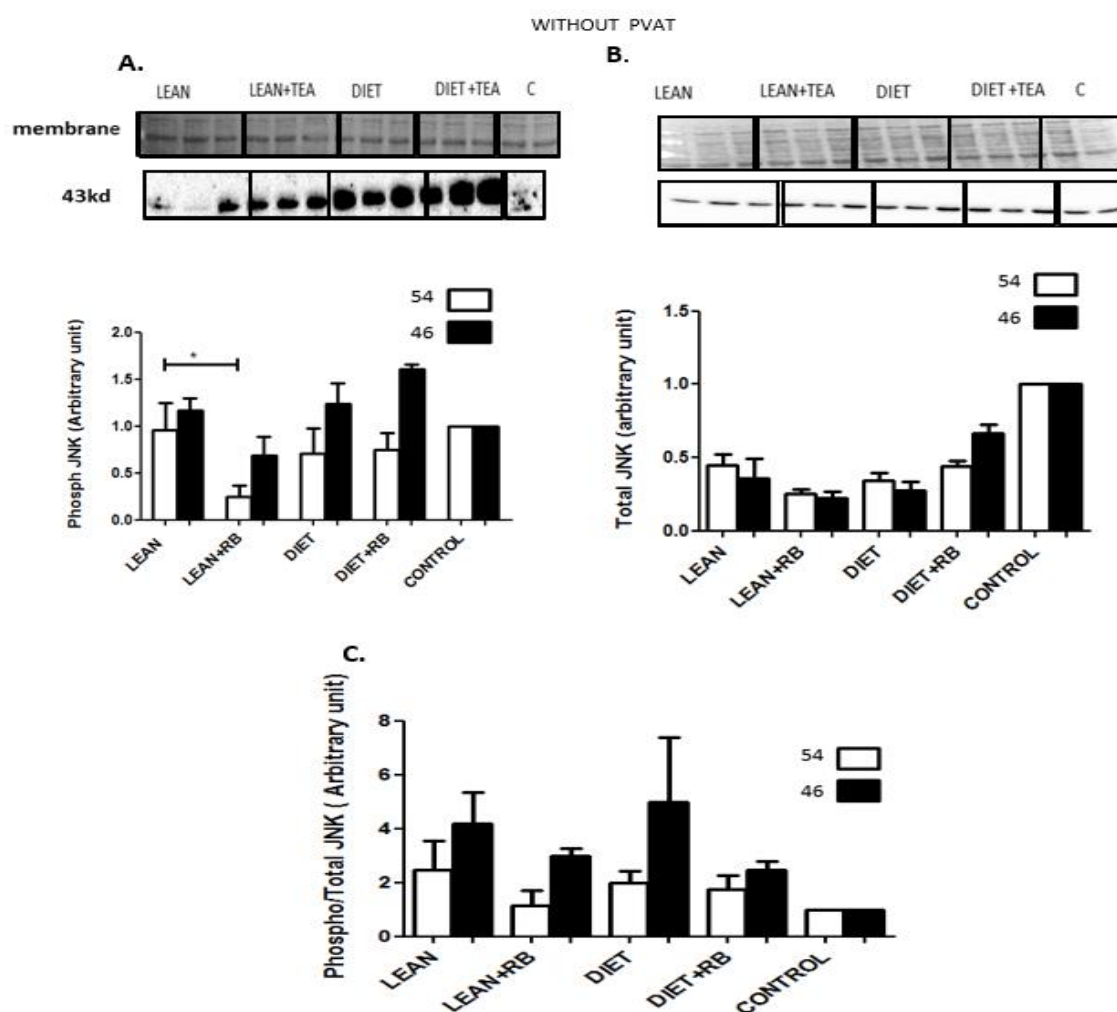


Figure 3.3.2.4: The effect of RB on JNK p54/p46 in aortic tissue without PVAT. Representative membranes (total proteins) and blots (exposed to antibody) with arbitrary units, of A. Phospho JNK p54/p46 B. Total JNK p54/p46, and C. Phospho/Total ratio of JNK p54/p46. Data expressed as mean \pm SEM. *P < 0.05.

In aorta without PVAT, figure 3.3.2.4 A, RB caused a significant decrease in the phosphorylation of p 54 JNK in the lean group, (lean with RB: 0.24 ± 0.11 vs lean: 0.95 ± 0.29 , $p < 0.05$). However, in figure 3.3.2.4 B and C there were no significant differences appeared in total and the phosphor/total ratio of JNK p54/p46.

3.3.2.5 The effect of RB on P38 MAPK signalling in lean and diet aortas with PVAT:

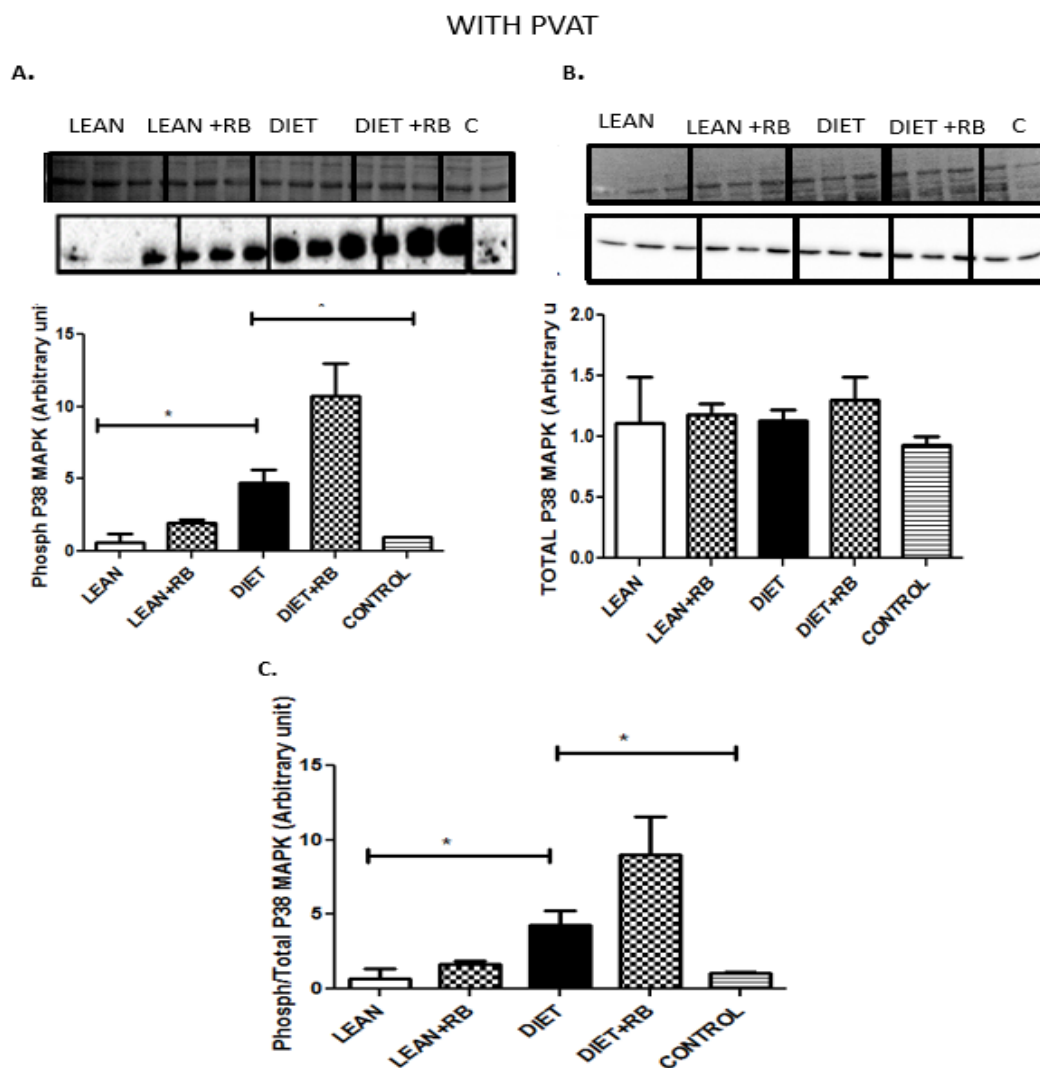


Figure 3.3.2 5: The effect of RB on p38 MAPK in aortic tissue with PVAT.

Representative membrane (total proteins) and blots (exposed to antibody) with arbitrary units, of A. Phospho-P38 MAPK, B. Total P38 MAPK, and C. Phospho/Total ratio of P38 MAPK. Data expressed as mean \pm SEM. *P < 0.05.

In aorta with PVAT, in figure 3.3.2.5 **A**, the only significant difference was an increase in the phosphorylation in P38 MAPK in diet group compared to the control group (control: 0.99 ± 0.005 vs diet: 4.77 ± 0.85 $p < 0.05$) as well as compared to the lean group, (diet: 4.77 ± 0.85 vs lean 0.63 ± 0.55 , $p < 0.05$). In figure 3.3.2.5 **C**, the changes in the phospho/total ratio of P38 MAPK confirmed by the increase in the phosphorylation.

3.3.2.6 The effect of RB on P38 MAPK signalling in lean and diet aortas without PVAT:

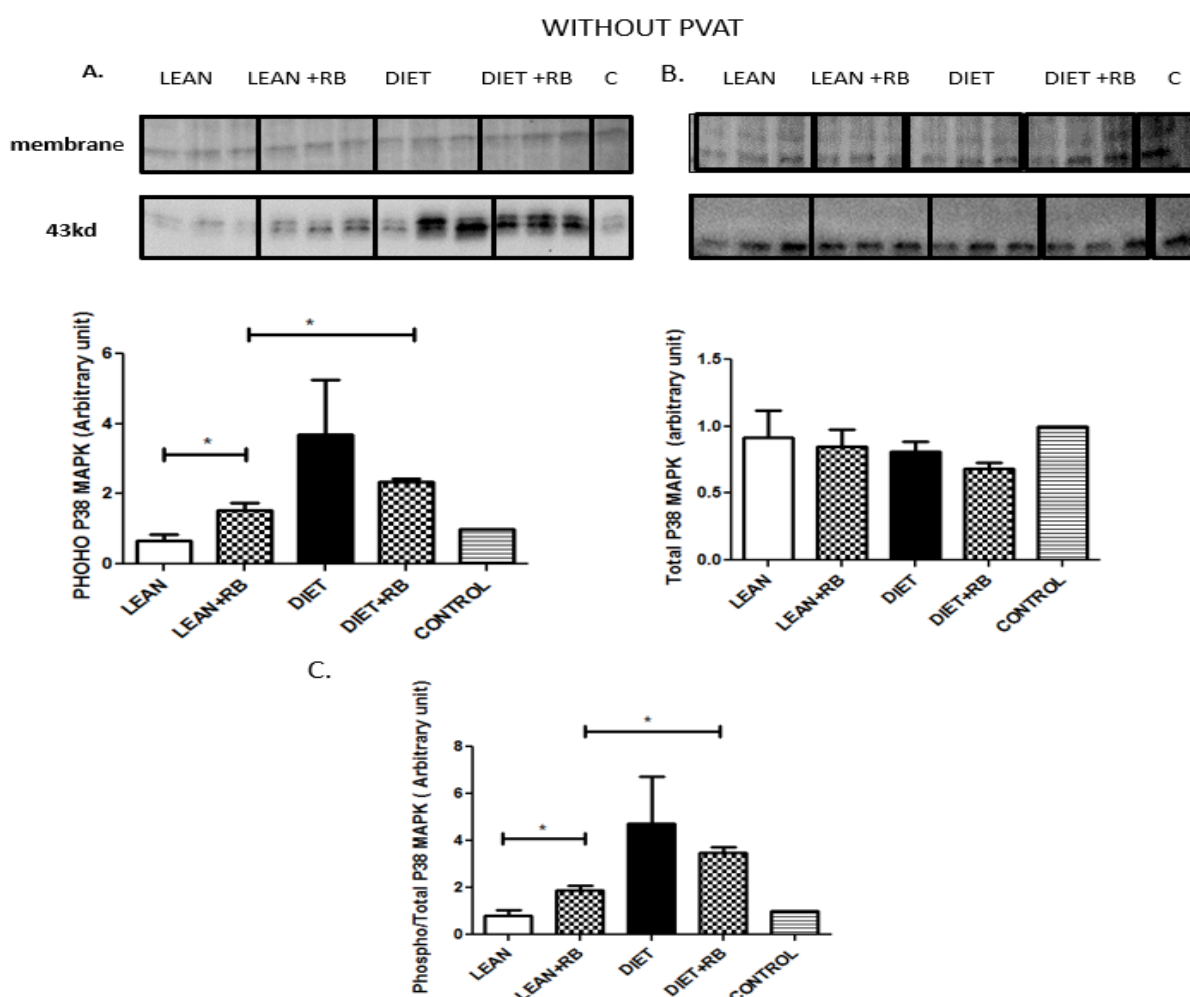


Figure 3.3.2.6: The effect of RB on p38 MAPK in aortic tissue without PVAT. Representative membranes (total proteins) and blots (exposed to antibody) with arbitrary units, of A. Phospho-P38 MAPK, B. Total P38 MAPK, and c. Phospho/Total ratio of P38 MAPK. Data expressed as mean \pm SEM. *P < 0.05.

In aorta without PVAT, in figure 3.3.2.6 **A**, there was an increase in the phosphorylation of P38 MAPK in diet with RB compared to lean with RB (diet with RB: 3.48 ± 0.25 vs lean with RB 1.54 ± 0.20 , $p < 0.05$). Furthermore, RB has increased in the phosphorylation of P38 MAPK in the lean with RB compared to the lean (lean with RB: 1.54 ± 0.20 vs lean: 0.66 ± 0.18 , $p < 0.05$). In figure 3.3.2.6 **C**, the phospho/total ratio of P38 confirmed increase in phosphorylation.

3.3.2.7 The effect of RB on CREB signalling in lean and diet aortas with PVAT:

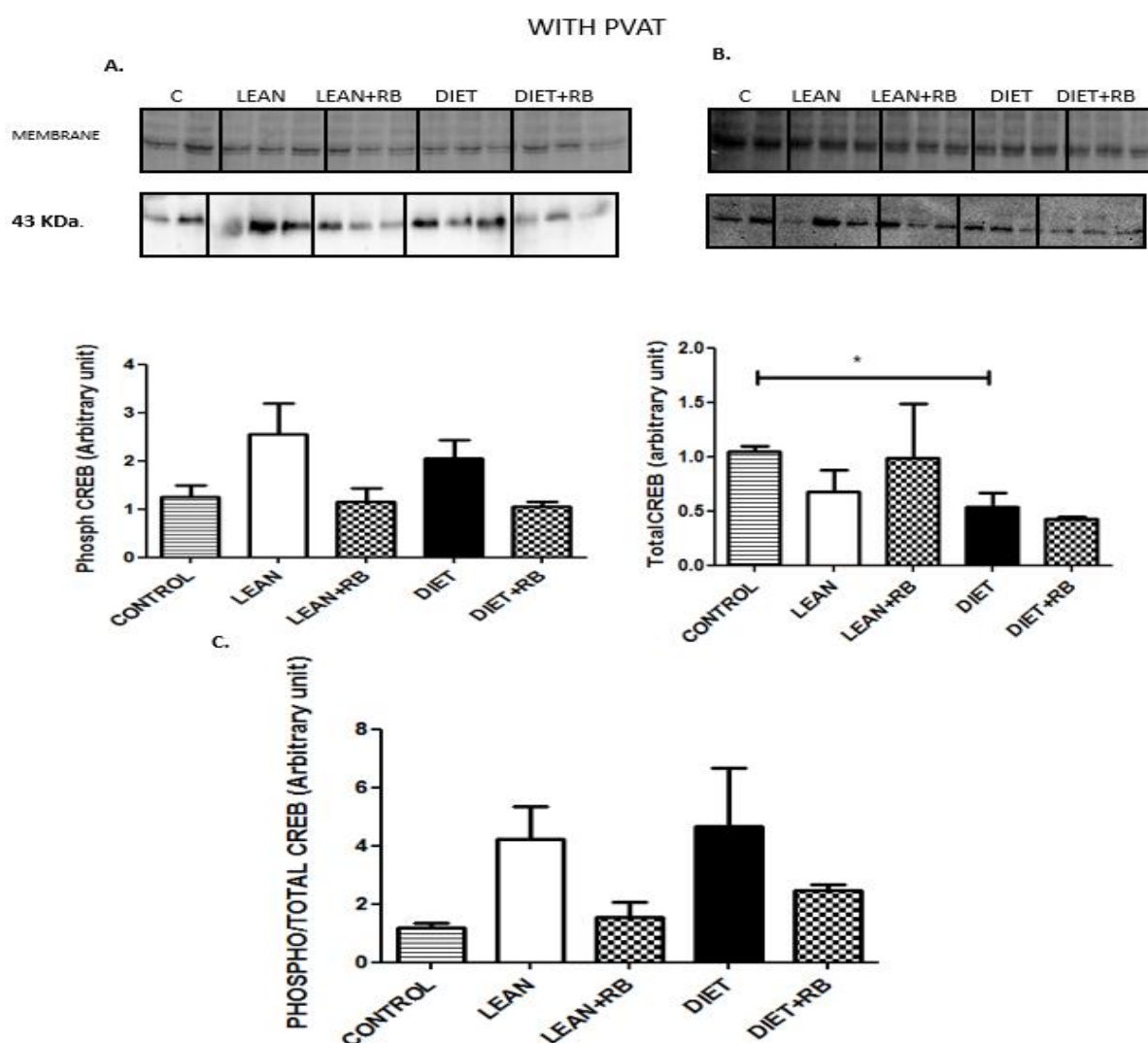


Figure 3.3.2.7: The effect of RB on CREB in aortic tissue with PVAT.

Representative membranes (total proteins) and blots (exposed to antibody) with arbitrary units, of A. Phospho- CREB, B. Total CREB, and c. Phospho /Total ratio of CREB. Data expressed as mean \pm SEM. *P <0.05.

In figure 3.3.2.7 **B** in the aortas with PVAT, there was a significant decrease in the total protein expression of CREB in the diet group compared to the control (diet: 4.65 ± 2.02 vs. control: 1.17 ± 0.17 , $p < 0.05$).

3.3.2.8 The effect of RB on CREB signalling in lean and diet aortas without PVAT:

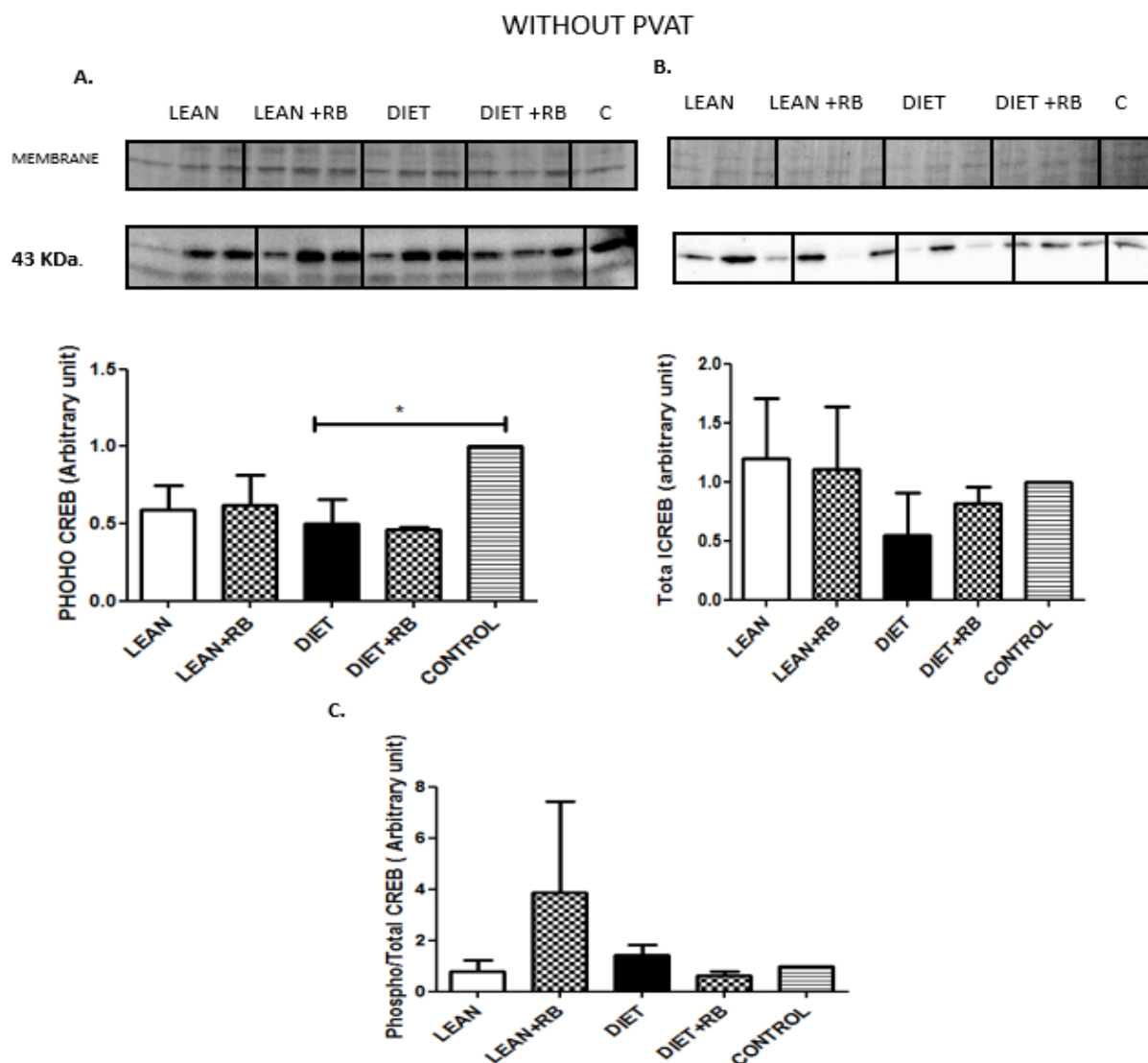


Figure 3.3.2.8 : The effect of RB on CREB in aortic tissue without PVAT.

Representative membranes (total proteins) and blots (exposed to antibody) with arbitrary units of A. Phospho- CREB, B. Total CREB, and c. Phospho /Total ratio of CREB. Data expressed as mean \pm SEM. * $P < 0.05$.

In figure 3.3.2.8 **A**, in the aortas without PVAT, there was a decrease in the phosphorylation of CREB in the diet group compared to the control (diet: 0.49 ± 0.15 vs. control: 1.00 ± 0.00 , $p < 0.05$).

3.3.2.9 The effect of RB on Nitrotyrosine signalling in lean and diet aortas with and without PVAT:

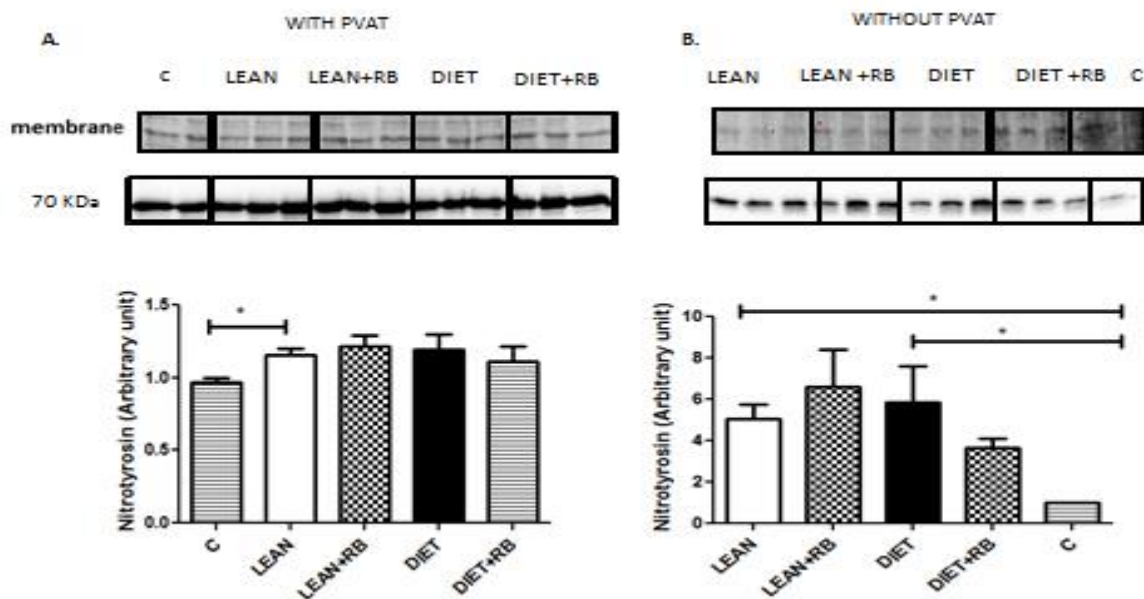


Figure 3.3.2.9: The effect of the tea on Nitrotyrosine in aortic tissue with and without PVAT. Representative membranes (total proteins) and blots (exposed to antibody) with arbitrary units of, A. Nitrotyrosine in Aortic tissue with PVAT, B. Nitrotyrosine in Aortic tissue without PVAT. Data expressed as mean \pm SEM. * $P < 0.05$.

In Aortas **with PVAT**, in figure 3.3.2.9 **A**, the only significant difference was an increase in the nitrotyrosin level in the lean group when compared to control group, (lean: 1.15 ± 0.04 vs control 0.96 ± 0.03 , $p < 0.05$).

In aorta **without PVAT**, in figure 3.3.2.9 **B**, a significant increase in the nitrotyrosine level appeared in the diet group compared to the control (diet: 5.87 ± 1.71 vs control: 1.00 ± 0.00), as well as in the lean group compared to the control (lean 5.06 ± 0.69 vs control: 1.00 ± 0.00 , $p < 0.05$).

Table 3. 2: Summary of the effect of diet induced obesity, PVAT, and RB on signalling: MAPK, CREB, and NITROTYROSIN

		Diet vs lean		Diet + RB vs diet		lean+RB vs lean	
		with PVAT	without PVAT	with PVAT	without PVAT	with PVAT	without PVAT
ERK:	phospho	↑	-	-	-	-	↓
	total	↑	↓	-	-	↑	↓
	phospho/total ratio	↑	↑	↓	↓	-	-
JNK	phospho	-	-	-	-	-	↓
	total	-	-	-	-	-	-
	phospho/total ratio	-	-	-	-	-	-
P38 MAPK	phospho	↑	-	-	-	-	↑
	total	-	-	-	-	-	-
	phospho/total ratio	↑	-	-	-	-	↑
CREB	phospho	-	-	-	-	-	-
	total	-	-	-	-	-	-
	phospho/total ratio	-	-	-	-	-	-
NITRO TYROSINE	total	-	-	-	-	-	-

↑ INCREASE, ↓ DECREASE, - NO EFFECT

3.4 Inflammatory markers:

The third aim of our study was to investigate the inflammatory markers using the multiplex kit by focusing on three main effects, (i) the effect of diet induced-obesity, (ii) PVAT, and (iii) rooibos (RB) on isolated rat aortas.

The inflammatory cytokines are classified into:

- 1- Pro-inflammatory markers which include: IL-1 α , IL-1B, IL-6, IL-12, IFN- γ , TNF- α .
- 2- Anti-inflammatory markers which include: IL-2, IL-4, IL-5, IL-10, IL-13.

3.4.1 Pro- inflammatory marker:

3.4.1.1 Pro-inflammatory marker: IL-1 α :

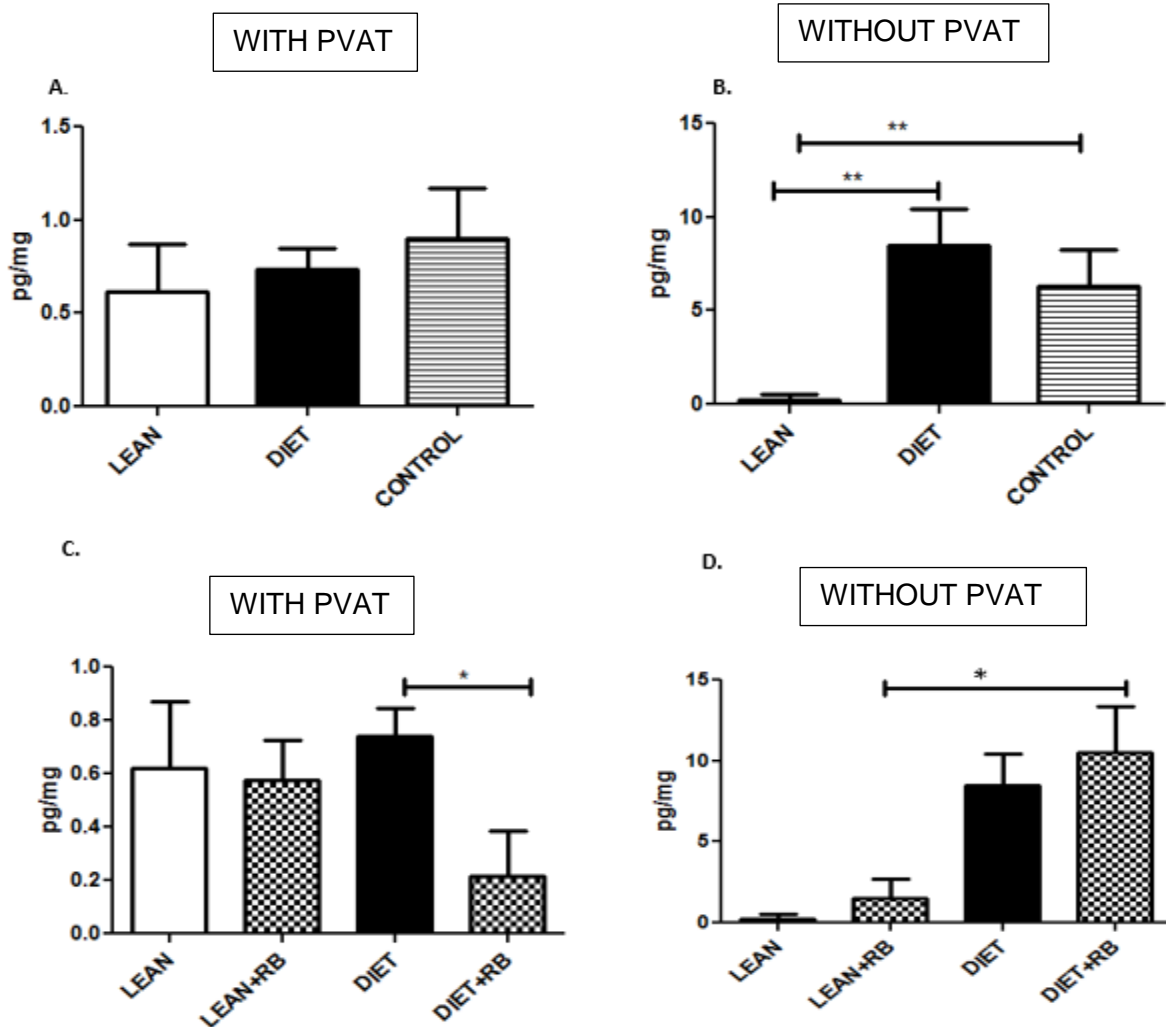


Figure 3.4.1.1: The effect of the diet on IL-1 α in aortas, A. with PVAT and B. without PVAT. The effect of the Rooibos (RB) on IL-1 α in aortas, C. with PVAT and D. without PVAT. Data expressed as mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, $n = 6$ per group.

The effect of diet on the IL-1 α in aortas with and without PVAT:

In figure 3.4.1.1 **A**, and table 3.6, the diet had no significant effect on IL-1 α in aortas with PVAT.

In figure 3.4.1.1 **B**, and table 3.7, when the PVAT was removed, IL-1 α was significantly decreased in the lean group when compared to both the diet and the control groups

(lean: 0.25 ± 0.25 pg/mg vs diet: 8.45 ± 1.20 pg/mg, $p < 0.01$), (lean: 0.25 ± 0.25 pg/mg vs control: 6.31 ± 1.93 pg/mg $p < 0.01$).

The effect of RB on the IL-1 α in aortas with and without PVAT

In figure 3.4.1.1 **C**, when RB was added it caused a significant decrease in IL-1 α in the diet with RB when compared to the diet alone. (Diet with RB: 0.21 ± 0.17 pg/mg vs diet: 0.74 ± 0.11 pg/mg, $P < 0.05$).

In figure 3.4.1.1 **D**, when the PVAT was removed, the IL-1 α in the lean with RB was significantly decreased compared to the diet with RB, (lean with RB: 1.50 ± 1.19 pg/mg vs diet with RB: 11.00 ± 3.00 pg/mg, $P < 0.05$).

3.4.1.2 Pro-inflammatory marker: IL-1 β :

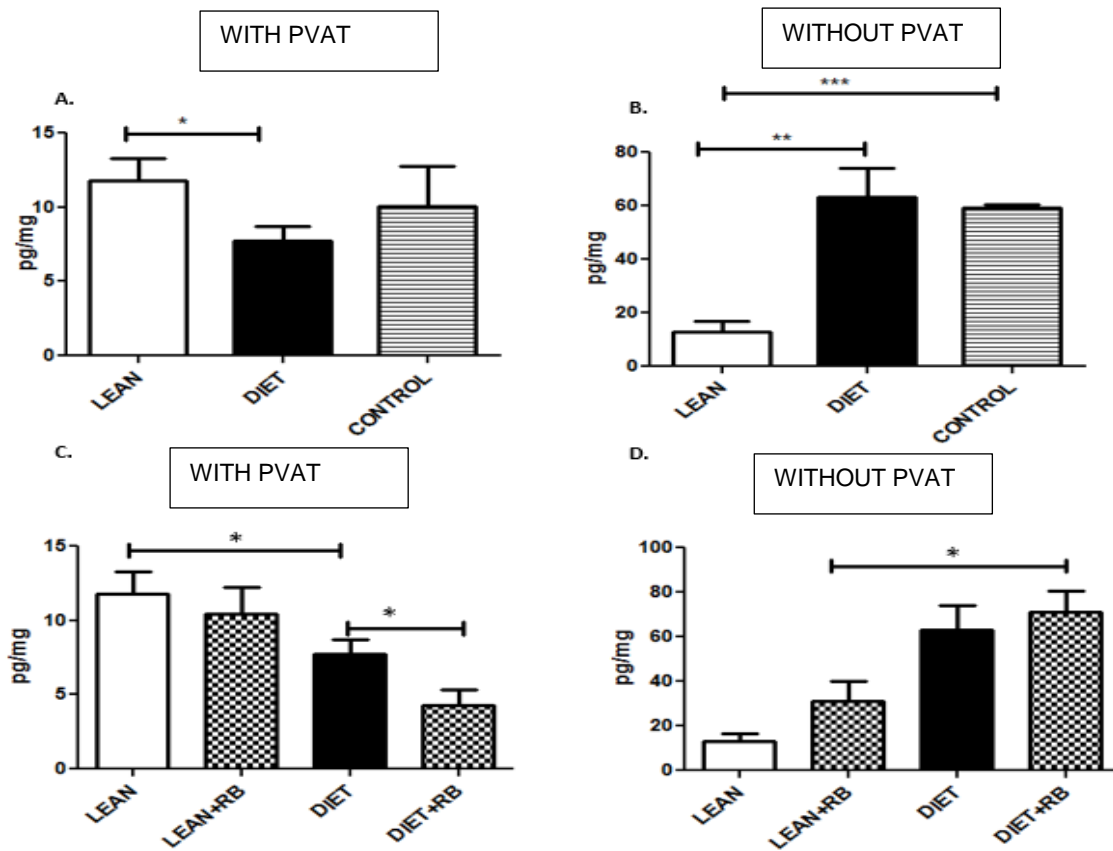


Figure 3.4.1.2: The effect of the diet on IL-1 β in aortas, A. with PVAT and B. without PVAT. The effect of the Rooibos (RB) on IL-1 β in aortas, C. with PVAT and D. without PVAT. Data expressed as mean \pm SEM. * $P < 0.05$, *** $P < 0.001$, $n = 6$ per group.

The effect of diet on the IL-1 β in aortas with and without PVAT:

In figure 3.4.1.2. **A**, the aortas with PVAT had a significant decrease in IL-1 β in the diet group when compared to the lean group, (diet: 7.71 ± 1.01 pg/mg vs lean: 12.00 ± 1.51 pg/mg, $p < 0.05$).

In figure 3.4.1.2. **B**, when the PVAT was removed, the IL-1 β was significantly increased in the diet group when compared to the lean group (diet: 63.07 ± 9.28 pg/mg vs lean: 13.00 ± 4.00 pg/mg, $p < 0.01$). Moreover, the lean group showed a significant reduction in IL-1 β when it was compared to the control group (lean: 13.00 ± 4.00 pg/mg vs control: 59.10 ± 9.33 pg/mg, $p < 0.001$).

The effect of RB on the IL-1 β in aortas with and without PVAT:

In the aortas with PVAT (figure 3.4.1.2.C) the RB significantly reduced IL-1 β in the diet with RB when it was compared to the diet alone (diet: 7.71 ± 1.01 pg/mg vs diet with RB: 4.27 ± 1.06 pg/mg, $P < 0.05$).

In figure 3.4.1.2.D, when the PVAT removed and RB was added, a significant increase in IL-1 β in the diet with RB was observed when compared to lean with RB (lean with RB 31.00 ± 9.28 pg/mg vs diet with RB: 63.07 ± 9.28 pg/mg, $P < 0.05$).

3.4.1.3- Pro-inflammatory marker: IL-6:

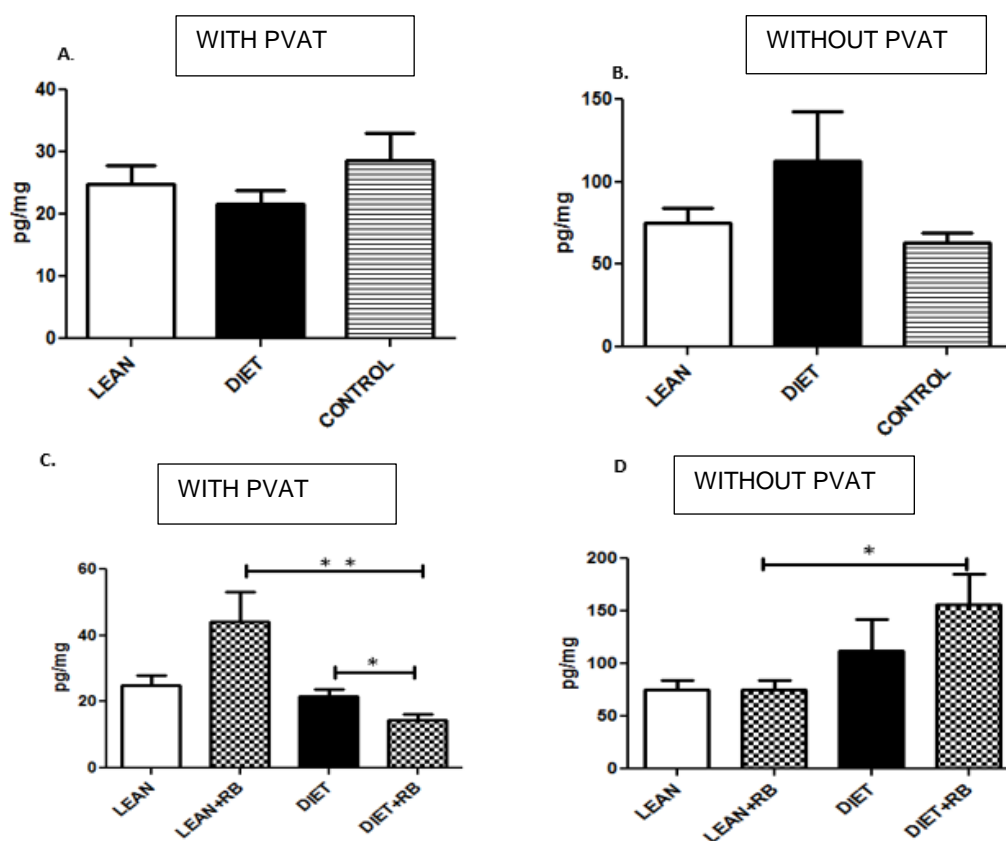


Figure 3.4.1.3: The effect of the diet on IL-6 in aortas A. with PVAT and B- without PVAT. The effect of the Rooibos (RB) on IL-6 in aortas, C. with PVAT and D- without PVAT. Data expressed as mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, $n = 6$

The effect of diet on the IL-6 in aortas with and without PVAT:

In figure 3.4.1.3 **A** and **B**, and tables 3.6 and 3.7 the diet had no significant effect on IL-6 in aortas with and without PVAT.

The effect of RB on the IL-6 in aortas with and without PVAT:

In aortas with PVAT (figure 3.4.1.3 **C**), when RB was added, IL-6 was significantly reduced in the diet with RB compared to the diet alone (diet with RB: 14.47 ± 2.00 pg/mg vs diet 22.01 ± 2.10 pg/mg, $P < 0.05$), as well as compared to the lean with RB (diet with RB: 14.47 ± 2.00 pg/mg vs lean with RB: 44.25 ± 9.00 pg/mg, $P < 0.05$),

In figure 3.4.1.3.D RB caused a significant elevation in IL-6 in the diet with RB compared to lean with RB in aortas without PVAT (diet with RB: 156.2 ± 29.24 pg/mg vs lean with RB: 75.00 ± 9.28 pg/mg, $P < 0.01$),

3.4.1.4- Pro-inflammatory marker: IL-12:

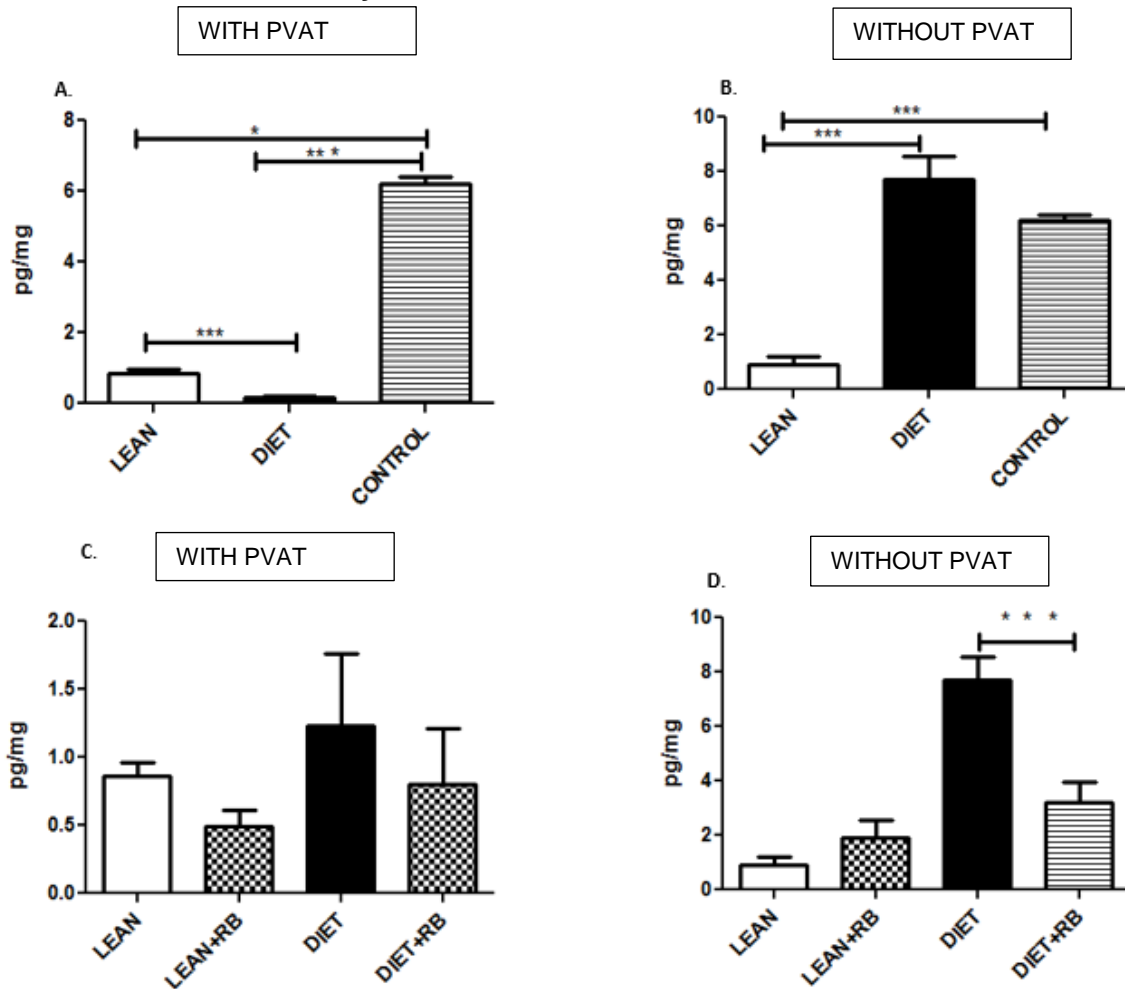


Figure 3.4.1. 4: The effect of the diet on IL-12 in aortas, A. with PVAT and B- without PVAT. The effect of the Rooibos (RB) on IL-12 in aortas C. with PVAT and D- without PVAT. Data expressed as mean \pm SEM. * $P < 0.05$, *** $P < 0.001$, $n = 6$

The effect of diet on the IL-12 in aortas with and without PVAT:

In figure 3.4.1.4.A, a significant decreased in IL-12 in the lean and diet group was observed when compared to the control group (lean: 0.86 ± 0.01 pg/mg vs control: 0.23 ± 0.03 pg/mg, $p < 0.05$) (diet: 1.23 ± 0.53 pg/mg vs control: 0.23 ± 0.03 pg/mg, $p < 0.001$). Furthermore in the diet group IL-12 significantly decreased compared to the lean group (diet: 1.23 ± 0.53 pg/mg vs lean: 0.86 ± 0.01 pg/mg, $p < 0.001$).

In figure 3.4.1.4.**B**, when the PVAT was removed, a significant increase in IL-12 in the diet group compared to the lean group was observed (diet: 8.00 ± 1.00 pg/mg vs lean: 1.00 ± 0.28 pg/mg, $p < 0.001$). The IL-12 was significantly reduced in the lean group when it was compared to the control (lean: 1.00 ± 0.28 pg/mg vs control 6.22 ± 0.10 pg/mg $p < 0.001$).

The effect of RB on the IL-12 in aortas with and without PVAT:

In figure 3.4.1.4.**C** when RB was added to aortas with PVAT, no significant differences were observed among all groups.

In figure 3.4.1.1.**D**, when the PVAT was removed, the IL-12 in the diet with RB was significantly decreased compared to the diet alone (diet with RB: 3.19 ± 1.00 pg/mg vs diet: 8.00 ± 1.00 pg/mg, $p < 0.001$).

3.4.1.5- Pro-inflammatory marker: IFN- γ :

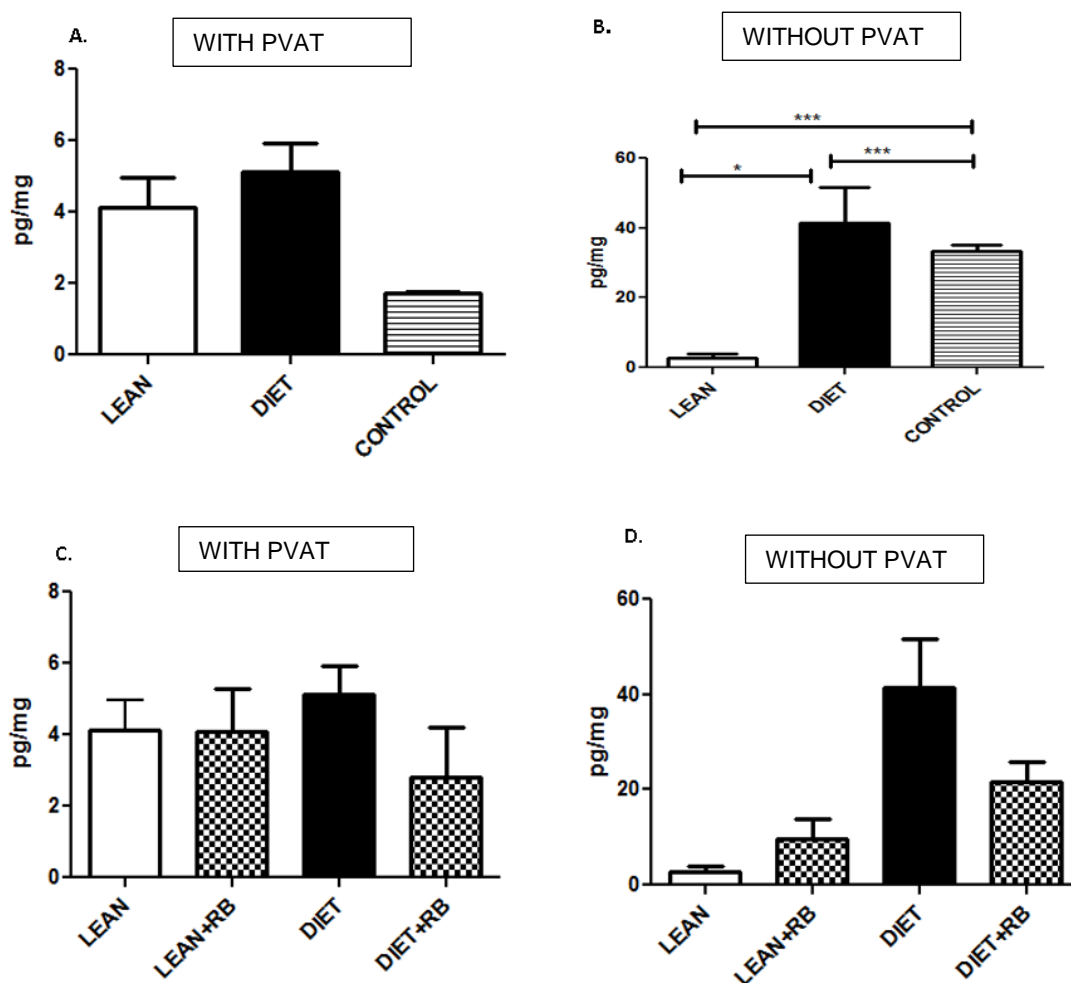


Figure 3.4.1. 5: The effect of the diet on IFN- γ in aortas, A. with PVAT and B. without PVAT. The effect of the Rooibos (RB) on IFN- γ in aortas, C. with PVAT and D. without PVAT. Data expressed as mean \pm SEM. * $P < 0.05$, *** $P < 0.001$, $n = 6$.

The effect of diet on the IFN- γ in aortas with and without PVAT:

In figure 3.4.1.5.A the different diet groups had no significant effect on IFN- γ in aortas with PVAT.

In figure 3.4.1.5.B. when the PVAT was removed, a significant increase in IFN- γ in the diet group compared to the lean group (diet: 41.60 ± 10.2 pg/mg vs lean: 3.00 ± 1.17 pg/mg, $p < 0.05$). However, a significant reduction in IFN- γ in the lean group and diet

compared to the control (lean: 3.00 ± 1.17 pg/mg vs control: 33.33 ± 2.00 pg/mg, $p < 0.001$), (diet: 41.60 ± 10.2 pg/mg vs control: 33.33 ± 2.00 pg/mg, $p < 0.001$)

The effect of RB on the IFN- γ in aortas with and without PVAT:

In figure 3.4.1.5 C and D when RB was added, no significant difference was observed among all groups whether PVAT was present or removed.

3.4.1.6- Pro-inflammatory marker: TNF- α

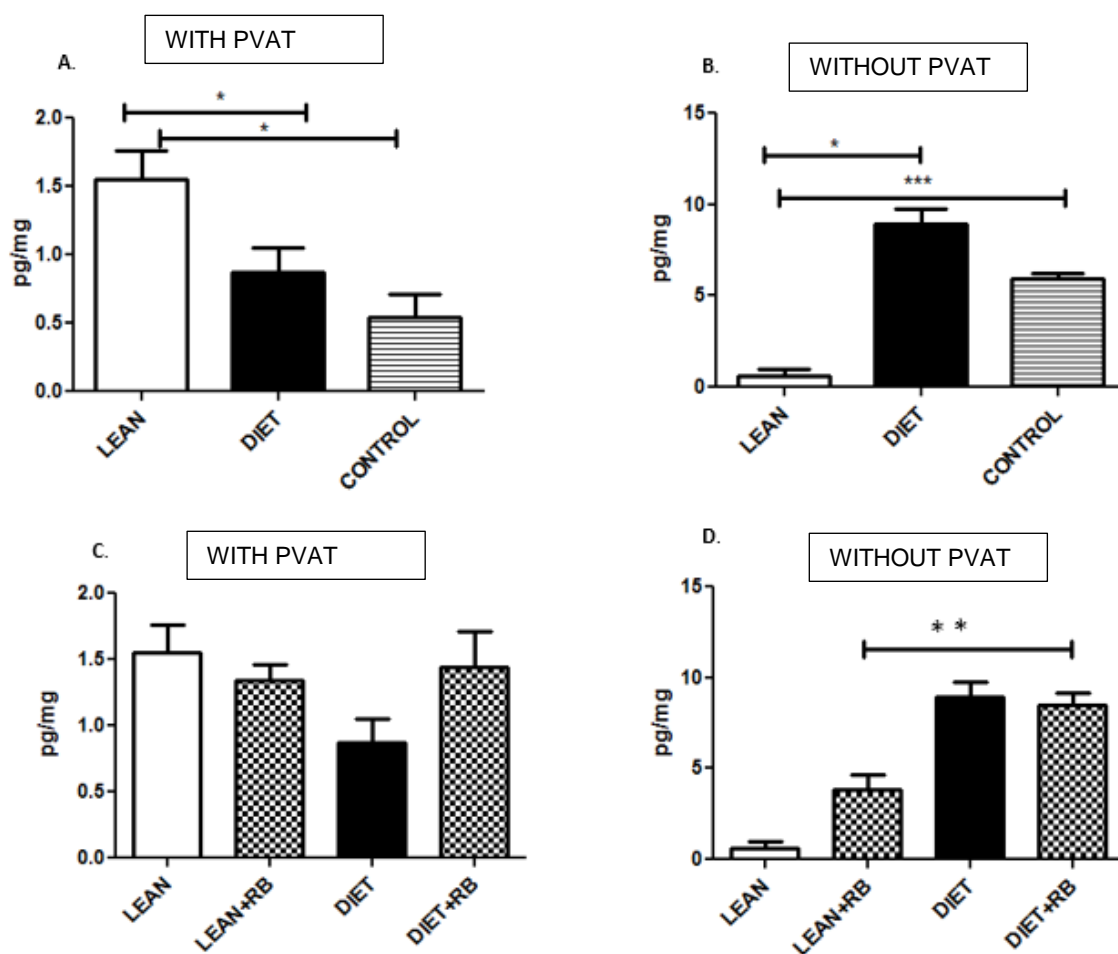


Figure 3.4.1. 6: The effect of the diet on TNF- α in aortas, A. with PVAT and B- without PVAT. The effect of the Rooibos (RB) on TNF- α in aortas, C. with PVAT and D. without PVAT. Data expressed as mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, $n = 6$.

The effect of diet on the TNF- α in aortas with and without PVAT:

In figure 3.4.1.6.A there was a significant reduction in TNF- α in the diet group compared to the lean group (diet: 5.00 ± 4.11 pg/mg vs lean: 1.60 ± 0.02 pg/mg, $p < 0.05$). . Furthermore, there was a significant increase in TNF- α in the lean group compared to the control group (lean: 1.60 ± 0.02 pg/mg, vs control: 0.53 ± 0.17 pg/mg, $p < 0.05$).

In figure 3.4.1.6.B. when the PVAT was removed, there was a significant increase in TNF- α in the diet group compared to the lean group (diet: 10.00 ± 1.00 pg/mg vs lean: 0.62 ± 0.32 pg/mg, $p < 0.05$). However, a significant reduction in TNF- α in the lean group compared to the control was also found (lean: 0.62 ± 0.32 pg/mg vs control: 5.00 ± 0.27 pg/mg, $p < 0.001$).

The effect of RB on the TNF- α in aortas with and without PVAT:

In figure 3.4.1.6.C RB did not show any effect among all groups. However, when the PVAT was removed in figure 3.4.1.6.D there was an elevation of TNF- α in diet with RB compared to lean with RB (diet with RB : 8.50 ± 0.68 pg/mg vs lean with RB : 4.00 ± 1.00 pg/mg, $P < 0.01$).

Table 3.6: Summary of the effect of diet induced obesity, PVAT, and RB on the Pro-inflammatory markers:

Pro-inflammatory	DIET (vs LEAN)		DIET+RB (vs DIET)		LEAN+RB (vs LEAN)	
	With PVAT	Without PVAT	With PVAT	Without PVAT	With PVAT	Without PVAT
IL-1 α	-	↑	↓	-	-	-
IL-1 β	↓	↑	↓	-	-	-
IL-6	-	-	↓	-	-	-
IL-12	↓	↑	-	↓	-	-
IFN- γ	-	↑	-	-	-	-
TNF- α	↓	↑	-	-	-	-

↑ Increase; ↓ decrease; - no effect.

In the aortas with PVAT the inflammatory markers decreased in the diet compared to the lean group, except for IL-1 α and IFN- γ that was not affected.

All of the inflammatory markers were elevated in aortas without PVAT of the diet compared to the lean group, except for IL-6 that did not change.

In the diet group the administration of RB has caused a decrease in IL-1 α , IL-1 β , IL-6 (in aortas with PVAT), as well as in IL-12 (in aortas without PVAT), with no effect on the other markers

RB had no effect on aortas, with or without PVAT, in the lean group.

3.4.2 Anti-inflammatory markers:

3.4.2.1 Anti-inflammatory marker: IL-2:

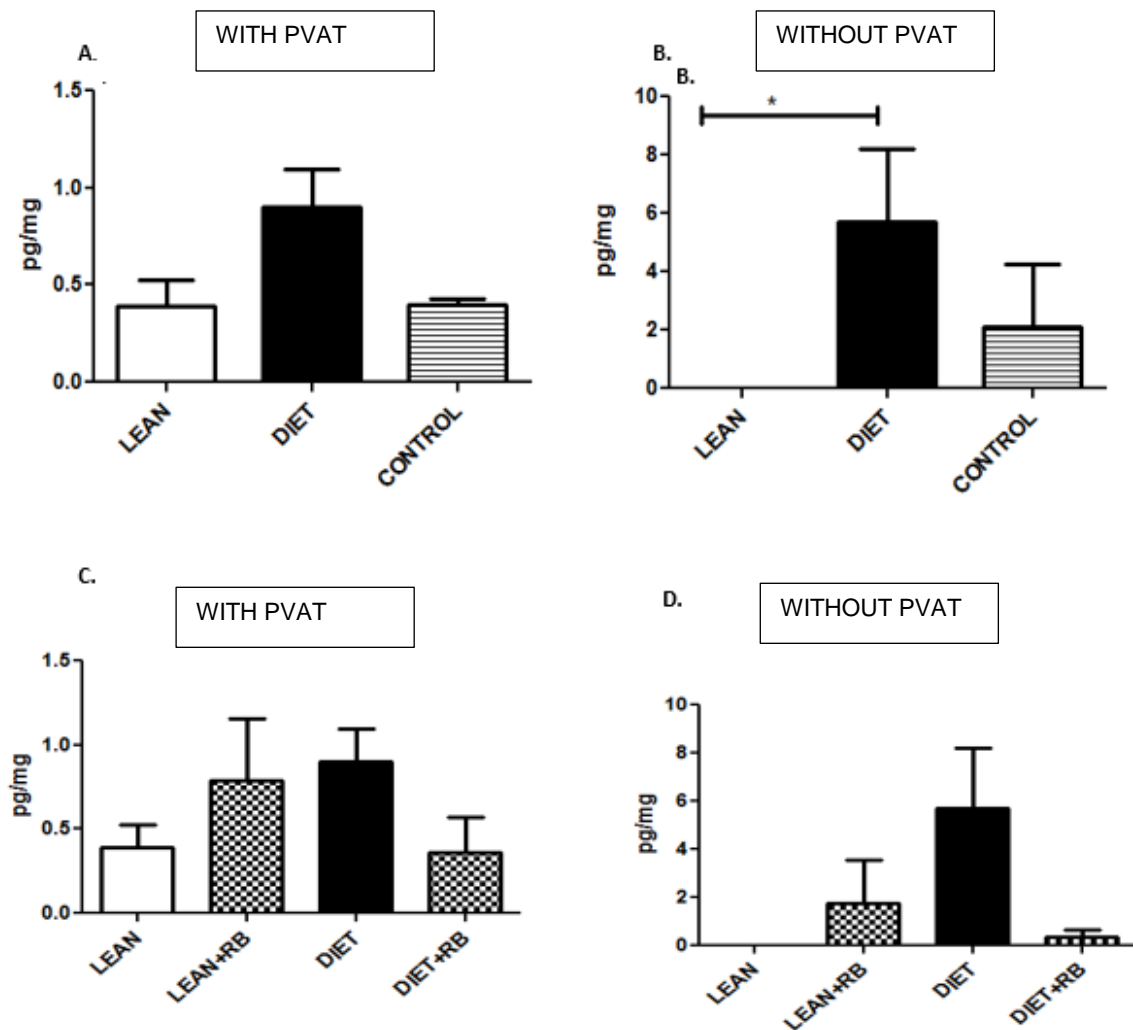


Figure 3.4.2 1: The effect of the diet on IL-2 in aortas A. with PVAT and B- without PVAT. The effect of RB on IL-4 in aortas, C. with PVAT and D. without PVAT. Data expressed as mean \pm SEM. * $P < 0.05$, $n = 6$

The only significant difference was in figure 3.4.2.1 **B**, an increase in IL-2 in aortas without PVAT in the diet compared to the lean group (diet: 6.00 ± 1.00 pg/mg vs lean: 0.00 , $p < 0.05$). Furthermore, RB administration did not caused any significant effect.

3.4.2.2- Anti-inflammatory marker: IL-4:

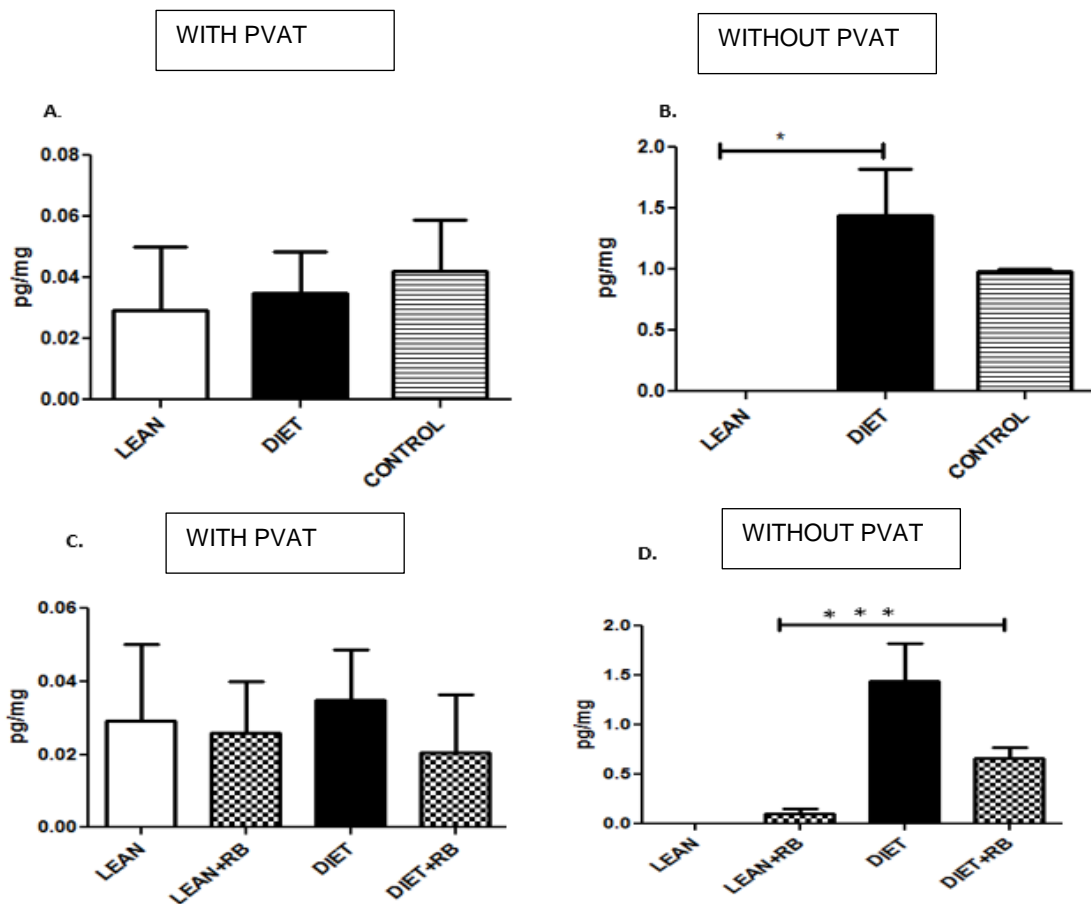


Figure 3.4.2 2: The effect of the diet on IL-4 in aortas, A. with PVAT and B. without PVAT. The effect of the RB on IL-4 in aortas C. with PVAT and D. without PVAT. Data expressed as mean \pm SEM. * $P < 0.05$, *** $P < 0.001$, $n = 6$

The effect of diet on the IL- 4 in aortas with and without PVAT:

In figure 3.4.2.2.A the diet had no significant effect on IL-4 in aortas with PVAT.

In figure 3.4.2.2.B. when the PVAT was removed, the IL-4 significantly increased in aortas of the diet group compared to the lean group (diet: 1.44 ± 0.38 pg/mg vs lean: 0.00, $p < 0.05$).

The effect of RB on the IL-4 in aortas with and without PVAT:

In figure 3.4.2.2 C when RB was added, no significant difference was observed among all group with PVAT. However when the PVAT was removed (in figure 3.4.2.2 .D) IL-

4 was higher in the diet with RB than lean or lean with RB, this is likely to be due the diet effect(diet with RB : 0.66 ± 0.10 pg/mg vs lean with RB : 0.00, $P < 0.001$).

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3.4.2.3: Anti-inflammatory marker: IL-5:

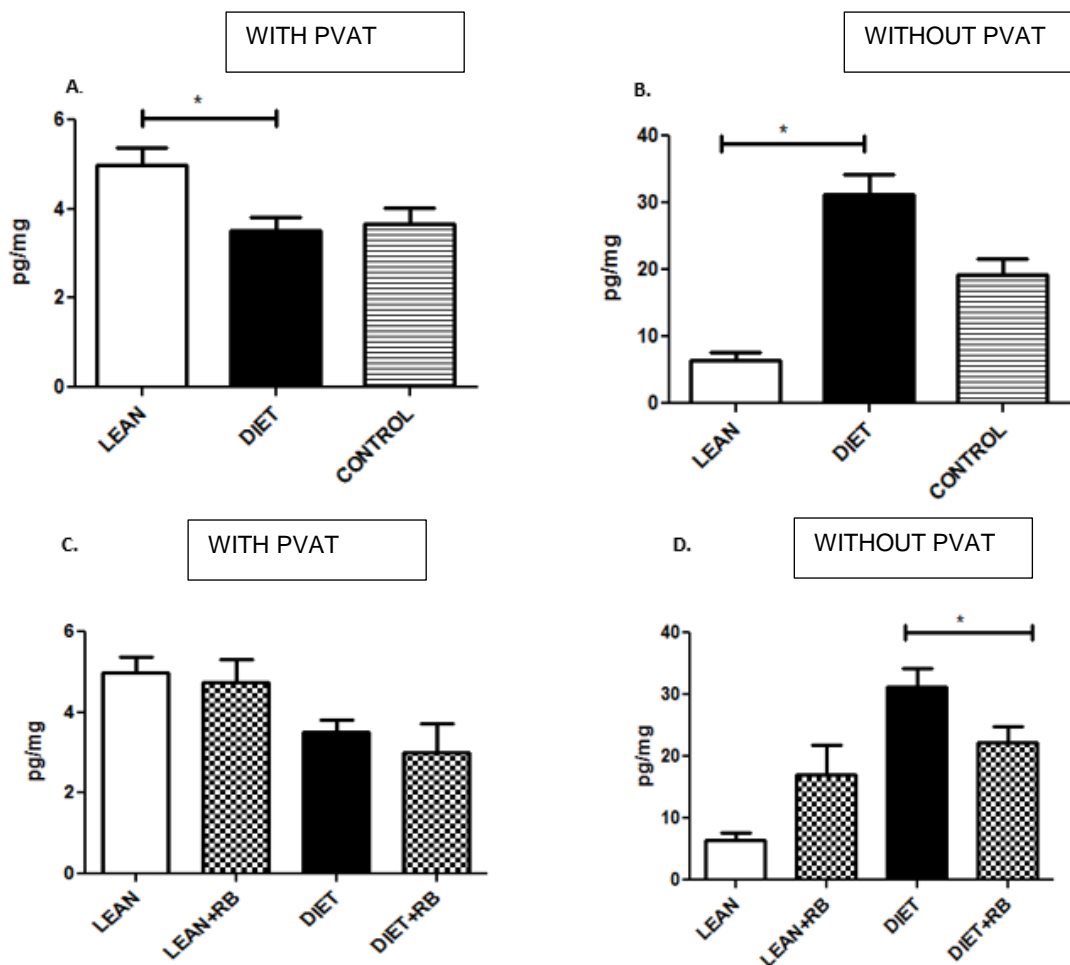


Figure 3.4.2.3: The effect of the diet on IL-5 in aortas, A. with PVAT and B- without PVAT. The effect of the RB on IL-5 in aortas, C. with PVAT and D. without PVAT. Data expressed as mean \pm SEM. * $P < 0.05$, $n = 6$.

The effect of diet on the IL-5 in aortas with and without PVAT:

In figure 3.4.2.3.A there was significant reduction in IL-5 in the diet group compared to the lean group (diet: 3.51 ± 0.28 . vs lean: 4.99 ± 0.36 , $p < 0.05$).

However, (In figure 3.4.2.3.B.) when the PVAT was removed, a significant increase in IL-5 was observed in the diet group compared to the lean group (diet: 31.15 ± 3.07 pg/mg vs lean: 6.45 ± 1.14 pg/mg, $p < 0.05$).

The effect of RB on the IL-5 in aortas with and without PVAT:

In figure 3.4.2.3.C when RB was added, no significant difference observed among all groups with PVAT. However when the PVAT was removed in figure 3.4.2.3. D, RB

significantly decreased IL-5 in the diet with RB group compared to the diet alone (diet: 31.15 ± 3.07 pg/mg vs diet with RB: 22.32 ± 2.42 pg/mg, $P < 0.05$).

3.4.2.4 Anti-inflammatory marker: IL-10:

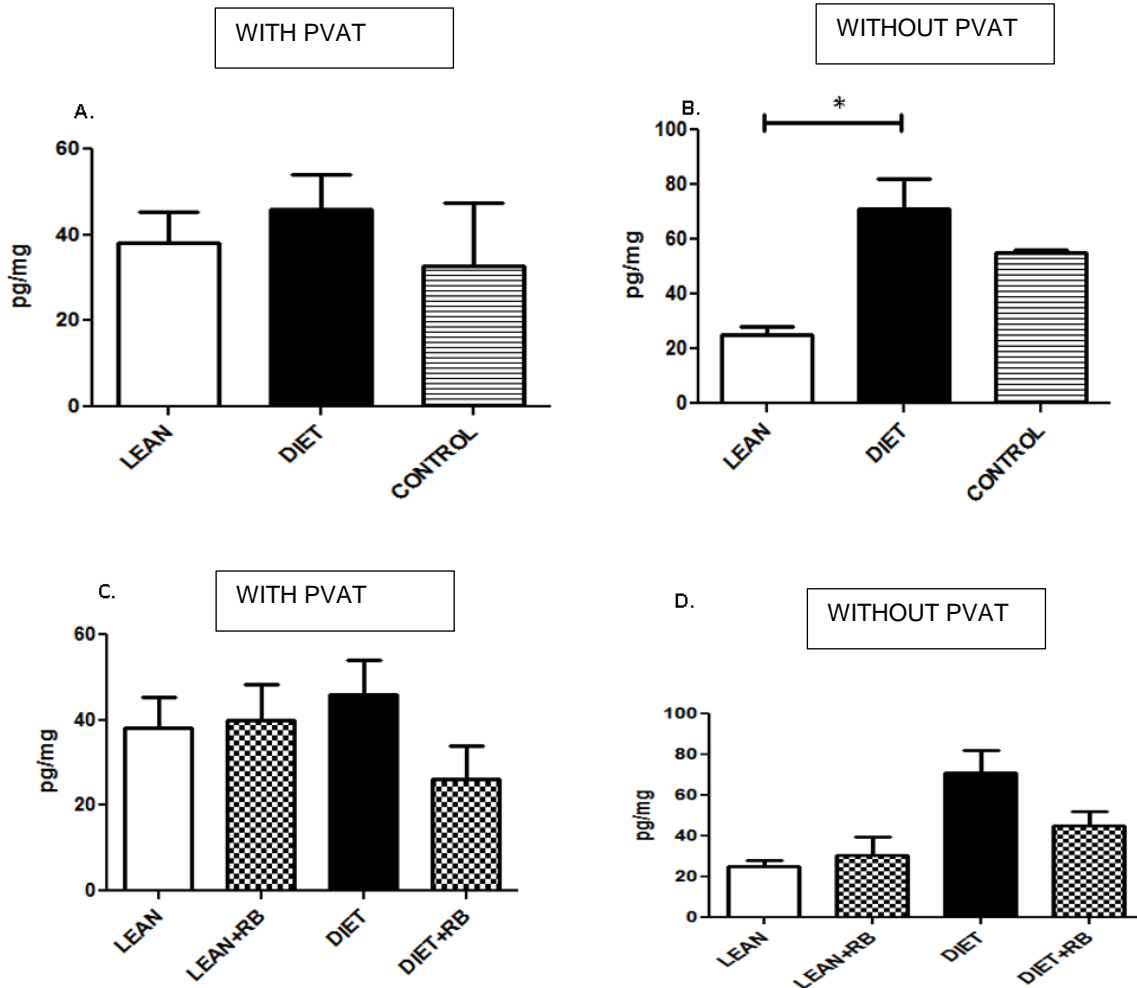


Figure 3.4.2.4: The effect of the diet on IL10 in aortas, A. with PVAT and B- without PVAT. The effect of RB on IL-10 in aortas, C. with PVAT and D. without PVAT. Data expressed as mean \pm SEM. * $P < 0.05$, $n = 6$.

In figure 3.4.2.4 the only significant difference was in figure 3.4.2.4.B, where IL-10 increased in aortas without PVAT in the diet group compared to the lean group (diet: 71.2 ± 11.00 pg/mg vs lean: 25.00 ± 3.14 pg/mg, $p < 0.05$).

3.4.2.5- Anti-inflammatory marker: IL-13:

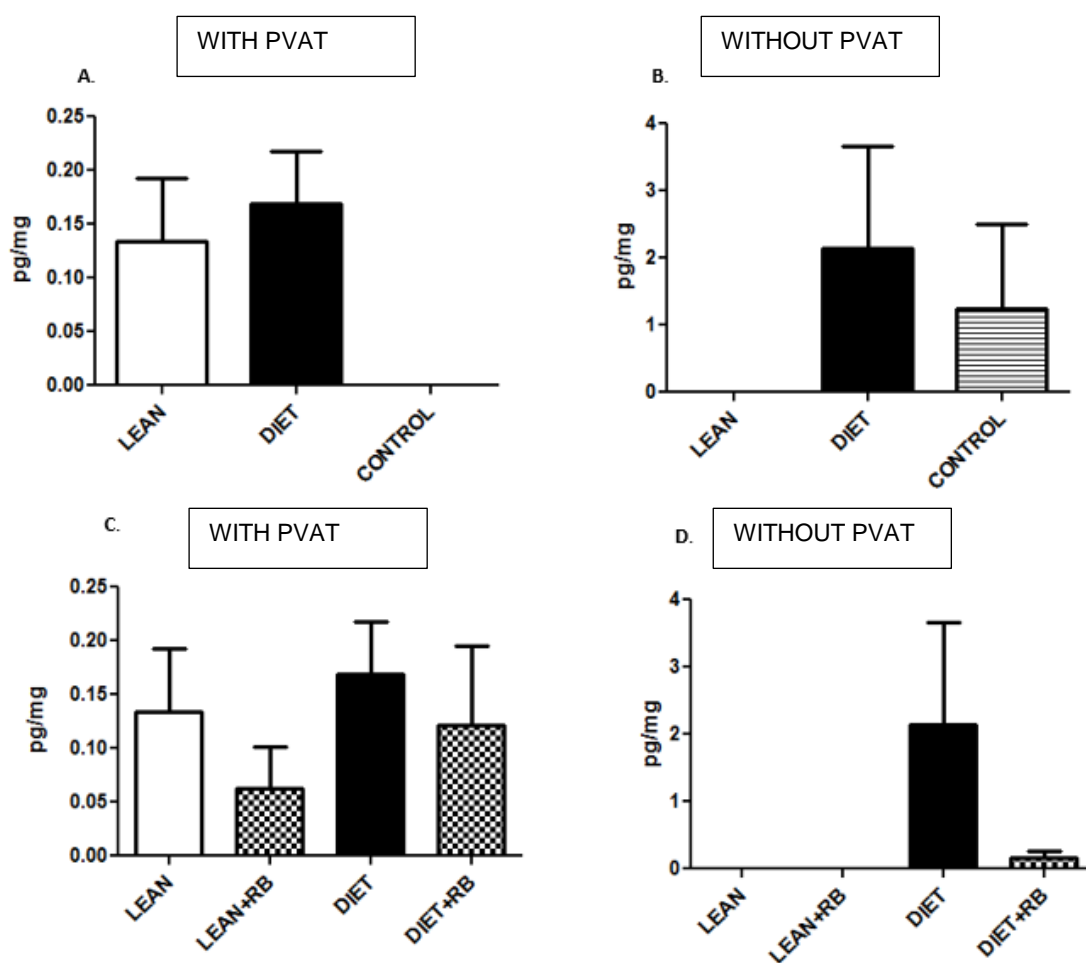


Figure 3.4.2.5. The effect of the diet on IL13 in aortas A. with PVAT and B- without PVAT. The effect of the Rooibos (RB) on IL-13 in aortas C. with PVAT and D- without PVAT. Data expressed as mean \pm SEM, n = 6.

There were no significant differences in IL-13 levels.

Table 3.7: Summary of the effect of diet induced obesity, PVAT, and RB on the Anti-inflammatory markers:

Anti-inflammatory	DIET (vs LEAN)		DIET+RB (vs DIET)		LEAN+RB (vs LEAN)	
	With PVAT	Without PVAT	With PVAT	Without PVAT	With PVAT	Without PVAT
IL-2	-	↑	-	-	-	-
IL-4	-	↑	-	-	-	-
IL-5	↓	↑	-	↓	-	-
IL-10	-	↑	-	-	-	-
IL-13	-	-	-	-	-	-

↑ Increase; ↓ decrease; - no effect.

There were no effect on any anti-inflammatory marker in the diet group with PVAT, except IL-5 which was decreased.

All of the anti-inflammatory markers were elevated in diet group without PVAT, except for IL-13 which did not change.

4. DISCUSSION

The literature indicated that obesity is linked to inflammation and oxidative stress by the release of the adipokines and different inflammatory cytokines associated with endothelial dysfunction (Vigili de Kreutzenberg et al. 2000). Furthermore, RB has been proposed to be an anti-oxidant (Marnewick et al. 2011b) as well as anti-inflammatory (Baba et al. 2009b), which could suggest that RB can protect against obesity induced endothelial dysfunction. The main aims of our project were to assess the effect of age and diet induced obesity through adding RB to the organ bath for assessment of the vascular function (through *ex vivo* aortic ring contraction and relaxation), intracellular signalling as well as the inflammatory markers.

4.1 Biometric data

In this study three different groups of rats were used: a young control (aged 16 weeks) and a 22 week old lean group on a normal rat chow diet, and 22 week old diet group on a 16 week high fat diet (HFD) with Holsum cooking fat. The obesity-inducing diet was linked to insulin resistance, as these animals presented with increased IP fat weight, basal glucose, fasting insulin as well as HOMA IR levels.

Our results have shown that the HFD induced obesity in our rat model, since there was a significant increase in the body weight and intraperitoneal fat in the diet group compared to the lean group, as shown in figure 3.1.1 and 3.1.2. This can be explained by the higher percentage of fat, sugar and carbohydrate composition (table 2.1) of the HFD compared to the normal rat chow diet. Clinical studies indicated that VAT around the aorta and mesenteric artery are linked to the development of CVD (Szasz, Bomfim & Webb 2013). In obesity and insulin resistance the fat volume of VAT and SAT in the upper part of the body is larger than the lower part (DeFronzo 2004). Therefore the HFD group may store their fat as VAT, in contrast to the lean animals, which may increase the risk for CVD in the HFD group.

We shared animals with another research group, therefore insulin and fasting glucose data were also obtained from them in order to reduce cost and animal usage. The insulin and fasting glucose levels as well as the HOMA index were significantly higher in the HFD group compared to the lean group. Therefore indicating the animals in the

HFD group were insulin resistant and pre-diabetic. This is consistent with previous studies which was done in animals on a similar diet (Kaskar 2015, Salie, 2014). In addition these studies also determined the lipid profile and peroxidation status of animals on the HFD. They observed an increase in triglyceride and free fatty acids, but no significant differences in lipid peroxidation in the HFD compared to lean groups. Therefore we could accept that our HFD group would also be dyslipidaemic.

4.2 The effect of lean and HFD on aortic ring function and intracellular signalling as well as the inflammatory markers:

One of the aims of the project was to investigate the effect of age and obesity on aortic ring function (contraction and relaxation), as well as the role of the presence or absence of PVAT.

Age-matched lean group animals showed no significant differences in aortic ring contraction with or without PVAT, compared to the controls. However, there was a decrease in the aortic relaxation with PVAT in the age-matched lean group compared to the young control group, as well as increased phosphorylation of JNK P54/P46, and elevation in IL-12 and TNF- α . This can be explained by the influence of aging on vascular inflammation and vascular dysfunction due to elevated basal stress and infiltration of peri-aortic tissue with macrophages, compared to younger animals (Bailey-Down et al 2013).

The HFD group did not show any difference in aortic ring contraction when compared to the controls (this group was young rats aged 16 weeks on a normal diet) However, when the diet group was compared to the lean group with the PVAT there was a significant decrease in the contractility, which disappeared after the PVAT was removed. Therefore it may be argued that the PVAT induced an anti-contractile effect in obese rat.

With regards to the aortic relaxation, there was no significant differences in the diet group with and without PVAT when it was compared to the control. However, the diet group relaxed more than the lean group with PVAT (pro-relaxant).

Moreover, analysis of eNOS and the upstream activators PKB and AMPK were done to gain insight in the involvement of these intracellular signalling pathways, there was

a significant increase in the phosphorylation of eNOS as well as the phospho/total ratio of eNOS in the diet group, both with and without PVAT, compared to the lean. However, there were no significant difference in the phosphorylation of PKB in aorta with and without PVAT. There was a significant increase in total expression of PKB in the diet PVAT group that led to a significant decrease in the ratio in the diet group compared to the lean. On the other hand, the AMPK did not show any significant changes in the diet group, with or without PVAT, compared to the lean,

Other signalling pathways were investigated such as the MAPKs (ERK1/2, JNK1/2, and P38MAPK) that has proven to play a role in metabolism, stress and gene expression. Similar to eNOS, the phospho/ total ratio of ERK1/2 p44 increased in aortas of the diet group, with and without PVAT. There was an increase in the phosphorylation and the phospho/total ratio of P38 MAPK in diet group, with PVAT, compared to the lean. However, there were no significant differences observed in JNK1/2.

We also investigated the effect of the diet (with and without PVAT) of the inflammatory markers which included the pro-inflammatory as well as the anti-inflammatory.

In diet aortas with PVAT there was a decrease in the pro-inflammatory markers with PVAT (IL-1 β , IL-12, and TNF- α) compared to lean. Surprisingly, when the PVAT was removed there was an elevation in all the pro-inflammatory markers (IL-1 α , IL-1B, IL-12, IFN- γ , TNF- α). Furthermore, all of the anti-inflammatory markers in aorta with PVAT were unchanged except the IL-5 which was decreased. On the other hand, when the PVAT was removed an opposite effect occur, with an increase in all of the anti-inflammatory markers except IL-13 which remained unchanged.

Since the aim of our study was to link the vascular function and mechanism as well as inflammatory markers, we propose the following:

The anti-contractile effect of diet group with PVAT that we have seen in our project correlates with another study from our laboratory (Loubser,2014) who had seen the anti-contractile effect of the diet group with PVAT, and it correlates as well with most of the literature (as summarised in table (1.2)). The anti-contractile effect of PVAT could be contributed to a combination of mechanisms such as the activation of perivascular-derived relaxing factors and the activation of K⁺- or Ca²⁺-channels (Szasz, Webb 2012).

The high percentage of relaxation (pro-relaxant) in the diet group with and without PVAT correlate with a high phosphorylation of eNOS which could be due to NO production and vasorelaxation (Forstermann, Munzel 2006b). In the present study, both the upstream activators of eNOS (PKB and AMPK) were unchanged, indicating a separate mechanism for eNOS phosphorylation. NO production can also result from the activation of eNOS by calcium/calmodulin complex (Zhao, Vanhoutte & Leung 2015)

Obesity is correlated to endothelial dysfunction due to the decrease in NO production, which is a compensatory mechanism that correlates with the enhanced superoxide and oxidative stress production (Prieto, Contreras & Sánchez 2014b). This is contradictory to our findings that the HFD increased the phosphorylation of eNOS and did not enhance nitrotyrosin (the oxidative stress marker). Superoxide production may be affected in our study by removal of the aorta from the rest of the tissues. Therefore this might be an indication of normal endothelial function in the vasculature of our diet induced obese model. This also correlates with other studies from our laboratory that indicated that rat hearts, from a high fat diet model, were protected from ischemia-reperfusion injury (Salie, Huisamen & Lochner 2014).

Furthermore, a previous study on the HFD model elicited a significant increase in the plasma lipid profile such as FFA and triglycerides, however, lipid peroxidation was not observed (Kaskar, 2015). Therefore this HFD model is dyslipidaemic but did not induce oxidative stress, which correlate with our finding that there was not any significant difference observed in nitrotyrosine. This is contradictory to a study by (Grattagliano et al. 2008) that revealed that obesity is linked to oxidative stress that increases the risk of cardiovascular disease.

As mentioned in the literature by Sethi & Vidal-Puig (2007) the association between obesity and endothelial dysfunction is inflammation, due to hypertrophy of the adipocyte and the releases of the pro-inflammatory cytokines that will lead to the increase in ROS production and in turn decrease the bioavailability of nitric oxide. In contrast, we found that in aortas with PVAT in the diet group compared to the lean, most of the pro-inflammatory cytokines were decreased, while the anti-inflammatory markers remained unchanged. Interestingly, removal of PVAT resulted in the expected increase in all the pro-inflammatory cytokines measured in aortas from the diet group

compared to lean. The elevation of pro-inflammatory cytokines in obesity indicate that inflammation is one of the mechanism that is responsible for the pathophysiology of obesity (Sethi, Vidal-Puig 2007)). A study by Jager and colleagues (Jager et al. 2007b) showed that the IL-1 β inhibited insulin stimulated glucose uptake which can mediate ERK activation, this corresponds with our finding that IL-1 β as well as ERK phosphorylation were elevated in obesity. Furthermore, TNF- α was shown to be elevated in obese humans (Hotamisligil et al. 1995). Moreover, IL-6 was also elevated in obesity and cause IR (McArdle et al. 2013). Interestingly, we found that when PVAT was present the adipokines were decreased in the aorta of the diet group, This may be due to the fact that the PVAT was masking the levels of the adipokines in the aortic tissue. It is also possible that the lack of oxidative stress in our model may be caused by this unexpected inflammatory results

On the other hand, when the PVAT was removed the anti-inflammatory markers surprisingly also increased in the diet group compared to the lean. This finding may explain why we did not see any endothelial dysfunction in the diet group and this also correlates with the increased phosphorylation of eNOS in the diet group.

Previous studies indicated an association between the inflammatory markers and eNOS signalling. A study was done by (Goetze et al. 2002, Vecchione et al. 2002) on isolated endothelial cell and human aortic rings showed that leptin stimulates eNOS phosphorylation at Ser 1177. Leptin was not measured in this study and might be one of the factors that influenced phosphorylation of eNOS in this study.

As described in the literature (Michell et al. 1999), there is a positive relationship between eNOS phosphorylation and upstream activators PKB and AMPK. However we found that the increase in eNOS phosphorylation in the diet group did not correlate with an increase PKB and AMPK phosphorylation. On the other hand we did find an increase in the expression of total PKB that might be the consequence of chronic high fat diet that is reflected in the increase in the phosphorylation of eNOS. Moreover, our finding with regards to the positive relationship between MAPKs (ERK and p38) and eNOS contradict other studies that had proven the role of MAPKs in decreasing eNOS activation (Salerno et al. 2014).

4.3 The effect of RB on aortic ring function and intracellular signalling as well as the inflammatory markers:

RB caused a decrease in the contraction of the lean group, with no effect on the relaxation, as well as increased the phospho and phospho/total ratio of P38 in aorta without PVAT, a previous study was done by Li et al 2014, showed the role of leptin in influencing vascular smooth muscle cell proliferation, through activation of P38 MAPK signalling pathway, and as mentioned in table 1.3 leptin plays an important role in endothelial dysfunction, the lack of leptin investigating in this study may be the underlying cause of activation of P38 MAPK.

RB caused increased the contractile effect on aortas with PVAT from obese rats, which may reverse the anti-contractile effect of HFD on aortas. With regards to the relaxation, RB had no effect on the aorta of the diet group with PVAT. In contrast, when the PVAT was removed, a decrease in the relaxation was observed relaxant compared to the lean group. This shows that RB is not deleterious when PVAT is present and may be beneficial to endothelial function in obese individuals.

We investigated the effect of RB on the signalling pathways in order to elucidate the underlying mechanism. The only significant effect was observed during the analysis of eNOS and ERK. RB caused a significant decrease in the phosphorylation to total protein ratio of both eNOS and ERK in aorta of the diet group (with and without PVAT) compared to the diet group without RB. These results correlate with the pro-contractile and anti-relaxation effect of RB on aortic function.

RB did not show any significant effect on the majority of the inflammatory cytokines, in spite of the fact that RB was pro-contractile. However, in the pro-inflammatory markers RB did significantly decrease IL-1 α and IL-1 β in diet group with PVAT and when PVAT was removed RB significantly decreased IL-12 levels. In addition, although RB did not have a significant effect on the majority of anti-inflammatory markers in aorta of the diet group with and without PVAT, it did decrease IL-5 and IL-6. To our knowledge this is the first study correlating the effect of RB on inflammatory cytokines in obesity. A previous study, showed that after RB supplementation, it reduced the injury from injection of LPS by reducing the pro-inflammatory markers in blood (IL-1 β , IL-6, and TNF- α) (Ajuwon, Oguntibeju & Marnewick 2014), this correlates with the decrease in IL-1 β and IL-6 that we have found with RB. These findings are

contradictory to the others, and this may be explained by the fact we measured the cytokines in the aortic tissue and not in the plasma or blood as indicated in the previous study.

4.4 Conclusion:

Administration of RB to the organ bath in *ex vivo* aorta was according to our knowledge, a novel study. In contrast, most other studies used RB supplementation to the diet.

The main findings of the thesis are as follow:

Age match lean group effect:

There was a decrease in the relaxation of the lean group compared to the control. Also, RB treatment decreased the contraction on the lean group compared to the control.

Diet and PVAT effect:

There was a decrease in the contractility in the diet group with PVAT compared to the lean due to the anti-contractile effect of PVAT. Moreover, the diet group (with and without PVAT) had increased contractility and decreased relaxation compared to the lean.

RB effect

Adding RB to the organ bath increased the contraction in the diet group (with and without PVAT), while it decreased the relaxation of the diet group (without PVAT).

The underlying cellular mechanism had shown an increased phosphorylation and phospho/ total ratio of eNOS and ERK1/2 (with and without PVAT) in the diet group compared to the lean.

Finally, this study demonstrated that RB may reverse the contractile inhibition in the aorta of obese rats. Aortas in which the PVAT was present showed no significant difference in relaxation. Furthermore, RB had showed an increase in aortic contraction, and a decrease in aortic relaxation, when no PVAT was present in obese

rats. This shows that RB is not deleterious when PVAT is present and may be beneficial to endothelial function in obese and lean individuals. Furthermore, RB caused a decrease in the phosphorylation of eNOS and ERK1/2 which correlated with the increased aortic relaxation.

4.5 Limitations:

The amount of animals in this study were limited due to lack of funding. Therefore it limited our ability to further elucidate the role of RB in aortic function (see suggested further studies), as well as in the Western blotting only a limited number of samples (n=3) per experiment could be compared with each other. To improve this analysis one could use a 36 well gel instead of the 12 well gel to compare more samples with each other.

4.6 Further studies:

Since this study is preliminary work that could direct future studies, such as:

- To evaluate the involvement of the endothelium-derived nitric oxide aortic rings could be denuded (by removing the intimal layer of the tissue with a blunt needle) or incubation of aortic rings with N ω -nitro-L-arginine methyl ester (L-NAME), a non-specific NOS inhibitor aortas.
- A dose-response with increasing doses of RB could determine the optimal effectiveness and potential detrimental effects of direct administration of RB to an organ.
- The administration of the separate compounds, like *Aspalathin* and *Nothofagin*, instead of using the crude RB extract could clarify the specific effect of RB.
- The analysis of the inflammatory markers in the plasma, not only in the tissue, would give a better indication of these compounds in the circulation.
- The separation of the PVAT from the aortic tissue after RB administration before freezing and analysing these tissues separately could clarify the role of RB in these tissues. .
- The analyses of the other cytokines, such as adiponectin, leptin and resistin could aid in elucidating the effect of RB in diet-induced obesity.

- The investigation of other pathway in signalling molecules such as the apoptotic pathway, could elucidate if RB plays a role in cell death.

5. ADDENDUM:

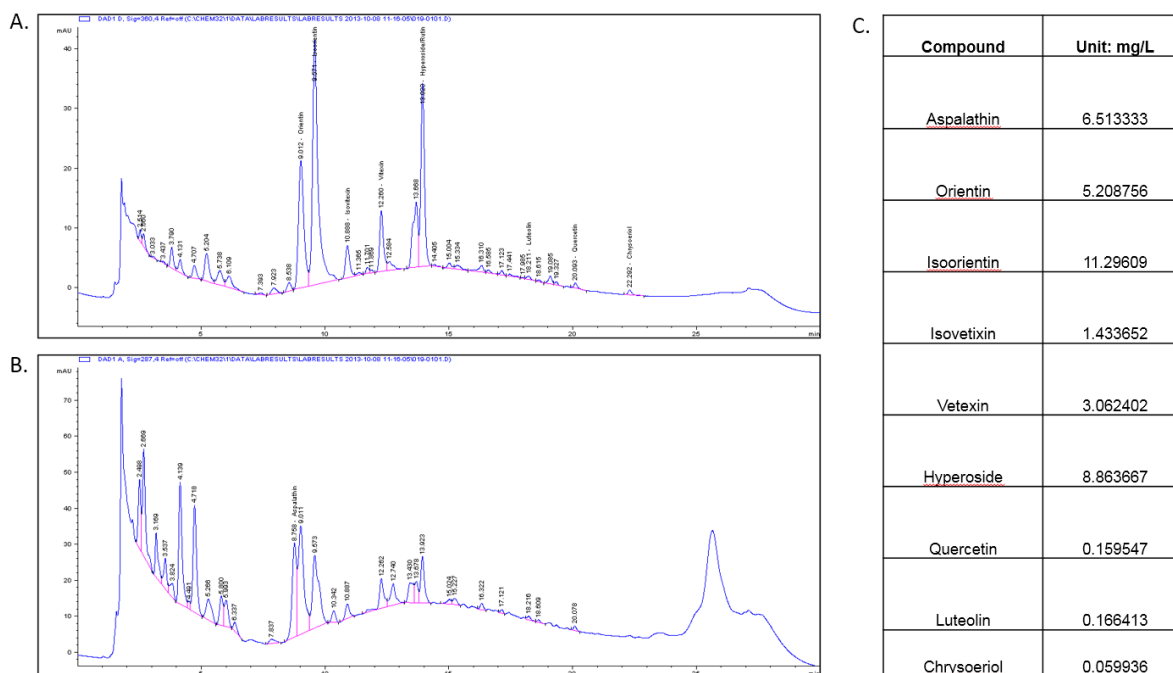


Figure 5.1: HPLC analysis of RB extract. A. Chromatogram at 360nm, B. Chromatogram at 287nm, C. Compound content of the RB extract (units: mg/L)

Table 5.3 : Insulin level, fasting blood glucose level and HOMA index in lean and diet group

	Fasting glucose level (mmol/L)	Insulin (mIU/ μ l)	HOMA index
Lean	4.71 \pm 0.0458 n=10	6.6837 \pm 2.2099 n= 6	1.55 \pm 0.70 n=6
Diet	5.4 \pm 1.534 n=10***	14.576 \pm 2.036 n=5*	4.68 \pm 0.87 n=5*

Significance differences are presented as *p<0.05 diet vs Lean; *** p<0.001 diet vs Lean.

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