# 中 國 醫 藥 大 學
## 臨床醫學研究所
## 碩士學位論文

# 大白鼠血管週邊脂肪組織對血管影響之新進展
# Novel vascular actions of rat aortic perivascular adipose tissue


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# 中文摘要

## 大白鼠血管週邊脂肪組織對血管影響之新進展

肥胖乃長久以來影響身體健康的一個問題，肥胖已被證實與很多疾病有關，諸如高血壓、糖尿病、冠心病、心衰竭、腦中風等。今天，由於社會的進步，飲食之多元化，使肥胖在全球之比率日益俱增。基於肥胖對身體之不良影響科學界已著手研究脂肪組織細胞，他們發現脂肪細胞其實是有多功能性的，因其內之 Adiponnectin 可分泌脂肪激酶 Adipokines，而引發出自體分泌，旁分泌及內分泌等種種作用。

血管是控制血壓的一重要角色，血管主要包含有三層，即內層的內皮細胞層、中層的平滑肌層，以及外層的外膜層，而外膜層又往往被二到三條的週邊血管脂肪組織所圍繞著，在最近 10 年中，學者們紛紛提出，此等週邊血管脂肪組織，是會釋出一些未知之因子，來調節血管功能，因而與血壓有關。在大多數之研究結果認爲，此等因子是有增加血管之舒張能力，而少數學者則認爲有增加血管之收縮能力，此因子暫定爲 是一種血管衍生舒張因子， (Adipose derived relaxing factor ADRF) 我們利用大白鼠之動脈及其週邊所黏附的脂肪組織在不同的情況下，諸如有脂肪黏附的主動脈血管環，去脂肪組織之血管環，及去脂肪組織而附加脂肪組織溶液之血管環，與不同的試劑，如苯腎上腺素 ( Phenylnephrine PE ) 氯化鉀 (Potassium Chloride KCl ) Carbocheol ( CCh )及血管收縮素 Angiotensin Ⅱ Ang. Ⅱ ) 進行反應，結果顯示，不論在有黏附脂肪組織的血管環，或在脂肪組織而附加脂肪組織溶液之血管環，在與 PE 及 KCl 反應下，皆有增加血管收縮之作用，又當我們用 $N^G$-Nito-L- arginine methyl ester ( L-NAME ) 來抑制內皮細胞釋放 Nitie Oxide ( NO ) 後，此等收縮現象依然存在。當洗去收縮劑 ( PE 及 KCl )後，那些有黏附脂肪組織的血管環，對血管舒張之速度，又比那些去脂肪組織的血管環有明顯緩慢性作用。因此，我們暫定此等脂肪組織，會釋放一種因子，命名爲『 脂肪細胞衍生因子 』 ( Adipose derived Factor ADF ) 此等因子是有提高血管收縮之能力的。

當去脂肪組織之血管環與內皮細胞舒張作用有關之 CCh 作用反應，比較其附加脂肪組織溶液之前及附加之後之變化。我們發現，在這些有附加脂肪組織溶液

前之血管環，其有良好之血管舒張作用。但在附加脂肪組織溶液後，則有明顯之減低舒張作用，而此一作用反應，在洗去脂肪組織溶液後，又可回復之前之血管舒張作用，在與血管收縮素 Ang. Ⅱ 作用時，發現在去脂肪組織之血管環中，那些有附加脂肪組織溶液的血管環，對血管收縮的能力，遠較那些沒有附加脂肪組織溶液的血管環爲高，此外，當脂肪溶液加熱到沸點時，則會喪失其血管收縮的作用，故可假想此等脂肪組織，爲一種蛋白質類之結構物，加熱後會被去自然 (De-nature) 而被破壞。

結論，經過多次實驗所得之結果，我們發現，週邊血管脂肪組織，確實是有增加血管收縮之作用，雖然此些發現與之前之文獻報告大有出入，但在現實生活中，肥胖人士容易產生高血壓及血管之病變，則又達到一合理共識之據點。

# Abstract


## A novel vascular action of perivascular adipose tissue

Obesity has been a long term problem in health. It was proved to have a high relationship to many systemic diseases, such as hypertension, diabetes mellitus, coronary vascular disease, heart failure and stroke. Large increase in the prevalence of obesity over the world have resulted largely from alterations in environmental factors that increase energy intake and reduce physical activity sedentary to lifestyle and work activities.

To learn the pathological effect of obesity to the body, scientists studied adipose tissue cells. They found that the adipose cells have multiple functions which release adipokines to exert autocrine, paracrine and endocrine effects. Blood vessel plays an important role in blood pressure control . They consist of three main layers: The inner endothelial layer, the media smooth muscle layer and the outer adventitia layer which is also surrounded by fat tissue called perivascular adipose (fat) tissues (PVAT). In recent years, investigators have been proposed that the perivascular adipose tissue could affect vascular reactivity via releasing some unknown factors and can thus provide the regulation of blood pressure. Most of the previous studies found that the perivascular adipose tissue exerts a vessel relaxation effect and a diffusible adipose derived relaxation factor (ADRF) was thus proposed although not yet identified.. We have studied the effects of PVAT on the rat thoracic aortic artery under different conditions, such in fat - intact aortic rings, fat - denuded aortic rings, fat denude aortic rings following transfer of solution incubated with fat (fat solution) or boiled fat solution. Aorta were stimulated with phenylnephrine (PE), potassium chloride (KCl), angiotensin II (Ang II), and the carbachol (CCh). In contrast to the previously study, we found that either the in situ aortic fat or the solution incubated with isolated fat can increase contractile response in the form of enhanced sensitivity to PE and KCl. This respondes persist even after the inhibition of the endothelium nitric oxide (NO) production with $N^G$-nitro-L arginine methyl ester (L-NAME). Upon wash out of PE and KCl, a slower relaxation rate was found in the aortic rings with intact perivascular adipose tissues. For the endothelium dependent relaxation to carbachol (CCh), it was reduced in the presence of fat pre-incubated solution and can be reversed after wash out of the fat solution. In response to Ang II, the aortic rings also showed an enhance

contractile response in the upon  transfer of fat pre-incubated solution. When the fat solution has been boiled, no enhance contractile response was he notion that obese people with high visceral fat content has a higher risk in developing high blood pressure and vascular complications characteristic of  metabolic syndrome

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Dedicated to my parents
and family

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## List of Abbreviations

| | |
|---|---|
| ADF | Adipose derived factor |
| ADP | Adenosine 5 diphophate |
| ADRF | Adipose derived relaxing factor |
| Ang.II | Angiotensin II |
| AT1 | Angiotensin II receptor 1 |
| ATP | Adenosine tri-phosphatase |
| ATP | Adenosine tri-phosphate |
| BMI | Body mass index |
| CaM | Calcium calmodulin complex |
| c-AMP | Cyclic adenosine monophosphate |
| CCh | Carbachol |
| c-GMP | Cyclic guanosine monophosphate |
| CNG | Cyclic nucleotide gated |
| DAG | 1-2, Diacylglycerol |
| DHPs | 1, 4-dihydropyridines |
| EDCF | Endothelial derived contractive factor |
| EDRF | Endothelial derived relaxing factor |
| eNOS | Endothelium nitric oxide synthase |
| ERK | Extracellular signal regulate kinase |
| ET | Endothelin |
| F - | Fat tissue denuded |
| F + | Fat tissue intact |
| F solution | Fat solution |
| FFA | Free fatty acid |
| GPCR | G protein coupled receptor |
| GTP | Guanosine triphosphate |
| iNOS | Inducible nitric oxide synthase |
| IP3 | Phosphoiniosite the 1,4,5 trisphosphate |
| IRS | Insulin receptor substrate |
| KATP | ATP sensitive potassium channel |
| KCl | Potassium chloride |
| LDL | Low density lipoprotein |

| | |
|---|---|
| L-NAME | $N^G$-nitro-L arginine methyl ester |
| LTCC | L-type calcium channel |
| MAPK | Mitogen activate protein kinase |
| MLC | Myosin light chain |
| MLCK | Myosin light chain kinase |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NCX | Sodium calcium exchange |
| nNOS | Neuron nitric oxide synthase |
| NO | Nitric oxide |
| ONOO | Peroxynitrite |
| PAI-1 | Plasminogen active inhibitor-1 |
| PDE1 | Phosphodiasterase-1 |
| PE | Phenylnephrine |
| PIP2 | Phosphatidyl inositol 4-5 bisphosphate |
| PKC | Protein kinase C |
| PLC | Phospholipase C |
| PVAT | Perivascular adipose tissue |
| RAS | Renin angiotensin aldosterone system |
| RyR | Ryanodine receptor |
| SEM | Standard error of the mean |
| SOD | Superoxide dismutase |
| SR | Sarcoplasmic reticulum |
| TNF | Tumor necrosis factor |
| TTCC | T-type calcium channel |
| VDCC | Voltage depend calcium channel |
| VSM | Vascular smooth muscle |
| VSMC | Vascular smooth muscle cell |

# Chapter 1

## Preface

Obesity is defined as an excess of body fat accumulation with elevated body mass index (BMI). Men and women with a BMI of 25.0 to 29.9 Kg/$m^2$ are considered over weight, and those with a BMI > 30 Kg/$m^2$ are considered obese (1).

Obesity is a global epidemic as a result of confluent socioeconomic condition and high caloric food intake, not only in developed countries, but also reaching epidemic proportion in the developing countries.

Large epidemiological studies have established that there is a strong inverse relationship between BMI and mortality (2). The etiology of obesity is complex and still not well understood. It can involve in genetic, life style, environmental and psychological factors (3). Under the normal physiological condition, body fat is stored as energy depot (Calories) in adipocytes. A lean adult has about 35 billion adipocytes. Each adipocyte contains about 0.4 to 0.6 μg of triglycerides (4). The triglyceride storage within adipose tissue constitutes the body major energy reserve. The pool average of a man and a woman with normal weight has a 10-20% and 15-25% adipose tissue, respectively (5). When this stored energy lost balance due to excessive food intake or insufficient use of energy, the excessive calories are stored as fat in adipose fat tissue resulting in obesity. Obesity is a significant risk factor for many systemic diseases, direct or indirectly associated with an increased incidence and prevalence of heart disease, diabetes, hypertension, stroke, cardiac arrhythmia and dyslipidemia (6). All these diseases involve structural and /or functional changes in blood vessels.

Today, physicians and health care professionals are facing the challenge combating against in the complications and sequelae of obesity. The blood vessels of many visceral organs are surrounded by perivascular fat tissues, but the roles of these fat tissues other than its insolating and protecting effects are not clear. To find out whether the perivascular fat tissue has any physiological impact on the blood vessel function, we designed different experiments to test the presence and absence of perivascular fat tissue on the contractility of rat aortic rings to different stimulants, such as phenylephrine, potassium chloride, carbachol and angiotensin II.

# 1. Background

## 1-1.Vascular tissues and functions

The basic constituents of the wall of blood vessels have 3 concentric layers, the intima, media and outer layers (figure 1). Beside these 3 layers, there are also tissue surrounding the outer vessel wall. For example, 2 to 3 strips of fat tissue are associated with rat aorta. In smaller arteries the entire vessel segments are surrounded by fat tissues.

The intima (endothelial layer) consists of a single layer of endothelial cells with minimal underlying sub-endothelial connective tissue. It is separated from the media by a dense elastic membrane called the internal elastic lamina. Functions of the endothelial layer are structural maintenance of vessel wall, homeostasis and normal circulatory function. As a semi-permeable membrane, endothelium controls the transfer of small and large molecules across the vascular wall. It also expresses adhesion molecule, cytokines and chemokines (7). These vasoactive molecules result either in vasodilatation or vasoconstriction functioning as relaxing factors, such as nitric oxide (NO) (8, 9) and contracting factors, such as endothelin (ET) (10).Under normal physiological condition, they exist in a balanced and co-ordinated manner.

The media (smooth muscle layer) consists of concentric sheets of smooth muscle cells embedded in the frame work of connective tissue, such as collagen and elastin fibers It receives oxygen and nutrients by direct diffusion from the vessel lumen.(11) The primary function of smooth muscle layer is to maintain vascular tone and to regulate peripheral vascular resistance. They are two types of contraction, phasic and tonic depending on the nature of the stimuli. These contractile and elastic compoments can resist the pressure generated by the heart pump (12).

The outer (adventitia) layer, a very complex layer consisting of connective tissues, collagen fibers, nerve fibers and small vessels. The collagen fibers may blend into those of the adjacent tissue. The function of the adventitia involved stabilizing and anchoring the blood vessels (13). The fibroblasts derived from adventitia also possess the capacity of contracting and relaxing (14). They have recently been shown to be a rich source of reactive oxygen species thus limiting nitric oxide bioactivity (15). The fibroblasts contains substantial amount of NADPH oxidase, which can produce reactive oxygen species that affect fibroblast proliferation, connective tissue deposition and vascular tone modulation (16) (figure 1).

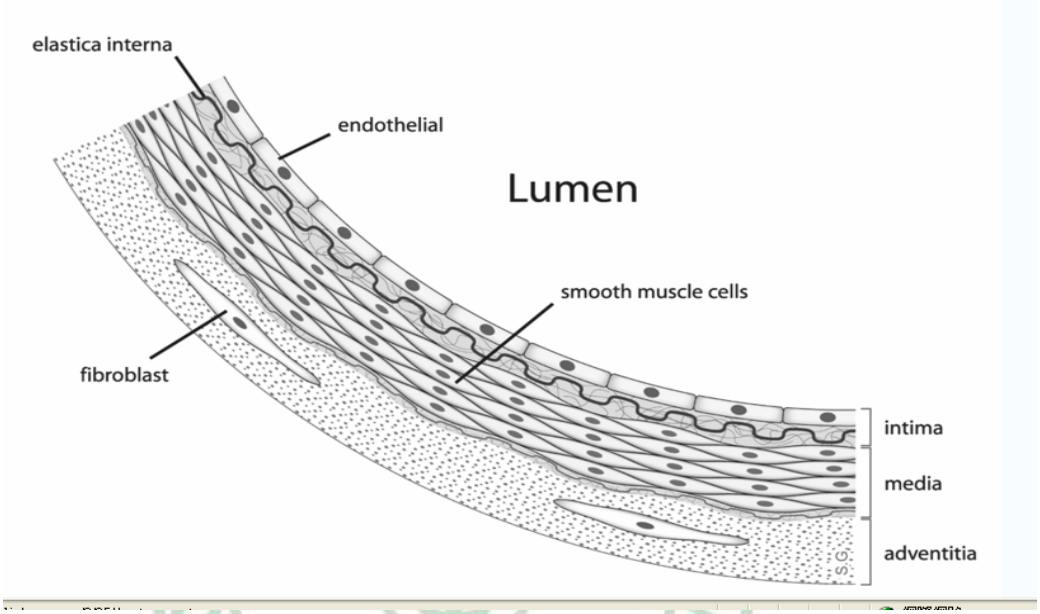


Figure 1. The basic structure of blood vessel. The inner layer called intima which consists of endothelium layer. The middle layer called media which full of smooth muscle cells. The outer layer called adventitia which consists fibroblast, collagen an nerve fibres.

**(Alberts. et al, molecular biology of the cell, 2002)**

## 1-2 Adipose tissue and function

Fat tissue (adipose tissue) is a loose connective tissue that contains many lipid filled cells called adipocytes. The formation of adipose tissue is controlled by the adipose gene. Adipocyte is derived from fibroblast-like precursor cells (pre-adipocytes) under appropriate stimulatory condition (17). Adipose tissue contains, leukocytes, macrophages, small blood vessels and pro-inflammatory cytokines and chemokines (18).Obesity is associated with enlarged fat cells and an increased number of macrophages in the adipose tissue surrounding individual adipocytes (19). Adipose tissue is commonly found beneath the skin, and is also found around the internal organs such as abdominal cavity, intestine and blood vessels. An increased mass of adipose tissue can occur by hyperplastic growth , which is an increase in number of adipocytes or by hypertrophic growth, an increase in size of adipocytes. Both increased number and size of adipose tissue can result in obesity (20)

The adipose tissue function as the major storage site for fat in the form of triglycerides. Approximately, 1 gram of fat can yield 9 Kilo.calories.(21) In mammals, adipose tissue is found in 2 different forms: The white adipose tissue and the brown adipose tissue, the latter found mainly in infants. White adipose tissue is distributed in multiple depots found both subcutaneously and internally. Approximately 60-85% of weight of white adipose tissue is lipid, it serves as heat insulation, mechanical cushion to protect organs from mechanical damage and energy source. With the discovery of leptin, white adipose tissues are recognized to secret several hormones. The function of adipokines involved lipid metabolism, insulin sensitivity, vascular function and blood pressure regulation (22). Brown adipose tissue which derives its color from rich vascularization and dense mitochondria is predominantly comprised of multilocular adipose cells. It stores nutrients in the form of triglycerides to generate heat in order to warm the body (23).

Adipose tissue also severs as an important endocrine organ (figure 2). for many secretory substance, such as adiponectin, leptin, resistin, tumor necrosis factors (TNF), plasminogen activate inhibitor (PAI-1), angiotensin (Ang), acylation-stimulating protein (ASP) and Interlukein-6 (IL-6), to name a few (24) (figure 2).

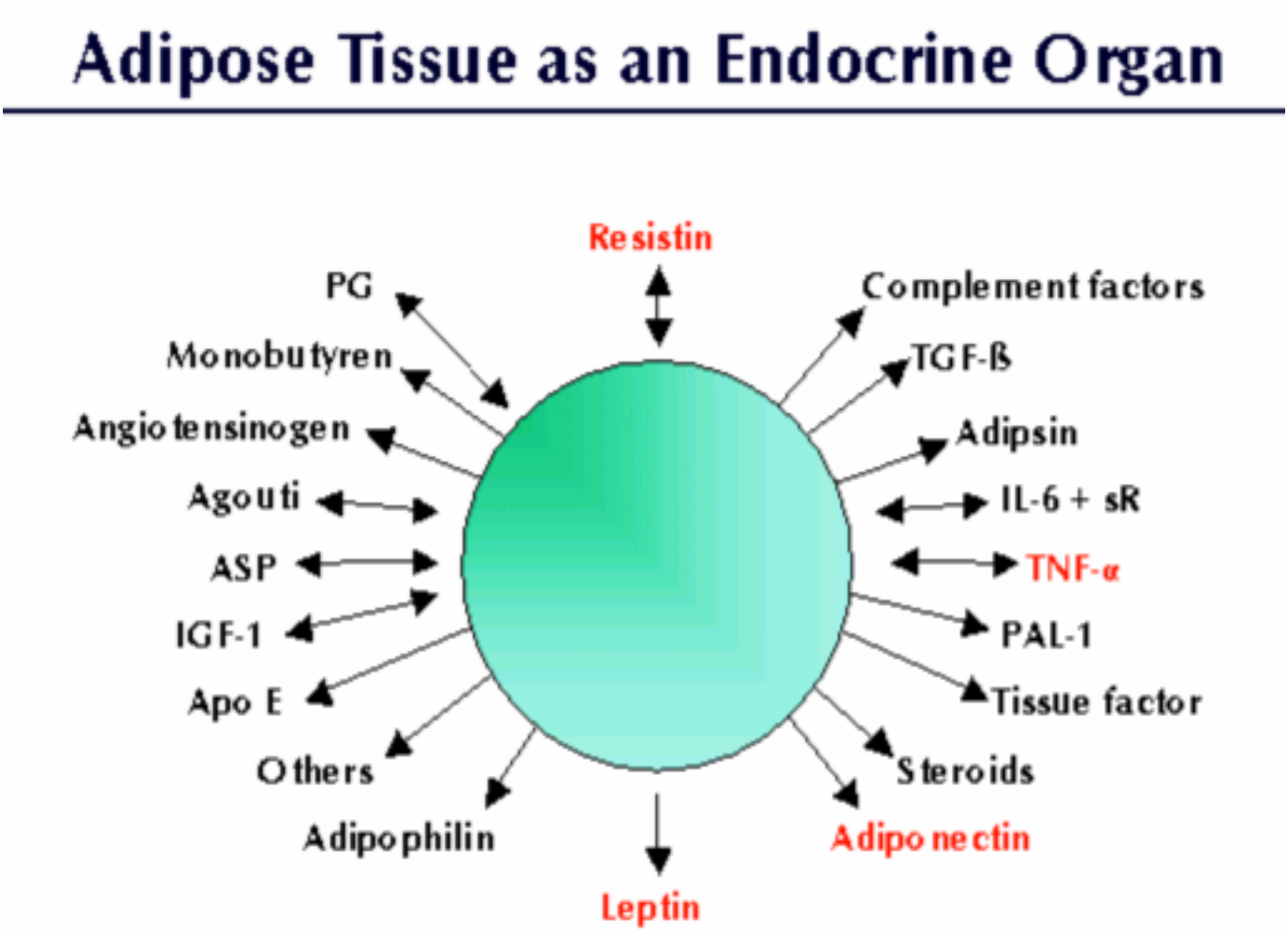


Figure 2: Adipose tissue can secret more than 20 different kinds of hormones, which play many important physiological functions.

**(Gema, Frobeck, Mattew. 2001)**

## 1-3. Diseases that are related to obesity

### 1-3-1: Metabolic syndrome:

European investigators recognized that abdominal adiposity was related to greater risk of cardiovascular events. The key components of metabolic syndrome include central obesity, insulin resistance, high triglycerides, low high density lipoprotein (LDL) and hypertension (25) (figure 3). The body mass index (BMI) is equal to the weight in kilograms divided by the height in meters squared. It is defined that normal BMI: 18.5 to 25 $Kg/m^2$. Overweight: 25 to 30 $Kg/m^2$. Obesity: > 30 $Kg/m^2$ (26) . Adipose tissue is and active secretory organ that elaborates a variety molecule known as adiopkinase. This kinase including tumor necrosis factor (TNF), α-interleukin-6, leptin, resistins and adiponectin,(24) all that mediated many of the metabolic change in the metabolic syndrome. The TNF α - interleukin-6 plays as an inflammatory marker in skeletal muscle and liver. Leptin can reflect adipose tissue mass and body energy balance, the plasma leptin level was found to be predictive of cardiovascular events. Resistin as a hormone that links obesity to diabetes by increasing insulin resistance. Adiponectin has antiatherogenic properties, can maintains coronary flow reserve, inhibit monocyte adhesion to endothelial cells, reduce macrophages that transform to foam cells and prevent the episode of thrombus formation (27, 28). However, adiponectin, which is produced only by the white adipose tissue. This hormone is reduced in states of insulin resistance, such as obesity and type II diabetes. And thus, in metabolic syndrome, patient is easily to get coronary artery disease, stroke or others vascular disease due to the reduce formation of adiponectin (29) (figure 3).

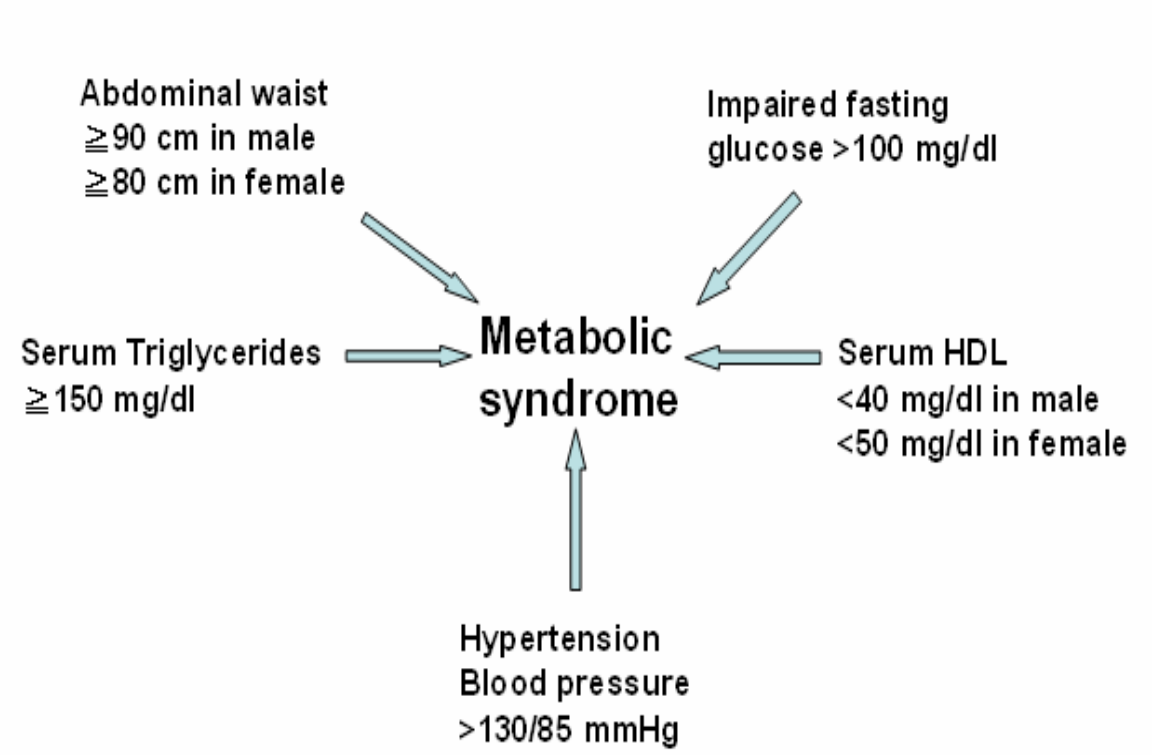


**Figure 3**: The metabolic syndrome, which includes hypertension, increase fasting serum glucose level, hyper-triglycerides, reduce HDL cholesterol level and increase abdominal waist. Only 3 out of 5 criterias can be said to have metabolic syndrome. ( Criterias from Taiwan health department )

## 1-3-2: Coronary artery disease

Coronary artery disease is due to chronic inflammatory response of the arterial wall initiated by injury to the endothelium, called atherosclerosis (30). Frequently also called cholesterol – dependent clogging of the arteries. In hyperlipidemia, particularly hypercholesterolemia, a high level of low density lipoprotein (LDL) may directly injure endothelial layer, and impair endothelial function through increase by reactive oxygen spieces accumulation –collectively, termed "oxidative stress" (31). In endothelial dysfunction, monocytes adhere to the endothelium and migrate into intima that differential into macrophage, at that it also increase permeability of leukocytes vascular cells adhesion molecule and lympocytes. All these substance migrate from smooth muscular layer to the endothelial layer and transform into macrophage. Marcophage then becomes activate, will engulf oxidative LDL form a foam cell (31). Accumulate of the foam cells then form a plaque When the blood pressure increases – resulting in increased shear stress to the blood vessels, the plaque ruptures, and further attracts a large amount of platelets and leukocytes which aggregate to the plaque and form a thrombus (atheroma) (32). When this thrombus enlarged, it will block the circulation of coronary blood vessel, which reduce the blood flow, form coronary artery disease. This process is demonstrated as shown in figure 4.

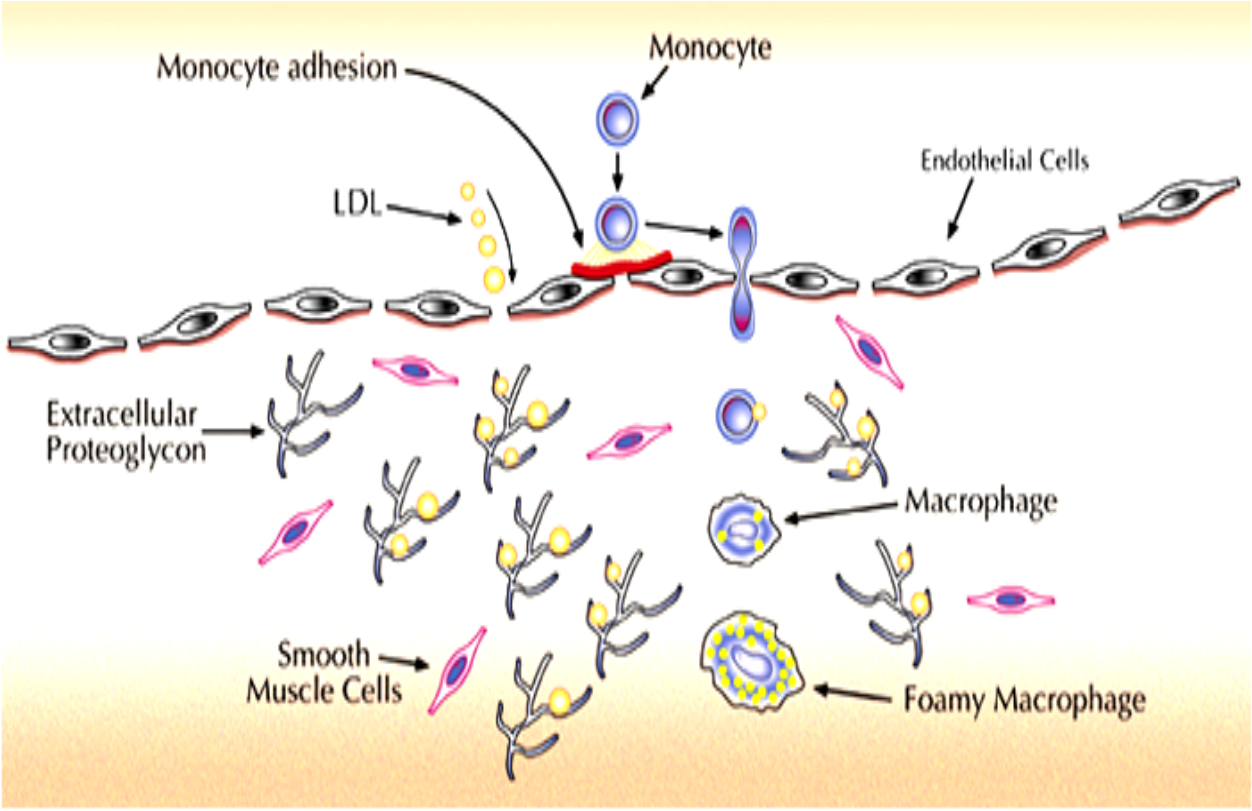


**Figure 4:** The formation of atheroma in coronary artery disease. In blood circulation, when LDL is increase, monocytes then adhere into the endothelial layer. Then migrate into the media layer and derived into macrophages. Macrophages engulf LDLs and become the foam cells. Large amount of foam cells accumulate to form a plaque. When the plaque rupture, lots of platelets, leukocytes aggregate into the plaque to form an atheroma.

**(Williams K,J. and Tabas I. Art. throm. vas. biol. 1995)**

## 1-3-3: Diabetes mellitus

Adipose tissues not only store lipid fuel but also release free fatty acids (FFAs) and glycerol into the circulation via lipolysis. Adipose tissue lipolysis is regulated primarily by insulin inhibition and catecholamines stimulation (33). Obesity is associated with several abnormalities of adipose tissue lipolysis, most notably resulting in higher FFA concentrations and insulin resistance. Insulin stimulates glucose disposal in muscle and suppress plasma FFA concentrations. Thus, Obesity is an important risk factor of type 2 diabetes mellitus. Hyperglycemia independently impairs endothelial function, probably acting in part through decreased generation of nitric oxide and in part through protein kinase C (PKC) and the formation of reactive oxygen species. The increase activity of PKC, activates the formation of vasoconstrictor endothelin-1 and decrease activity of the vasodilator endothelial nitric oxide synthase (eNOs), (34). Increase production of tumor necrosis factor – α (TNF-α) by adipocytes can reduces insulin sensitivity, reduces activity of insulin receptors substrate (IRS-1) and enhance production of pro-coagulant molecule plasminogen activator inhibitor (PAI-1) which leading to increase insulin resistance, formation of free fatty acid and reduce fibrinolysis that induces vascular occlusion disease (35) (figure 5).

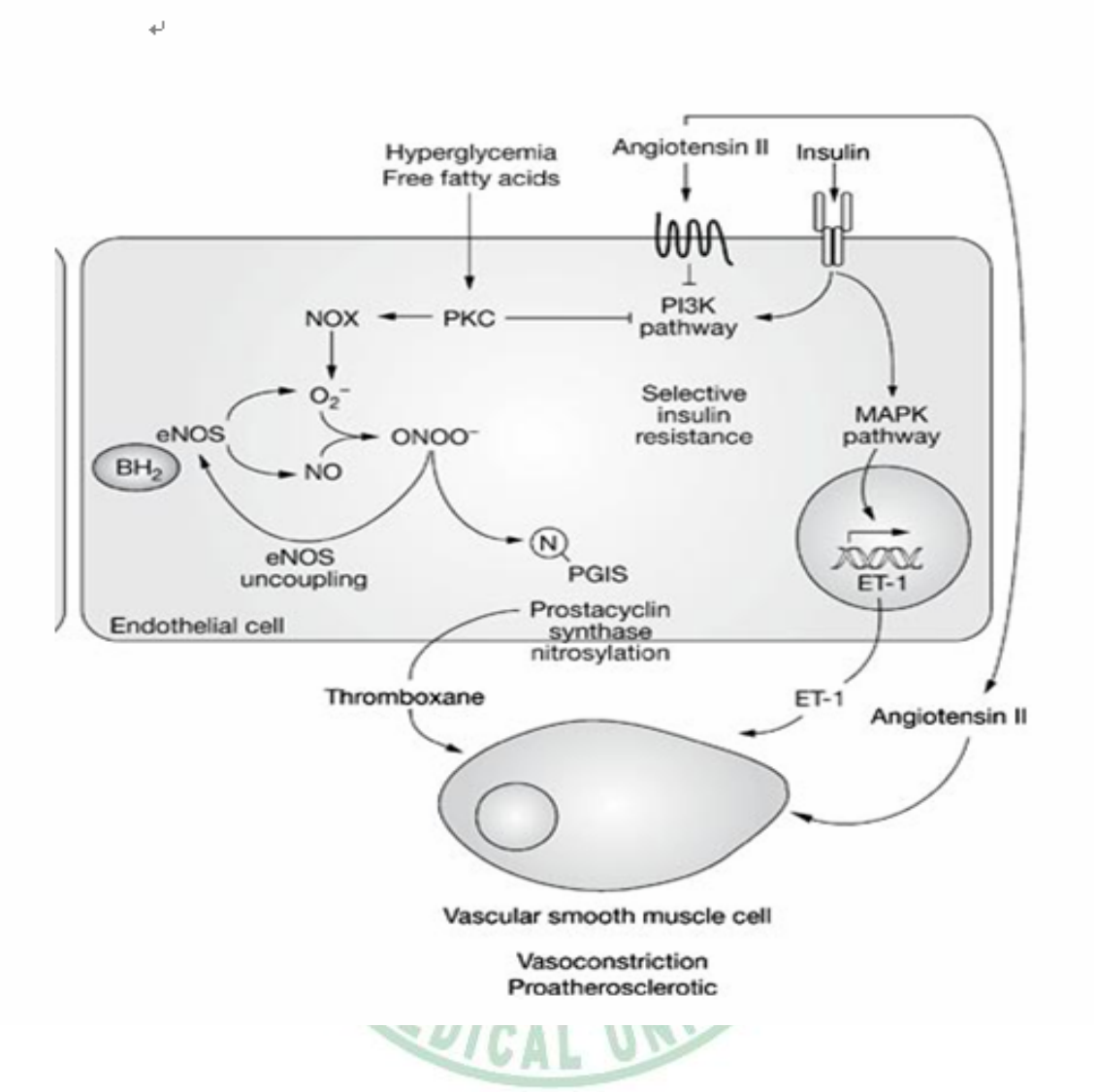


**Figure 5**: In diabetes patient, hyperglycemia increases free fatty acid. This can active protein kinase C (PKC), PKC blocks PI3K pathway, reduces NO formation and increase endothelin-1 (ET-1). All these result in vasoconstriction. Also, adipocytes secrete tumor necrosis factor-1 ( TNF-1) which reduce insulin sensitivity.

**( Jose Luis, Gonzalez-Sanchez. Drugs new perspect. 2007 )**

## 1-3-4: Hypertension

Blood pressure can be increased by a number of interactive and complicated mechanisms. It includes, increased circulating blood volume, abnormal vasoconstriction, decreased vascular relaxation and increased cardiac output (36). The causes of essential hypertension are not clear. It may relative to many factors, such as ethnic origin (black > white), environments and diet (high salt diet), gender (male > female), psychological problem (anxiety and stress), age, obesity, heavy drinking (37). All these can increase peripheral vascular resistance. The secondary hypertension are usually due to hormonal factors. The increase adrenalin or angiotensin. cause vessel constriction. In adrenal tumor, such as pheochromocytoma, has an excessive secretion of catecholamines (38). In renal disease, such as renal artery stenosis, which induce large amount of renin-angiotensin secretion (39). In parenchymal renal disease, often associated with sodium and water retention (40).The abnormalities of vascular resistance may also contribute to the patho-physiology of obesity-related hypertension. Elevated free fatty acids (FFAs) have been found to cause increased vasoconstriction and reduced nitric oxide mediated vaso-relaxation (41, 42). It has also been suggested that there is an increased activity of the sympathetic nervous system by leptin in some obesity phenotypes, adipose tissue can secrete renin-angiotensin-aldosterone system (RAAS), which activates the formation of angiotensin II, fatty acid increases aldosterone secretion from adrenal gland (43), all these may contribute to the vasoconstriction, observed in the obesity-associated hypertension (figure 6).

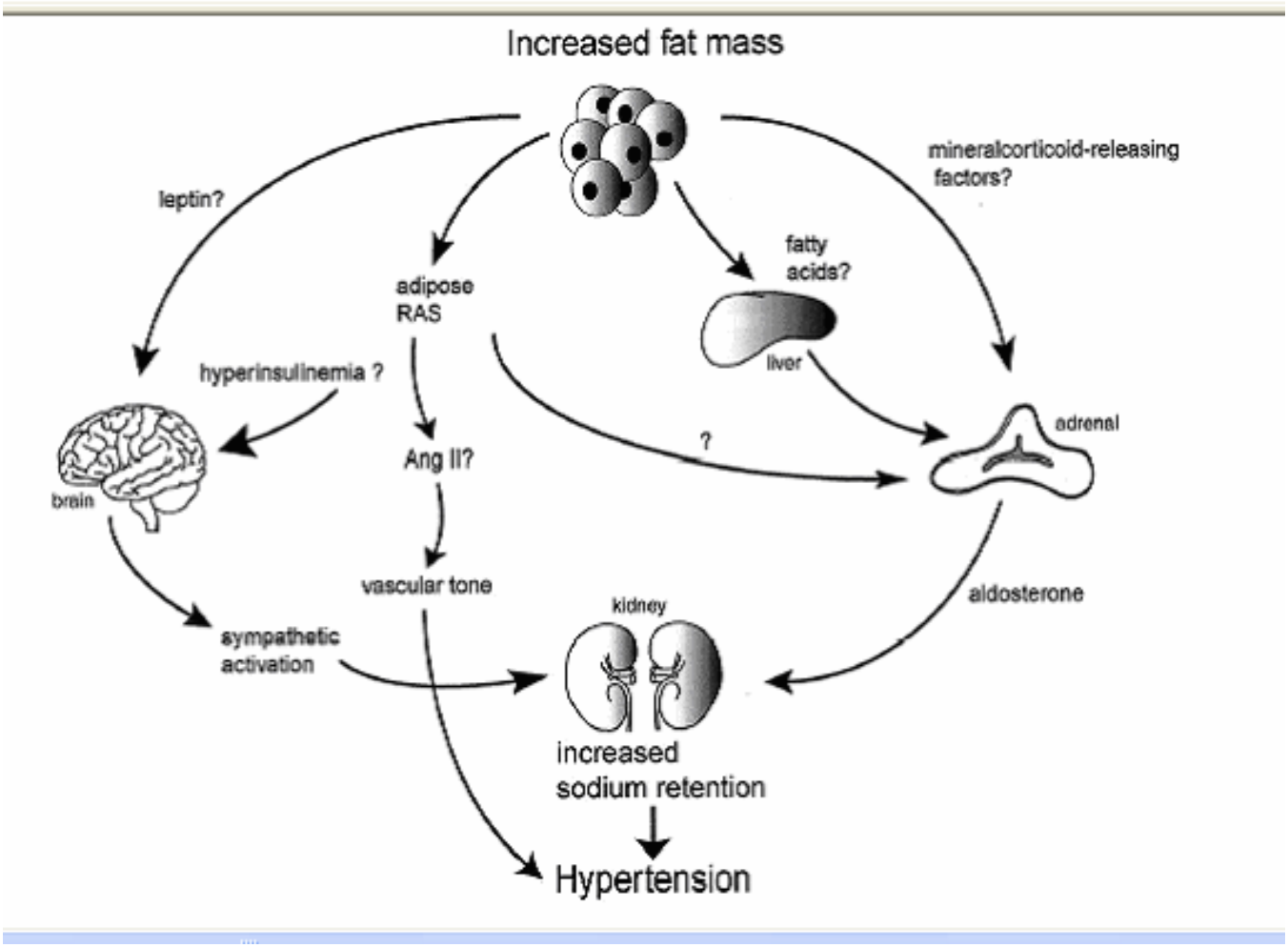


**Figure 6:** The adipose tissue has several factors to induce vasoconstriction. Through leptin to increase sympathetic activity, fatty acids to increase aldosterone secretion all cause sodium retention. The adipose RAS can increase angiotensin II secretion. All these can cause vasoconstriction..

**(Lamounier Zeptor et al. Obesity-related hypertension. 2004)**

## 1-4. Physiology of blood vessel contraction and relaxation

The blood vessel tone is elicited by smooth muscle in the medial vessel wall. Smooth muscle cells are responsible for the contractile capabilities of blood vessels (large and small arterials, veins), respiratory tract, gastrointestinal tract, bladder, male and female reproductive tracts.

Being different from the skeletal muscle and cardiac muscles, smooth muscle is non-striated muscle. There are two types of smooth muscle arrangements in the body: multi-unit and single-unit. The single-unit type, also called unitary smooth muscle, is far more common. Whereas the former presents itself as distinct muscle fibers that are usually activated by their own nerve fibers, the latter operate as a single unit and are arranged in sheets or bundles (44). Smooth muscle cells have, in general, single nuclei and a plethora of mitochondria. In order to contract, the cells contain thick actin filaments and a thick element called myosin. Whereas the filaments are essentially the same in smooth muscle as they are in skeletal and cardiac muscle, the smooth muscle cell contains less protein than a typical striated muscle cell and much less myosin. The actin content is similar, so the ratio of actin to myosin is ~6:1 in striated muscle and ~15:1 in smooth muscle ( 44, 45). Smooth muscle does not contain the protein troponin; instead calmodulin (which takes on the regulatory role in smooth muscle), caldesmon and calponin are significant proteins expressed within smooth muscle (44).

Unlike in striated muscle, the actin and myosin in smooth muscles are not arranged into distinct sarcomeres that form orderly bands throughout the muscle cell. The Actin filaments attach to the sarcolemma by focal adhesions or attachment plaques and attach to other actin filaments via dense bodies (acting much like Z-lines in striated muscle). Evidence indicates that smooth muscle myosin is not bipolar with a central bare zone as in striated muscle, but is either side-polar or row-polar, and has no bare zone (45).

The sarcolemma possess microdomains specialized to cell-signaling events and ion channels called caveolae. These invaginations in the sarcoplasma contain a host of receptors (prostacyclin, endothelin, serotonin, muscarinic receptors, adrenergic receptors), second messenger generators (adenylate cyclase, Phospholipase C), G

proteins (RhoA, G alpha), Protein kinase C, Protein Kinase A, ion channels (L type Calcium channels, ATP sensitive Potassium channels,) in close proximity. The caveolae are often in close proximity to sarcoplasmic reticulum or mitochondria, and have been proposed to organize signaling molecules in the membrane (44, 45).

## Smooth muscle contraction:

Smooth muscle contraction is caused by the sliding of myosin and actin filaments (a sliding filament mechanism) over each other (46). Myosin functions as an ATPase utilizing ATP to produce a molecular conformational change of part of the myosin and produces movement. Movement of the filaments over each other happens when the globular heads protruding from myosin filaments attach and interact with actin filaments to form crossbridges. The myosin heads tilt and drag along the actin filament a small distance (10-12 nm). The heads then release the actin filament and adopt their original conformation. They can then re-bind to another part of the actin molecule and drag it along further. This process is called crossbridge (46). Unlike cardiac and skeletal muscle, smooth muscle does not contain the calcium-binding protein troponin. Contraction is initiated by a calcium-regulated phosphorylation of a heavy meromyosin, rather than a calcium-activated troponin system (47).

Crossbridge cycling cannot occur until the myosin heads have been activated to allow crossbridges formation. It needs the presence of ATP. The myosin heads are made up of heavy and light protein chains. When the light chains are phosphorylated, they become active and will allow contraction to occur (47, 48). The enzyme that phosphorylates the light chains is called myosin light-chain kinase (MLCK). In order to control contraction, MLCK will work only when the muscle is stimulated to contract. Stimulation will increase the intracellular concentration of calcium ions. These bind to a molecule called calmodulin, and form a calcium-calmodulin complex. It is this complex that will bind to MLCK to activate it, allowing the chain of reactions for contraction to occur. The phosphorylation of the light chains by MLCK is countered by a myosin light-chain phosphatase, which dephosphorylates the myosin light chains and inhibits the contraction (49) (figure 7).

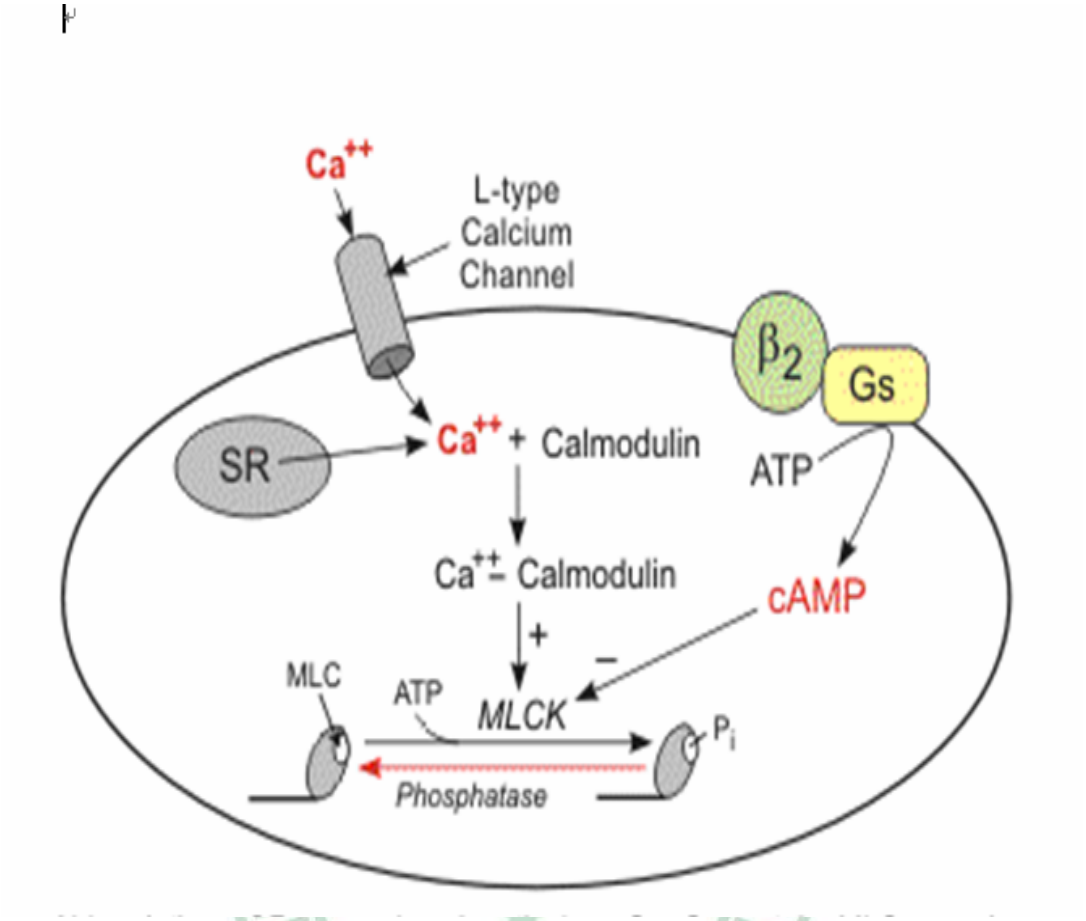


**Figure 7: I**n smooth muscle cell, increase level of cytosol calcium can be increased through calcium influx L-type calcium channel in sarcolemma and calcium release from sarcoplasmic reticulum (SR) inside cell. The calcium binds to calmodulin. This complex then active myosin light chain kinase (MLCK), which increase myosin-actin interaction form muscle contraction.

**(Richard E. Klabundle, CV physiology concept, 2007)-**

## 1-4-1 Factors in controlling vascular smooth muscle contraction

1-4-1-1: Calcium signalling in vascular smooth muscle: In the plasma membrane, there is L-type calcium channel, a voltage gated calcium channels. When an α1 stimulation occur, form an active membrane potential that depolarize cell membrane. This L-type calcium channel then open, allowing calcium ions influx into cell and bind to the Ryanodine receptor (RyR) of the sarcoplasmic reticulum (SR). The RyR open, release large amount of calcium from SR through the RyR (50).

α1 also form an active signal to the G-protein coupling receptor (GPCR). GPCR then activate the formation of IP3, the IP3 then releases calcium from SR through the IP3 sensitive calcium release channels. The calcium ions which are release in cytosol then binds to an protein-calmodulin and form calcium/calmodulin complex. This complex binds to myosin light chain kinase (MLCK), to activate MLCK, then phosphorylate myosin light chain and increase the myosin binds to actin cause smooth muscle contraction (50).

1. Voltage-dependent calcium channel (VDCC):

This channel are activate at depolarized membrane potential. High selective for calcium that allowing substantial amounts of calcium to enter the cells when membrane is depolarized and do not conduct the sodium or potassium. In voltage depend Ca. Channels, There are five subtypes: such as L,T, N, P and R types. The L and T types are predominant in cardiac muscles, where the L type are predominant in smooth muscle. The others are in nerve terminals (51).

(a). L type calcium channels (LTCC): represent a long lasting opening channel, are characterized by a large single–channel conductance are sensitivity to the 1,4-dihydropyridines (DHPs) and activation at larger depolarizations (52). Opening of the L-type calcium channel causes influx of extracellular $Ca^{2+}$ into cytosol, which then binds to calmodulin The activated calmodulin molecule activates myosin light chain kinase (MLCK),which phosphorylates the myosin thick filaments These channels are particularly important in regulating the contraction of cardiac and smooth muscle. It composed of three main subunits (53).

α1 subunit**:** is the major regulatory site with a large membrane protein pore-forming subunit. It is capable of mediating the L-type $Ca^{2+}$ current ($I_{Ca,L}$) without the involvement of other subunits.

β subunit: is a small protein without transmembrane - spanning domains. It influences the behavior of the LTCC and serve as a molecular chaperone to direct the α subunit on the surface membrane, a high affinity to the dihydropyridine site.

δ subunit: Mostly in skeletal muscle, function unknown

The calcium influx through the LTCC involves voltage and calcium dependent transitions in channel conformation that cause the pore-forming α1 subunit to transiently allow permeation of calcium into the myocyte. At resting potential (-80 mV), the LTCC is primarily in a closed but available in open state. Depolarization leads to the opening of the α1 subunit pore, and calcium. moves into the cell down its electrochemical gradient. On the other hand, further depolarization also leads to LTCC inactivation (54)

(b). T type calcium. channel (TTCC): a transient/short type. Widely distributed in cardiac pacemaker and atria tissue. It is hyperpolarization sensitive that cause calcium. influx into cells and becomes cells depolarization. When in cells depolarization, This channel rapid to inactivate. T channels mediate calcium. entry into neurons, and thereby control various calcium. dependent functions such as regulation of other channels, enzymes (55).

(2). Sodium/Calcium exchange (NCX):This channel can active transport of calcium outwards across the cell membrane and inward across the membrane of sacroplasmic reticulum, dependent on the activity of calcium. dependent ATPase. It also extruded calcium. from cell in Na/Ca exchange. This exchanger transfers three sodiums. for one calcium and, therefore, produces a net hyperpolarizing current when it is extruding calcium. The energy for calcium extrusion comes from the electrochemical gradient for sodium, but not directly from the ATP hydrolysis (56).

**1-4-1-2: α1 adrenergic receptors**

α1 adrenergic receptors cause contraction and promotes the growth of vascular smooth muscle cells. There are three subtypes of α1 receptors (α1a, α1b, α1d) (57). The α1a receptor is the predominant receptor causing vasoconstriction in many vascular beds including mammary, mesenteric, splenic, hepatic, pulmonary and coronary. It is also the major subtype in vena cava, the saphenous and pulmonary vein. α1b receptor is the most abundant subtype in the heart and α1d receptor is the predominant receptor causing vasoconstriction in the aorta (57).

All the adrenergic receptors are link to guanine nucleotide binding protein (G protein) that target the release of catecholamines, α1 adrenergic receptors are predominant in smooth muscle of blood vessels. They are members of G protein coupled receptor super family. Upon activation, α1 adrenergic receptor results in the regulation of multiple effectors system. The primary signal transduction involves activation of the α1 to couples to Gq. GTP then binds to Gq and moves to phospholipids Cβ **(**PLCβ**)**. PLCβ becomes activated, it can cleave phosphatidyl inositol 4-5-bisphosphate (PIP2) to inositol trisphosphate (IP3) and Diacylglycerol (DAG) in the plasma membrane (58). The IP**3** then diffuse from the plasma membrane to the sacroplasmic reticulum (SR). SR then binds to specific IP3 receptors and cause calcium release from SR to the cytosol. When the intracellular calcium rises to about $10^{-6}$ M (1 μM), calcium that bind to a variety of calcium regulate proteins including calmodulin (CaM) – an acidic protein with four high affinity calcium binding sites (59). The binding of calcium to calmodulin, activates the CaM kinase. This kinase than phosphorylates a number of target enzymes. In smooth muscle, CaM kinase can activate myosin light chain kinase (MLCK) that phosphorylated myosin light chain (MLC), and activates smooth muscle contraction as previously described (figure 7).

### 1-4-1-3: Angiotensin II

Angiotensin II is derived from angiotensinogen. When body sodium level falls in the distal renal tube, or a fall in renal perfusion pressure, the renal sympathetic nerve becomes activated. Where the β-adrenoceptor agonist and the prostaglandin I 2 are all stimulate rennin secretion. Renin stimulates angiotensinogen release from liver cells. The angiotensinogen then circulate through the lung and cleave to form angiotensin I. An enzyme, angiotensin convertive enzyme (ACE) that converts angiotensin I to angiotensin II (60). The receptor of angiotension II, especially $AT_1$ subtype, is involved in several physiological effects, such as the sympathetic activities, cells growth, water and sodium retention and vasoconstriction (figure 8).

There are 2 subtypes of angiotensin receptors: $AT_1$ and $AT_2$ receptors. The $AT_1$ receptors activate a large array of signal transduction system to produce effects but the function of $AT_2$ receptors are poorly defined, but may exert anti-proliferative, pro-apoptotic, vasodilatory, proliferation and anti-hypertensive effects (61). The AT1 receptors couple to several heterotrimeric G protein, including Gq, G12, G13 and Gi, but in most cell types, $AT_1$ receptor coupled to Gq and thus active phospholipase C β (PLCβ). PLCβ then cause formation of $IP_3$ and then calcium release from sacroplasmic reticulum. The pathway is similar to that for the α1 adrenergic receptor. On the other hand, angiotensin II also exhibits an inhibitory effects on c-GMP in vascular smooth muscle cells (VSMC) by activate of Ca/Calmodulin, which stimulated by phosphodiesterase (PDE5) (62), where the PDEs play critical roles in controlling intracellular c-GMP level by converting c-GMP to 5'-GMP. Chen et.al (63) found that $PDE_1A_1$ in VSMC is rapidly activated by angiotensin II, probably by way of increasing calcium. Angiotensin II – mediated activation of PDE1A1 contribute to the effects of angiotensin II on attenuation of c-GMP accumulation. The increase of intracellular calcium, activates MLCK, activate of myosin phosphorylation, cause VSM contraction (figure 9).

Activation of Ang.II receptor also enhance the vascular production of reactive oxygen species (ROS) in part through the activation of membrane-bound NADH and NADPH oxidases (64), which are present in endothelial cells, vascular smooth muscle cells and fibroblasts. The resultant increase in nitric oxide degradation or in-activation by ROS. ROS produces oxygen radicals ($O_2^-$). This oxygen radicals reacts

with in-active nitric oxide leads to the production of peroxynitrate ($ONOO^{-}$), a potent oxidant that contributes to vasoconstriction and vascular injury.( 64 ) (figure 10).

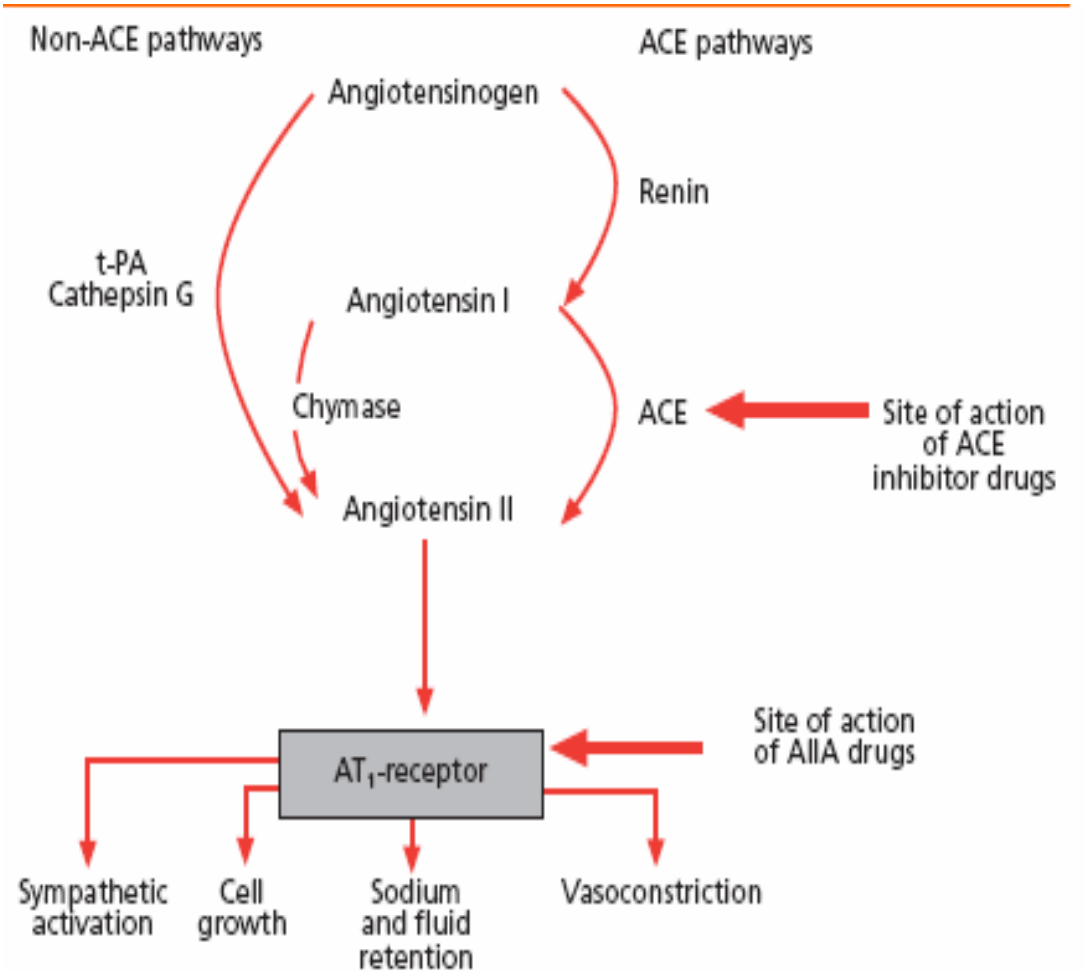


**Figure** 8 :The pathways of angiotension II formation. The ACE pathway is stimulates by renin which converts angiotensinogen to angiotensin I, then converts to angiotensin II by angiotensin converting enzyme. The angiotensin II receptor- AT1 plays several physiological effects.

**( Sherborne Gibbs Ltd. Br.J. of cardiol. 2004 )**

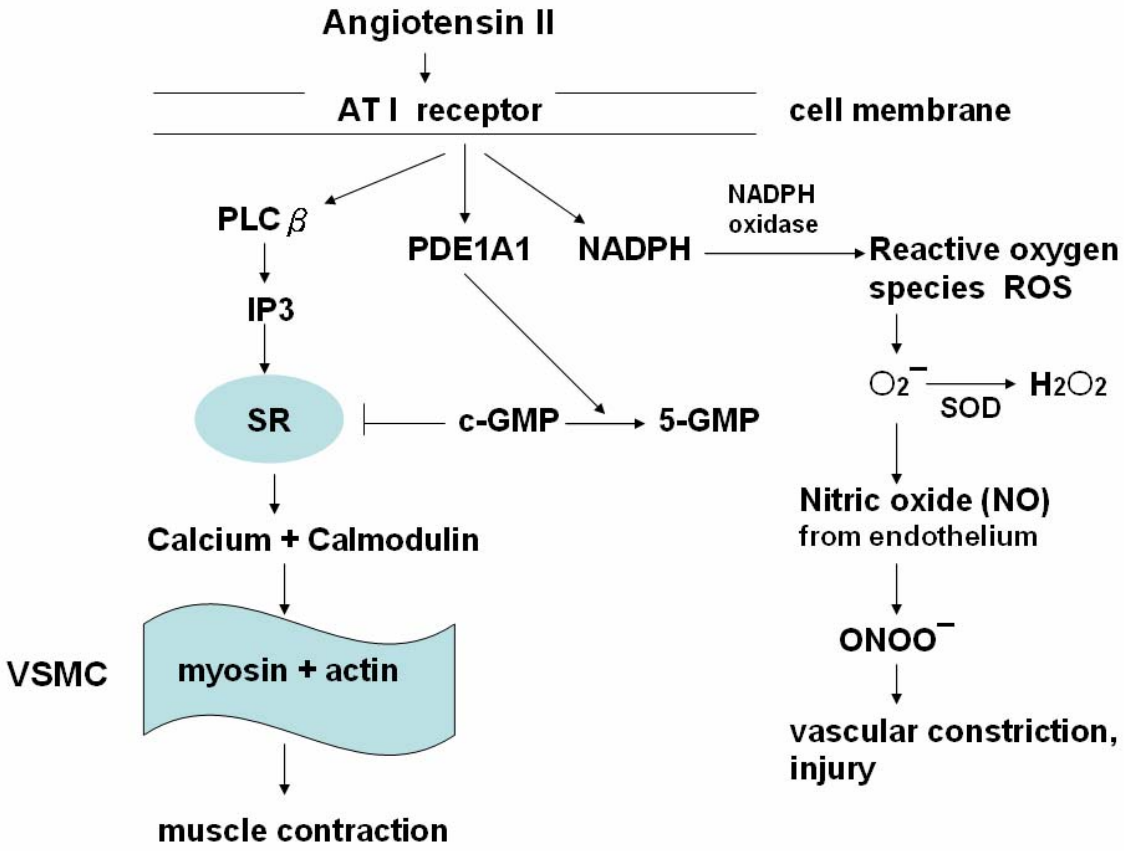


**Figure 9** :The angiotensin II AT1 receptor (AT1) induce vascular smooth muscle contraction and vascular injury. Induce calcium release from sarcoplasmic reticulum (SR) through IP3 pathway. Attenuate c-GMP by phosphodiasterase 1A1(PDE1A1). Calcium then binds to calmodulin to form CaM complex. CaM promotes myosin binding to actin in vascular smooth muscle cell (VSMC) cause smooth muscle contraction. AT1 activates NADPH oxidase with the formation of reactive oxygen species (ROS). The production of $O_2^-$ which react with NO to form the $ONOO^-$, a potent oxidant that cause vascular injury.

### 1-4-1-4: Endothelin (ET)

Endothelin is a strong vasoconstrictor produced in Endothelium. It is a 21 amino acid peptide that causes an extremely potent vasoconstriction in most vascular smooth muscle cells (VSMC). Stimuli of endothelin synthesis include many noxious factors, such as angiotensin II, thrombin, vasopressin, hypoxia et al (65). Within the endothelium, a large inactive precursor molecule, called preproendothelin, under the action of endothelin converting enzyme, is converted to an active ET-1. ET-1 exerts its vascular effects by binding to two specific receptors: the ET-A and ET-B, both belong to the superfamily of G-protein coupled receptors. The ET-A receptors are present on VSMC that promote vasoconstriction and smooth muscle proliferation. ET-B receptors are located on endothelial cells and mediate endothelium-dependent dilation by releasing nitric oxide (NO). When ET-1 binds to ET-A receptor, ET-A receptor becomes activated. This ET-A mediated responses include vasoconstriction, bronchoconstriction and aldosterone secretion (66, 67). ET-A receptors are coupled to phospholipase C, which stimulate Na-H exchange. Active of G-protein (Gq) then bound to GTP and activate phospholipids C (PLC). PLC then cleaves the phosphatidyl inositol 4-5 bisphosphate to inositol triphosphate ($IP_3$) and Diacylglycerol. The IP3 is a water soluble compound, diffuse from the plasma membrane to the endoplasmic reticulum (ER). When it binds to a specific receptor and cause calcium channels open, release of calcium from ER into the cytosol. When the calcium concentration rise up, calcium binds to Calmodulin and thus cause muscle contraction, as the previously described (figure 10).

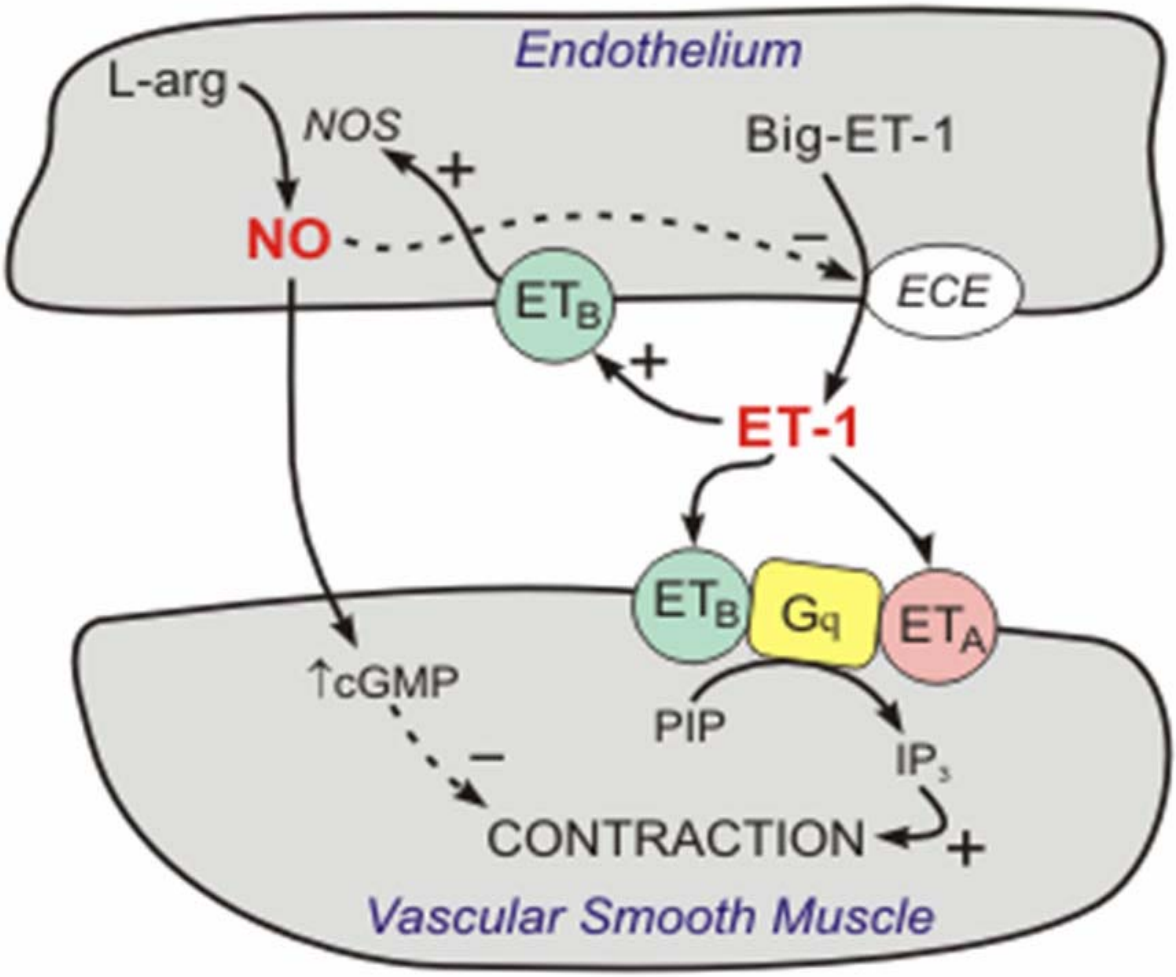


**Figure 10** ：In endothelium, ET-1 binds to a special receptor-ET**B** that release nitric oxide (NO), this NO can activate C-GMP in vascular smooth muscle and inhibits muscle contraction. The ET-1 also binds to ET**A** in vascular smooth muscle that activate the formation of $IP_3$, causing calcium release and muscle contraction.

**( Richard E. Klabundle. Cardiovascular pharmacology concepts. 2007 )**

## 1-4-2: Factors affecting vascular relaxation

### 1-4-2-1: β adrenergic receptor

β adrenergic receptors are members of a large super-family of receptors linked to guanine-nucleotide-binding protein (G protein). G-protein are signal transducer that conveys information from the receptors. A large super-family of receptors called G-protein coupled receptor (GPCR) is widely used to target for many receptors and drugs. The β adrenergic receptor is an integral protein with seven hydrophobic regions of 20 to 28 amino acids. Three subtypes of β adrenergic receptors are: $\beta_1$, $\beta_2$ and $\beta_3$.(57). All the β adrenergic belong to the super-family of G-protein coupled receptor. $\beta_1$ receptor is present in on cardiac muscle, and is responsible for the positive inotropic and chronotropic effects of catecholamine. $\beta_2$ receptor is present on smooth muscle cells and so responsible for smooth muscle relaxation in many organs. β3 receptors is present on brown adipose tissue and gallbladder. It appears to have a role in promoting lypolysis and heat generation in fat (57).

Adrenal gland release adrenaline which bind to β -adenoceptors ($\beta_1$, $\beta_2$) on the smooth muscle cell membrane. This can stimulate G-protein. G-protein than binds to guanosine 5' di-phosphate (GDP) following hydrolysis of guanosine 5' tri-phosphate (GTP). Then a subunit of G-protein (Gs) moves to the adenylyl cyclase and activate it. This activates adenylyl cyclase then catalyzes the formation of adenosine 3'-5' cyclic monophosphate (c-AMP) from adenosine tri-phosphate (ATP) (68).

C-AMP also directly regulates the activity of plasma membrane cation channels-the cyclic nucleotide gated (CNG) channel (69). CNG ion channels have been found in heart, vascular smooth muscle, kidney, testis and central nervous system. These channels open and so directly binding of intracellular cyclic nucleotides and contribute to cellular control of membrane potential and intracellular calcium level when accumulation of c-AMP activates protein kinase A (PKA).

In vascular smooth muscle, $\beta_2$ adrenoceptors activate the formation of c-AMP, which then phosphorylates myosin light chain kinase (MLCK) to form MLCK-$(PO_4)_2$. This inactive form of MLCK loss the function to phosphorylate MLC (49). Thus, inhibit the binding of myosin to actin cause muscle relaxation (figure 11).

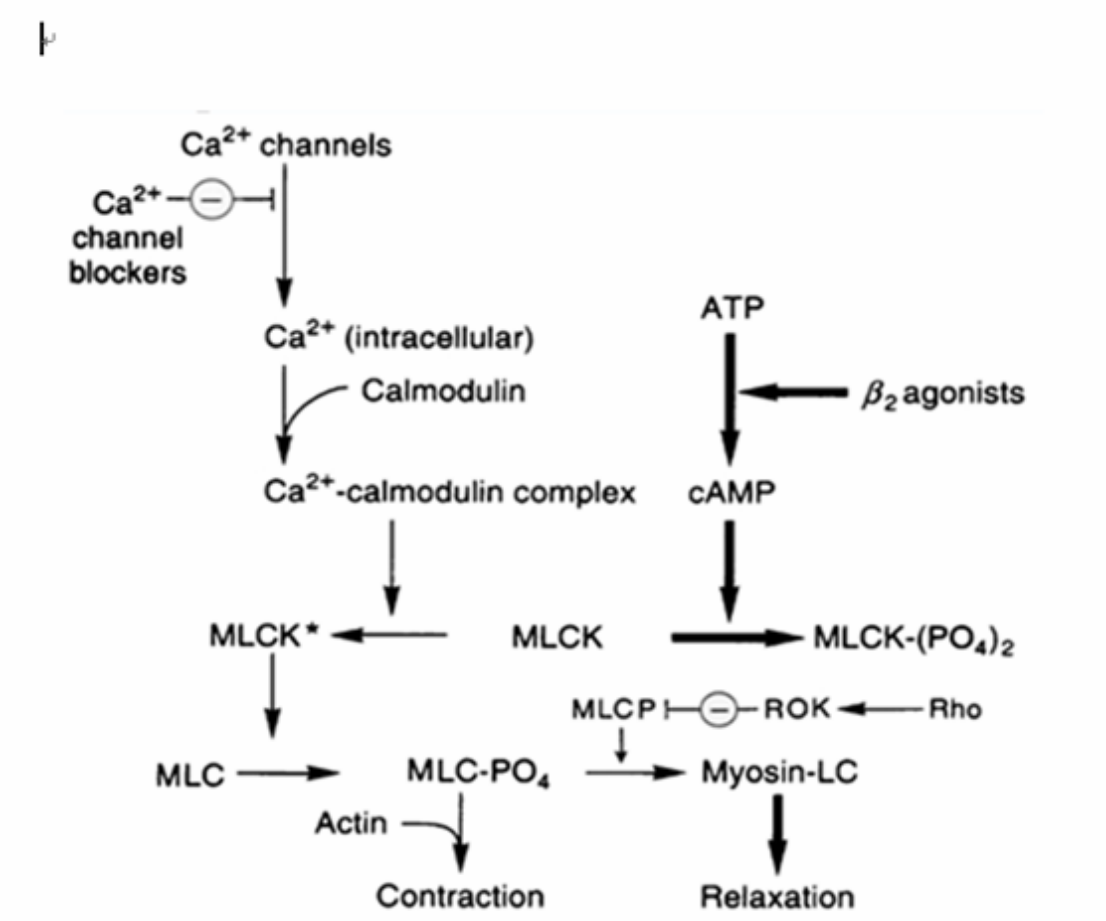


**Figure 11**: Contraction is triggered by influx of calcium through transmembrane calcium channel. The cytosol calcium ions bind to calmodulin to form a complex. This complex activated myosin light chain kinase (MLCK) that phosphorylated myosin light chain (MLC), this active the binding of myosin to actin cause muscle contraction. On the other hand, β adrenergic receptor can active adenylyl cyclase that catalyzes the formation of c-AMP. C-AMP phosphorylate MLCK becomes an inactive form – $MLCK(PO_4)_2$ cause muscle relaxation

**( I.F Ghalayini. Int. J of impotence research, 2004 )**

**1-4-2-2: Nitric oxide (NO)**

NO is a free radical gas molecule , which was discovered in endothelial cells (70). It plays as a signaling molecule in cardiovascular and nervous system as an endogenous activator of soluble guanylate cyclase, which leads to the formation of cyclic guanosine monophosphate (c-GMP), and serves as a second messenger in many cells including nerves, smooth muscle, monocytes and platelets.

NO synthase (NOS) is central to the control of NO biosynthesis. There are three known isoforms of NOS.

1. The neurons NOS (nNO or NOS-1) , a calcium/calmodium dependent enzyme was originally identified in nervous system. It is also important in smooth muscle, skeletal muscle, cardiac muscle and renal mesangial cells (71).

2. The inducible NOS (iNOS) or NOS-II), a calcium/calmodulin independent enzyme that expressed in macrophages, neutrophil, fibroblast and vascular smooth muscle . This enzyme binds calmodulin tightly. It catalyses rapid NO generation ( 71).

3. The endothelium NOS (eNOS or NOS-III), a calcium / calmodulin dependent enzyme that expressed constitutively in endothelial cells and synthesizes the NO for the regulation of blood pressure (71).

Formation of NO: The endogenous NO is produced from L-arginine in an NADPH-dependent reaction and catalyzed by nitric oxide synthase (NOS). NO is an unstable molecule, its formation is stimulated by interaction of nitric oxide synthase with the calcium/calmodulin complex. On the membrane of endothelial cell, the endothelium dependent relaxation stimulators: such as acetylcholine, substance P, bradykinin etc, increase cytoplasmic calcium concentration in the endothelial cells. Calcium than binds to calmodulin to form a calcium/calmodulin complex (CaM). This complex can activate NOS, which cleaves arginine into NO and citrulline. NO is found to be a strong vasodilator. (70).

In smooth muscle cell, the muscle tone is dependent on the cyclic GMP mediated NO. In cell membrane, NO activate guanylate cyclase (GC). The activated of GC than convert GTP to c-GMP. c-GMP activates Protein kinase G (PKG) – a c-GMP dependent protein kinase. Both the c-GMP and PKG relax vascular smooth by many ways:

The increase of c-GMP contributes to the endothelium –dependent relaxation, including:

(a) stimulation of Na/K ATP, the activated Na/K ATP release endothelial derived relaxation factor (EDRF) causes opening of the ATP sensitive K channel allow large of K diffusing out of cell, leading to cell hyperpolarization (71).

(b) inhibition of calcium release from endoplasmic reticulum (ER) leading to reduce intracellular calcium concentration (71).

(c) inhibition of phospholipase C and thus decreasing the production of phosphoinositide (IP3), and reducing calcium release from the ER (71).

(d) direct opening of Ca. dependent K channels, accelerating K efflux to cell and causing cells to hyperpolarize (71).

(e) Protein kinase G activates a signaling pathway that result in phosphatase myosin light chain, then turn myosin light chain in-activated and inhibit the myosin-actin interaction. Relaxation of the vascular smooth muscle cells causes blood vessels to dilate (71) (figure 12).

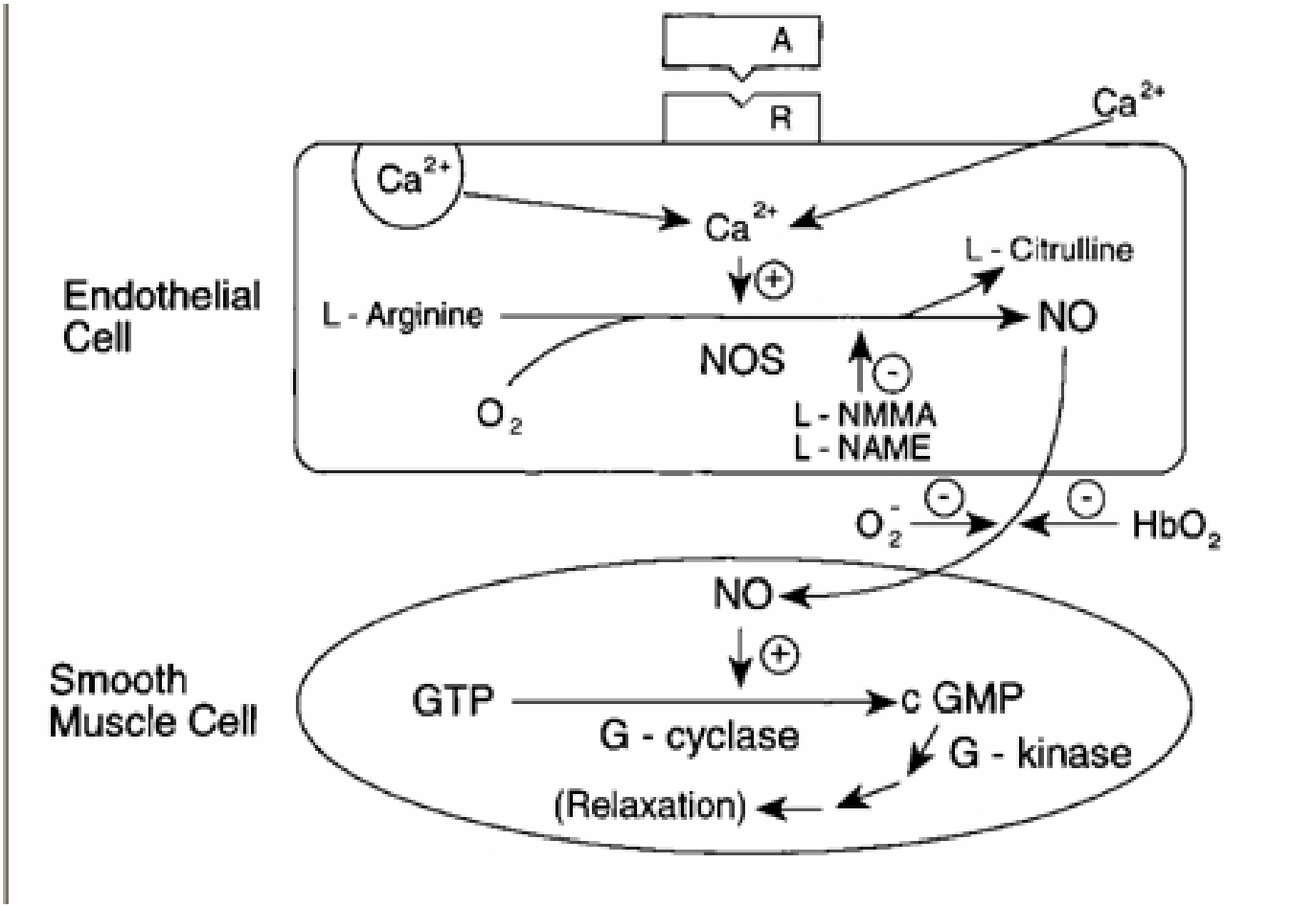


**Figure 12** : In endothelial cell, activating a receptor to acetylcholine, bradykinin or substance P can cause calcium influx into cytosol. Calcium ions binds to calmodulin to form CaM. CaM then actives an enzyme - NOs and catalyses L-arginine to form the Nitric oxide (NO). NO diffuse to smooth muscle cells that activate c-GMP to form a G-kinase, this kinase inhibit calcium release from sarcoplasmic reticulum to form relaxation.

**( Robert F. Furchgott. Blood vessels. 1991 )**

## 1-4-2-3: Potassium channels

### 1. ATP sensitive potassium channels (KATP)

K**ATP** Found in various tissues including pancreatic β cells, skeletal and smooth muscle cell. These channels are inhibited by intracellular adenosine -5' triphosphate (ATP) and activated by adenosine -5' diphophate (ADP).(72).The current voltage relationships of K**ATP** channel have a weak inward rectification, they allow a large K influx than do efflux, meaning that the outward current being smaller than the inward current. The inward rectification of K**ATP** channels is due to a voltage-dependent block of outward current by internal cations, such as $Mg^{2+}$ and $Na^{+}$ (72). ATP has two effects on K**ATP** channels: (a) Inhibiting channels activity. (b) maintaining the channels in an operative state.(72) When ATP is not hydrolyzed or in the absence of $Mg^{2+}$, the increased activity of ATP turn K**ATP** channel inactivated (inhibited). Closure of this channel leads to influx of $K^{+}$ into cells and depolarizes the cells membrane, enhances the membrane excitability. When ATP is hydrolysis, or in the presence of $Mg^{2+}$, this channel is in an actives or operative state. Opening this channel allow a large $K^{+}$ efflux hyperpolarizing the cell membranes (72).

In vascular smooth muscle (VSM), K**ATP** contributes to the resting membrane conductance controls $Ca^{2+}$ entry through the voltage-dependent $Ca^{2+}$ channels. The activity of vascular KATP channels is governed by the degree and the sites of phosphorylation and de-phosphorylation. In VSM, K**ATP** can be opened by adenosine dependent protein kinase (PKA) and closed by $Ca^{2+}$ dependent protein kinase (PKC). Under some situation of metabolic compromise through PKA, active C-AMP than open of these channels allow $K^{+}$ efflux and membrane becomes hyperpolarization. Leading to the closure of voltage-gated $Ca^{2+}$ channels and, hence, vaso-relaxation (73).

**2. Voltage-dependent potassium channels:**
The role of this channel is to allow influx of potassium if the concentration inside the cell drops below some minimal value. It conducts $K^+$ ions across the cell membrane in response to changes in the membrane voltage (74)) cell membrane voltage regulates the opening of a Kv channel, which interfaces the voltage-sensor domains to the pore. the convert voltage-sensor motions directly into gate opening and closing. As the charged ions flow across the membrane, they generate an electric current. The amount of current flow is determined by two factors. First, when the gate of an ion channel opens, ions flow down the concentration gradient from high to low across the membrane, which is typical of the passive transport mechanism. Second, the flow of ions is controlled by the voltage difference across the membrane. Thus, the total ion flow is influenced by the concentration gradient of the ions, the voltage difference across the membrane, and the permeability of the ions.( 74)

**3.Calcium sensitive potassium channel:** This channel is part of the delayed rectifier super family. When the membrane calcium channel opens, large of calcium influx into cytosol. The conductance $Ca^{2+}$ activated $K^+$ channels contribute to action potential repolarization and restrict the excitability of detrusor smooth muscle. In addition, the activation of voltage dependent $K^+$ channels is involved in repolarization and after-hyperpolarization, and it has a fundamental role in stabilizing detrusor smooth muscle excitability. The increase of cytosol calcium leading this calcium sensitive channel open. This result in a large efflux of potassium ions out of the cell, causing cell hyper-polarization. At last membrane calcium channel close, less of calcium influx into cytosol. Vascular smooth muscle then relaxation- vasodilation (75).

**1-4-2-4: The effect of PVAT to the blood vessels**
**The previous literatures review**

Since 1991, the PVAT was found to have a significant inhibition of the contraction induced by phenylephrine (PE). (76). Later, many investigators studies the PVAT in blood vessels and found that PVAT can cause relaxation or enhanced contraction in blood vessels..

(a) Vessel relaxation

Matthias Lohn et al (77) studied rats thoracic aortas, used transferred fat solution from isolated aortic perivascular fat tissue to the vessel without PVAT. They found that the transferred fat solution has an adventitial derived relaxing factor which plays an anti-contractile effect to the angiotensin II, serotonin and PE.

Stefan Verlohren et al (78) used rats mesenteric artery rings with either intact periadventitial adipose tissue (F+) or removed (F-) to test the contractile response to serotonins, PE and endothelin I. They found that the vasoconstriction to all stimulants were markedly reduced in intact vessels (F+) compared with vessels without periadventitial fat (F-).

Gao et al (79) found the PVAT in human thoracic artery can releases a transferable relaxation factor that acts through the activation of calcium dependent potassium channels. This relaxing factor can attenuated the maximal contraction to U46619 ( a thromboxane $A_2$ / prostaglandin $H_2$ agonist) and PE. This findings were present in either vessel with intact PVAT or transfer solution incubated with PVAT to vessel without PVAT

(b) Vessel contraction

Gao et al (80) found that in rat superior mesenteric artery with intact PVAT, a vaso-active factors can be released in response to perivascular nerve activation and showed a greater contractile response to electrical field stimulation (EFS) than that without PVAT . Their results show the PVAT enhance arterial contractile response to perivascular nerve stimulation through production of superoxide mediated by NAD(P)H oxidase. This enhancement involves activation of tyrosine kinase and MAPK/ERK pathways.

## 2. Aim of study and hypotheses

According to the previous studies, investigators found the PVAT exerts a relaxing effect in both small and large arteries.

We wonder whether this finding is true because obese people tend to have high propensity to develop hypertension and vascular diseases compared to the non-obese people.

We have the following objectives or experimental strategies:

a. Use novel methods which are different to the previous studies. such as used fat intact／fat denuded aortic rings, to transfer fat solution or boiled fat solution to the aortic rings, use nitric oxide inhibitor to inhibit NO activity. All the aortic rings are stimulate by phenylnephrine (PE), potassium chloride (KCl), angiotensin II (Ang.II), some of them are relaxing by carbachol (CCh). To find out whether the perivascular adipose tissues (PVAT) can release a factor, "Adipocyte derived factor" (ADF), which affects vascular tones, either in vascular smooth muscle contraction, relaxation or both.

b. Since obesity is an important risk factor for increase of blood pressure, atherosclerotic change of blood vessels (81). We hypothesize that PVAT accumulation enhance blood vessels contraction. We carry out this study in order to find out whether our findings are consistent with our hypothesis or not.

# Chaper 2
# Materials and Methods

## 1. Materials

**Animals and tissues preparations**

Male Sprague Dawley rats (320-350 g body weight), kept in university experimental animal facilities under standard animal housing conditions were anesthetized by ether and killed instantly by cervical dislocation under the university regulation for the use of experimental animals. The thoracic aorta from 1-2 rats in each experiment were quickly removed and rinsed with Ringer's solution to remove blood and blood clots. The entire thoracic aorta with intact PVFT and partially fat-denuded aorta are shown in figure 13

Fat intact (F +**)** and fat denuded (F -) aortic rings were obtained from the same rat and studied in parallel in each experiment. The loosely attached connective tissues were gently removed with tweezers and scissors. Then the vessel was cut into ring segments of 3 - 4 mm in width. The fatty tissue strips remained either intact or denuded. The composition of the Ringer's solution was (mM): NaCl, 119; $NaHCO_3$, 25; KCl, 4.7; $KHCO_3$, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 1.6; and Glucose, 1.1. Ringer's solution was kept at 36.5 C and pH 7.3. For prolonged experiments and during equilibration, the Ringer's solutions were replaced every 20-30 minutes. At the end of the experiment, the wet weights of aortic rings before and after removal of PVFT were measured in order to estimate the amount of fat tissues expressed as tissue wet weight..

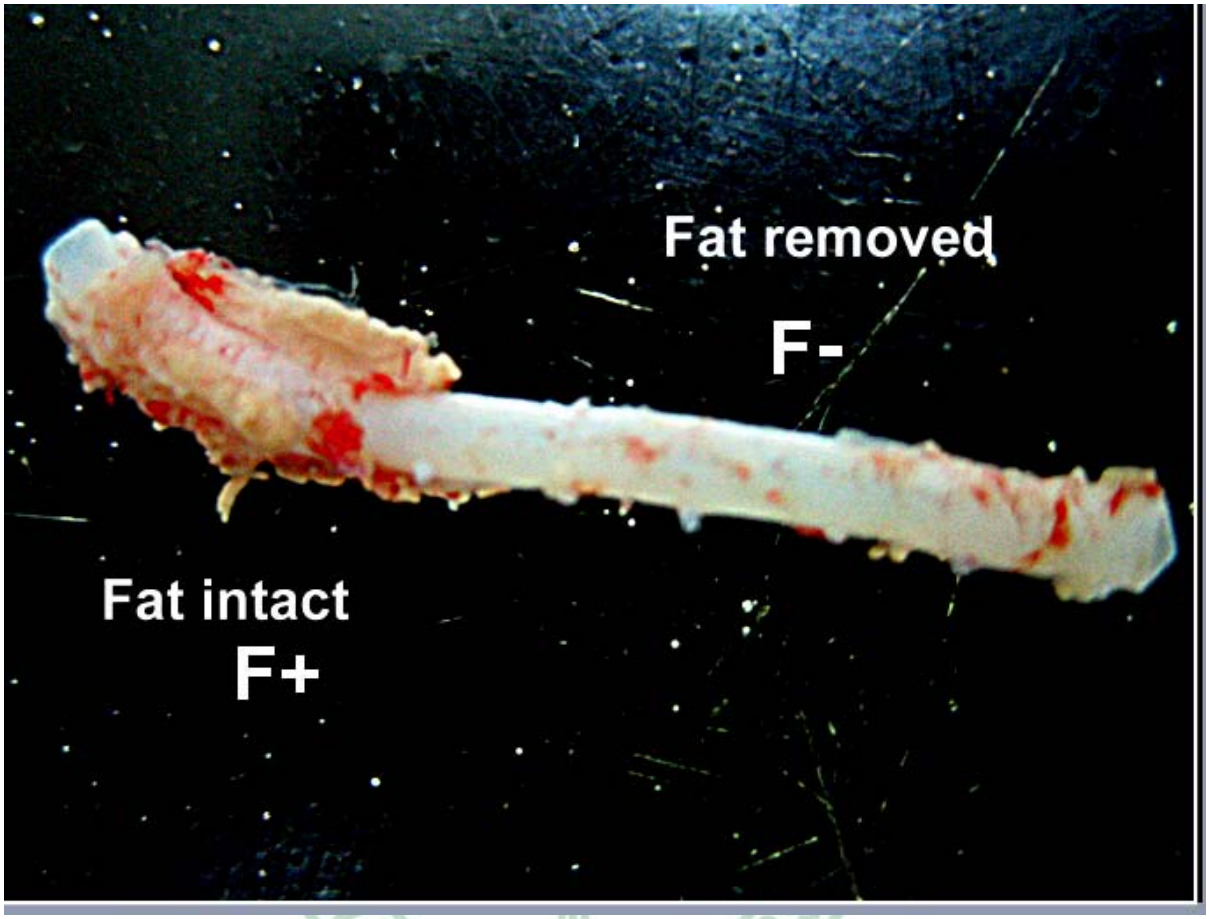


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**Figure 13 :** The rat thoracic aorta with fat tissue intact ( F +) and fat tissue denuded (F -)

**2. Methods**

Contractility measurements

General conditions: After tissue dissection, all the aortic rings were equilibrated in a 5 ml organ bath solution for an hour with a pre-determined stable 2 g (usually 1.5-2.5g) optimal resting tension. After equilibration, the rat aortic rings were then stimulated with 80 mM KCl 2-3 times until they reached a stable plateau contraction. The tension is expressed as a percentage of the steady-state tension (100%) (77). At the end of the experiment (usually after 5-6 hours), the contractile responses to 80 mM KCl remained about 80-90% of the original KCl contraction. Some aortic rings were denuded of fat tissues which were then transferred to and incubated in aerated Ringer's solution for approximately 2 hours before use (to release the adipocyte – derived factors). The clear solution after removal of the fat tissue just before use is operationally termed " F solution" (Fat solution). These F solutions were either keep at room temperature or heated to boiling (to deactivated or destroy any heat sensitive factors) and subsequently cooled down to room temperature before adding to fat-denuded aortic rings. (Figure 14)

Unless otherwise indicated, most experiments were performed in endothelium-denuded ring preparations. The functional intactness of endothelium was checked against the degree of endothelium-dependent relaxation to 3 uM CCh after reaching plateau contraction in response to 1 uM PE In most cases, the endothelium – dependent relaxation reached a magnitude of > 60% of the PE contractile response. Relaxation to CCh of < 20% of the PE contractile response was considered to be endothelium denuded.

Preparation of F solution: Rat's thoracic aortic rings, divided into 6 strips, usually weighing from 9.0 mg to 12 mg in each strip. Removed of fat tissue from those aortic rings and the fat tissue were transferred to a Petric dish containing 20 ml of Ringer's solution with continuous aeration. The incubation time of at least 2 hrs was allowed to ensure a high release of adipocyte-derived factors (ADF), For control groups, fat denuded aortic rings were stimulated with PE or KCl to construct the concentration-contraction curves. For the test groups, 2 different ways were used to apply the fat solutions.

(1) Fat-denuded aortic rings were stored in this organ bath with 5 ml of Ringer's solution. Transferred the same volume of F-solution into the organ bath, then incubated with the rings for at least 30 minutes (77). After incubation, the rings

were stimulated with PE or KCl to construct the dose response curves. Figure 15, tracing 1

(2) Fat-denuded aortic rings were stored with 5 ml of Ringer's solution, then increase the concentration of PE or KCl and added to it under the dose response curves. When a suboptimal contractile level were obtained, the Ringer's solution were replaced by the same volume of F-solution which contained the same concentration of PE or KCl. Figure 15, tracing 2

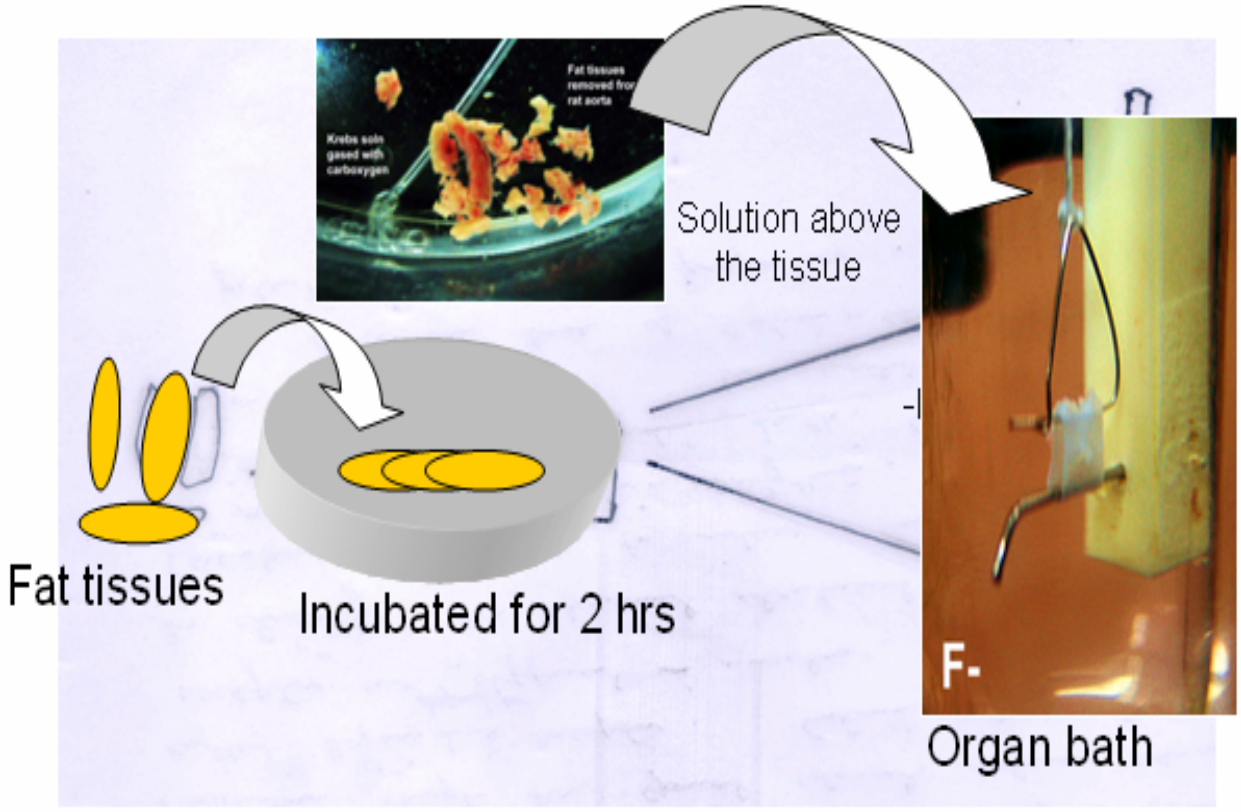


**Figure 14 :** The preparation and transfer of the solution pre-incubated with PVAT to the organ baths. First, fat tissues were removed from the thoracic aorta of the rat, and then transferred to a Patrick dish containing 20 ml of Ringer's solution and let stand with aeration for at least 2 hrs. The solution following the removal of fat tissue by centrifugation is called the fat solution for brevity. The fat solution is then transferred to the organ baths which contained the fat denuded aortic rings

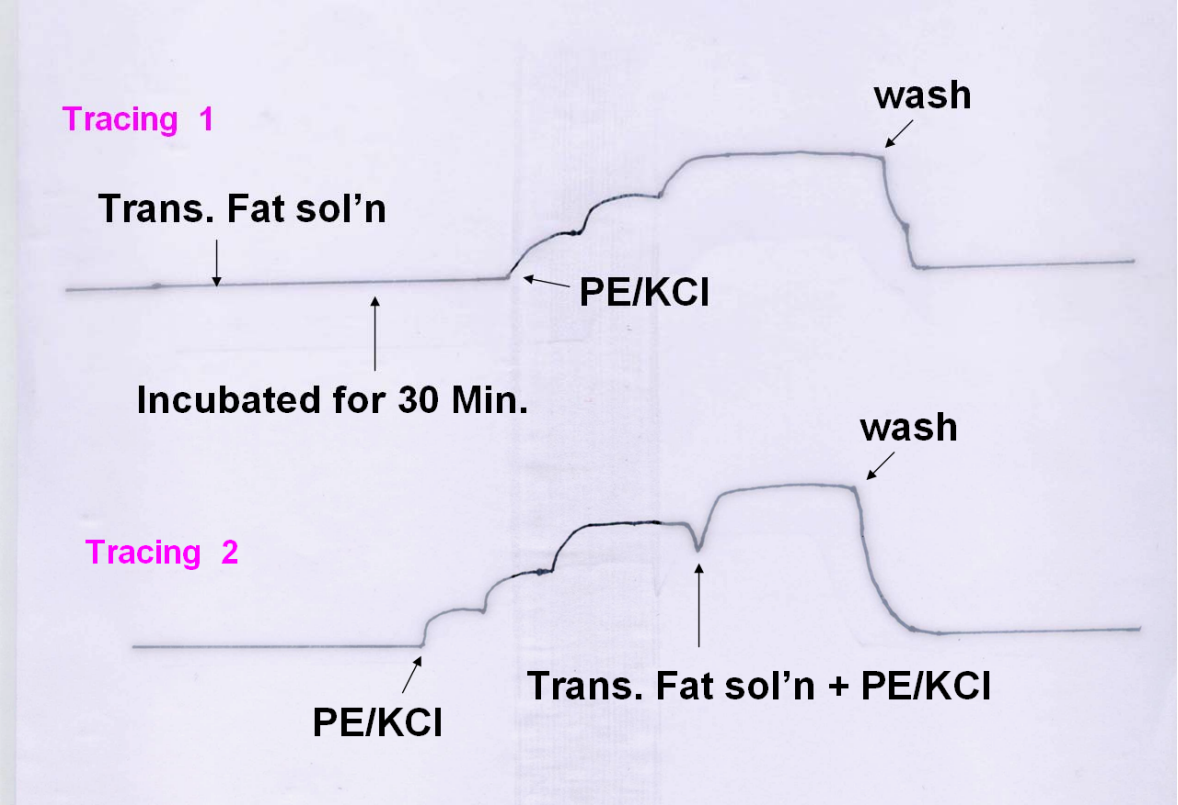


**Figure 15 :** Two methods in transfer fat solution to the F- aortic rings. One is to transfer fat solution into the organ bath, after 30 minutes incubation, the rings were stimulated with PE or KCl.(tracing 1) Another way is to stimulate with PE or KCl . When the contractile reaches the plateau, the fat solution containing the same concentration of PE or KCl was introduced (tracing 2)

In some study groups, the fat tissues were placed in 2.5 ml (half volume of organ bath) of distilled water. After 2-hr incubation, the F-solution was heated till boiling. When the solution was cooled to room temperature, 2.5 ml of doubly concentrated Ringer's solution was added and stored at a temperature of 36.5 C with continuous aeration shortly before use. This protocol helped minimize the change of the pH of the solution following heating, which tends to reduce the $HCO3^{-}$ in the solution causing altalination.

**3. Chemicals**

The phenylephrine (PE), potassium chloride (KCl), carbachol (CCh) and L-N-nitro-L-arginine methyl ester (L-NAME) were all purchased from Sigma chemical Co. (St. Louis, Mo USA). They were all dissolved in distilled water. PE was a 10 mM stock solution. KCl stock solution of 3 M. CCh stock solution of 330 mM. L-NAME stock solution of 30 mM

**4. Statistical analysis**

The results were expressed as the mean ± standard error of the mean (SEM). The Student's t test or ANOVA test was used when comparing two or multiple values as appropriate: n represents the number of rats. A value of $P<0.05$ is considered statistically significance.

# Chapter 3
# Results

### 3-1 Effect of perivascular adipose tissue (PVAT) on contraction to KCl and PE

To learn whether the PVAT has an effect on vascular tone in situ. We used the fat intact aortic rings and the fat denuded aortic rings and compared their contractile responses to cumulatively added KCl and PE. When the fat-intact aortic rings were stimulated with KCl and PE, it showed a significantly enhanced sensitivity to KCl and PE manifested as increased contraction to KCl and PE as compared to the fat denuded aortic rings. At low concentration, the maximal contractile response were not changed. Also, a slower rate of relaxation was observed in the fat intact aortic rings compared to the fat denuded aortic rings upon KCl and PE wash out. The mean standard error of half time maximal relaxation (t1/2) for F + after KCl wash out is 4.21 ± 1.45 min. in compared to 1.85 ± 0.24 min. for F – in these experiments. figure.16,17.

A separate experiment stimulated by PE is shown in figure.18,19. Where the t1/2 for F+ after PE wash out is 7.3 ± 1.23 min. in compared to 3.42 ± 1.54 min. for F-.

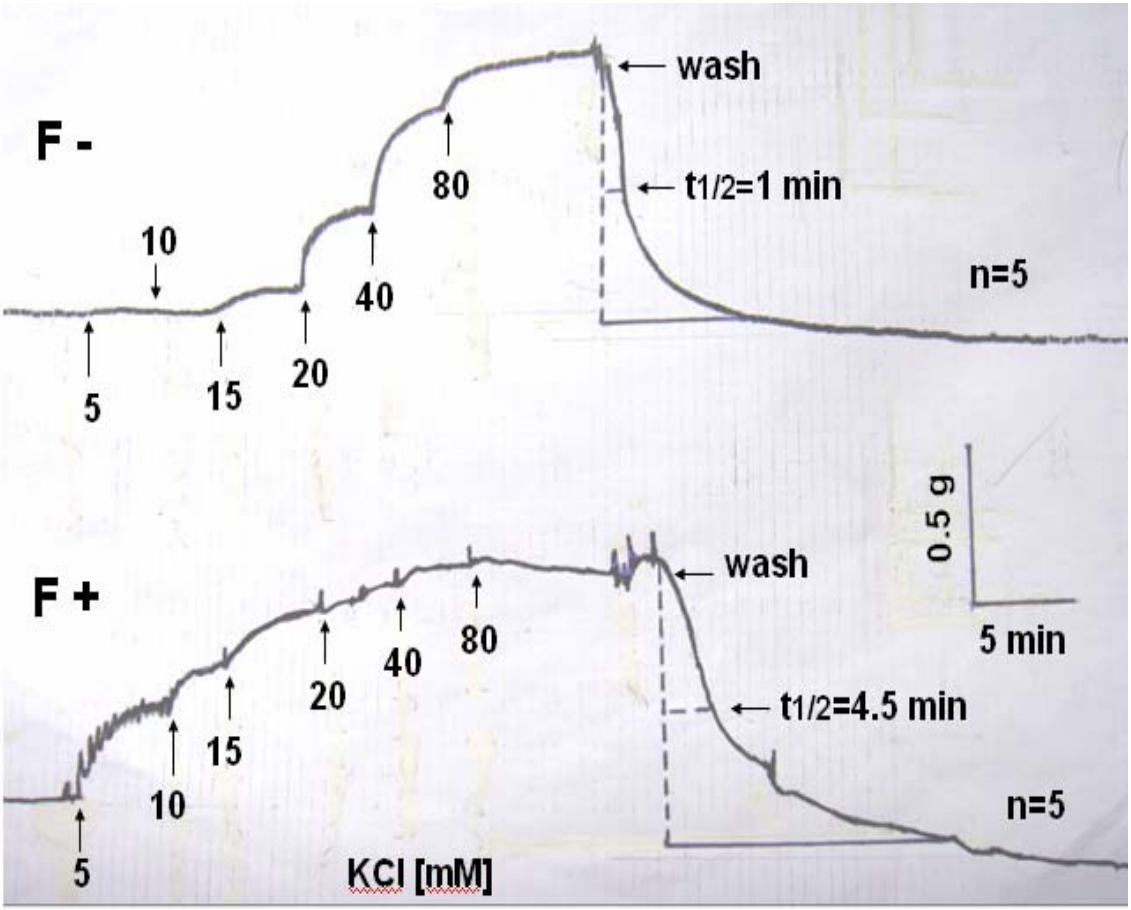


**Figure16**: Representative concentration-contraction curve of KCl induced contraction in rat aortic ring with fat intact (F+) or fat denuded (F-) aortic rings. Note the delayed onset of relaxation and the slow relaxation rate following washout of KCl in F+ compared to F-. t1/2 =half time maximal relaxation in minute.

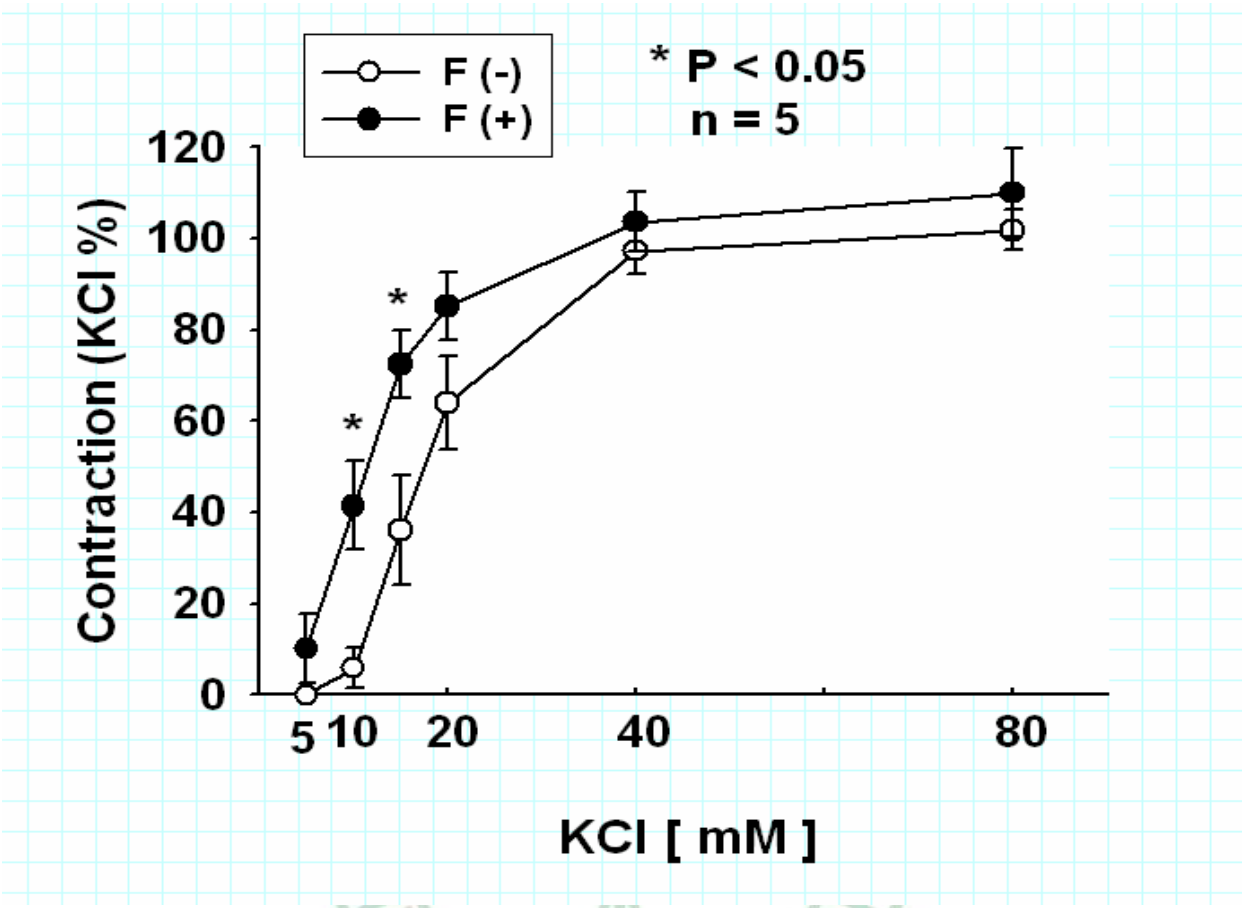


**Figure 17:** The Concentration-contraction relationship of KCl-induced contraction of the fat intact (F+, white) and fat denuded (F-, black) aortic rings. At low concentrations of KCl, the F+ showed a higher contractile response compared to F-. At the optimal concentrations of KCl, both F+ and F- showed the same level of maximal contraction. * P < 0.05

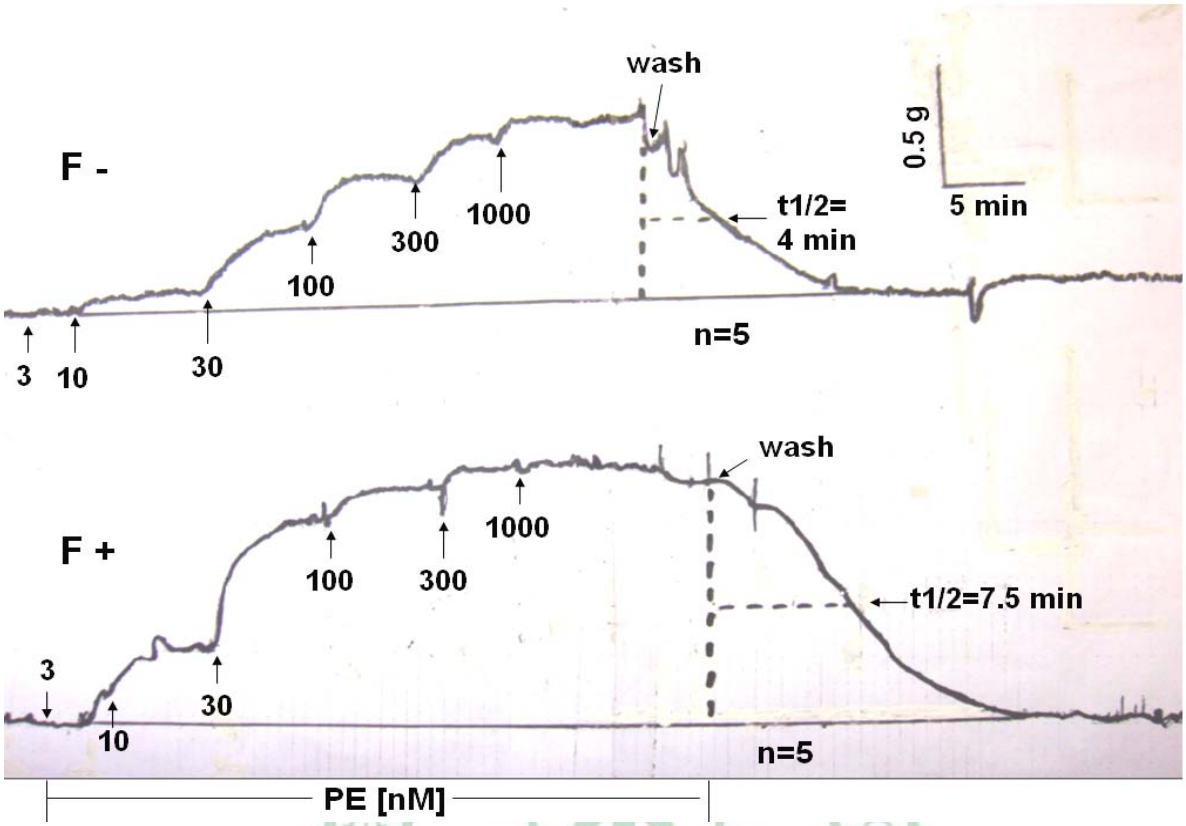


**Figure 18** ：The fat intact aortic rings F + has a higher contractile response in low concentration ( 30 nM ) of PE than the Fat denuded aortic rings F-. Upon PE washout, F + rings also shows the presence of a slow relaxing rate.
t1/2=half time maximal relaxation in minute

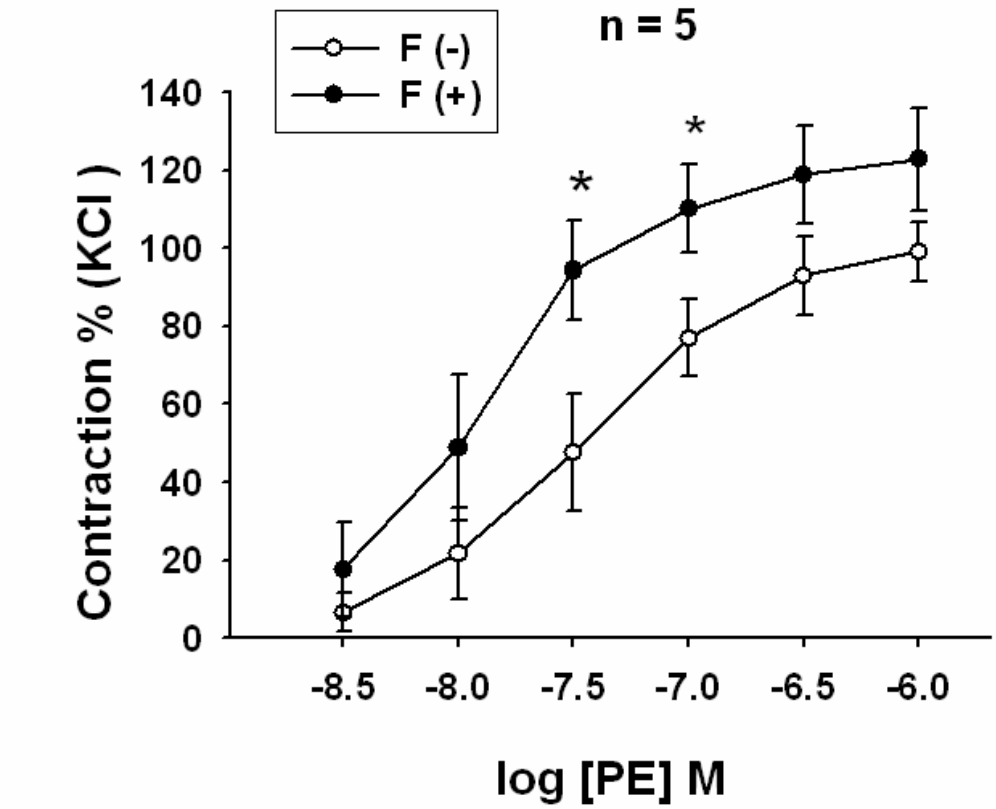


**Figure 19** ： The effects of PE to the fat intact aortic rings (F +, white) and fat denuded aortic rings (F -, black). At low concentrations ( 30-100 nM ) of PE , the F + have a higher contractile effect to PE, compared to F -. At high concentrations (300-1000 nM) of PE, both F + and F – showed the same maximal contraction.

* $P < 0.05$

## Effect of solution following pre-incubation with perivascular fat ( F solution)

### 3-2 Effect of F solution on aortic contraction to PE and KCl

To learn whether the effect of fat in fat intact aorta is due to factors released and diffused out from fat tissues, we studied the effect of the F solution, Ringer's solution on the contraction of the fat-denuded aortic rings. In the groups which were exposed to the fat solution, they showed an enhanced contractile response curves at low concentration of PE and KCl, comparable to the control groups. The mean standard error of half time maximal relaxation (t1/2) for F - in control group after PE wash out is 1.7 ± 0.75 min. and in study group is 2.1 ± 0.44 min. The mean standard error of half time maximal relaxation (t1/2) for F- in control group after KCl wash out is 1.9 ± 0.42 min. and in study group is 2.1 ± 0.55 min.

The stimulation by PE are show in figures 20, 21

The stimulation by KCl are show in figures 22, 23.

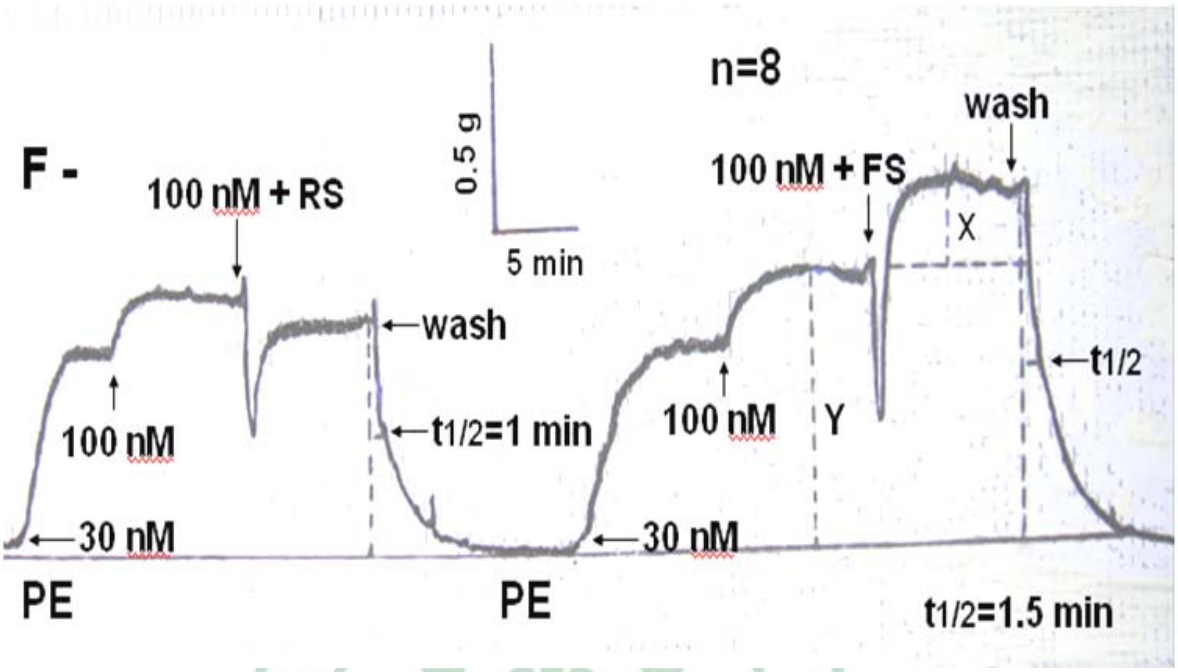


**Figure 20:** After transferred the F solution to the fat denuded aortic rings (F-) in response to PE ( 0.1 uM), it showed a significant increase reactivity to PE with a rise up baseline compared to the control groups. (in before transfer F solution or upon addition Ringer solution)

The percentage of rise up baseline is 31% ( X/Y=2.2/7=31% )

RS:Ringer's solution FS:Fat solution

Y: the magnitude in before transfer of F solution (control)

X: the increment about the control

t1/2=half time maximal relaxation in minute

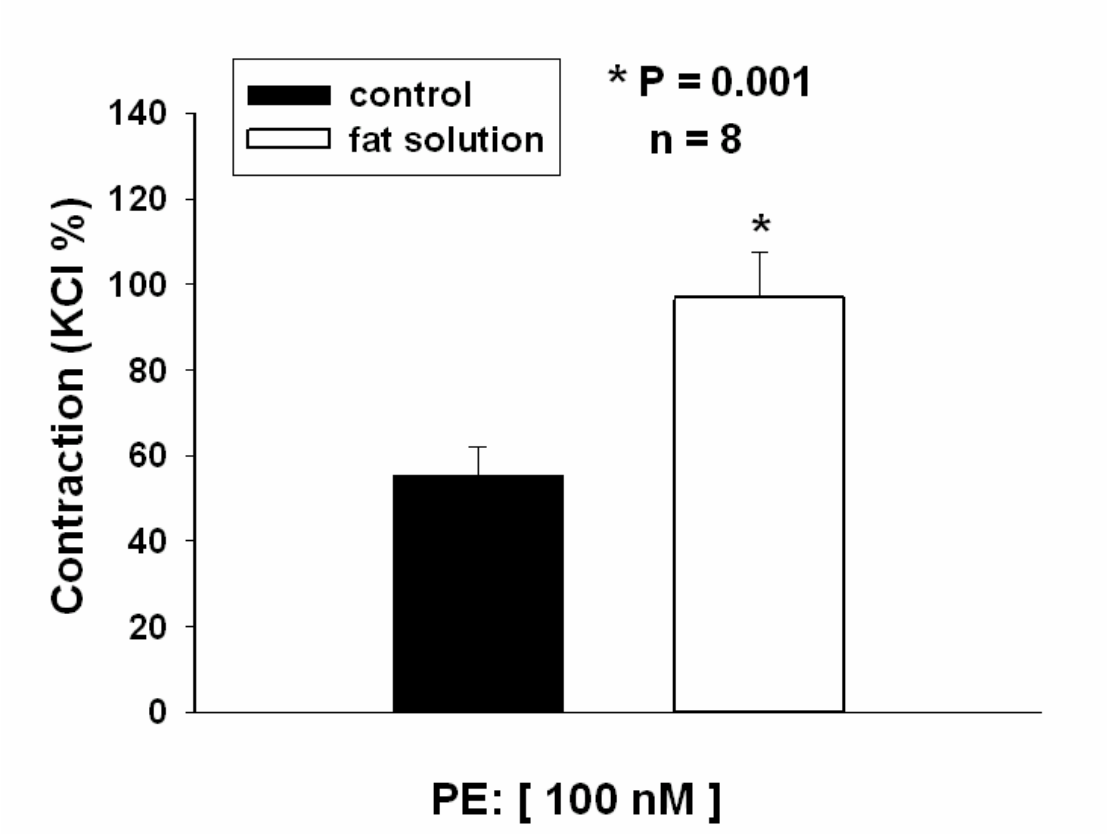


**Figure 21:** Transferred of F solution with the PE [100nm] showed a significant enhance vascular contractile response compared to the control group * $p < 0.005$

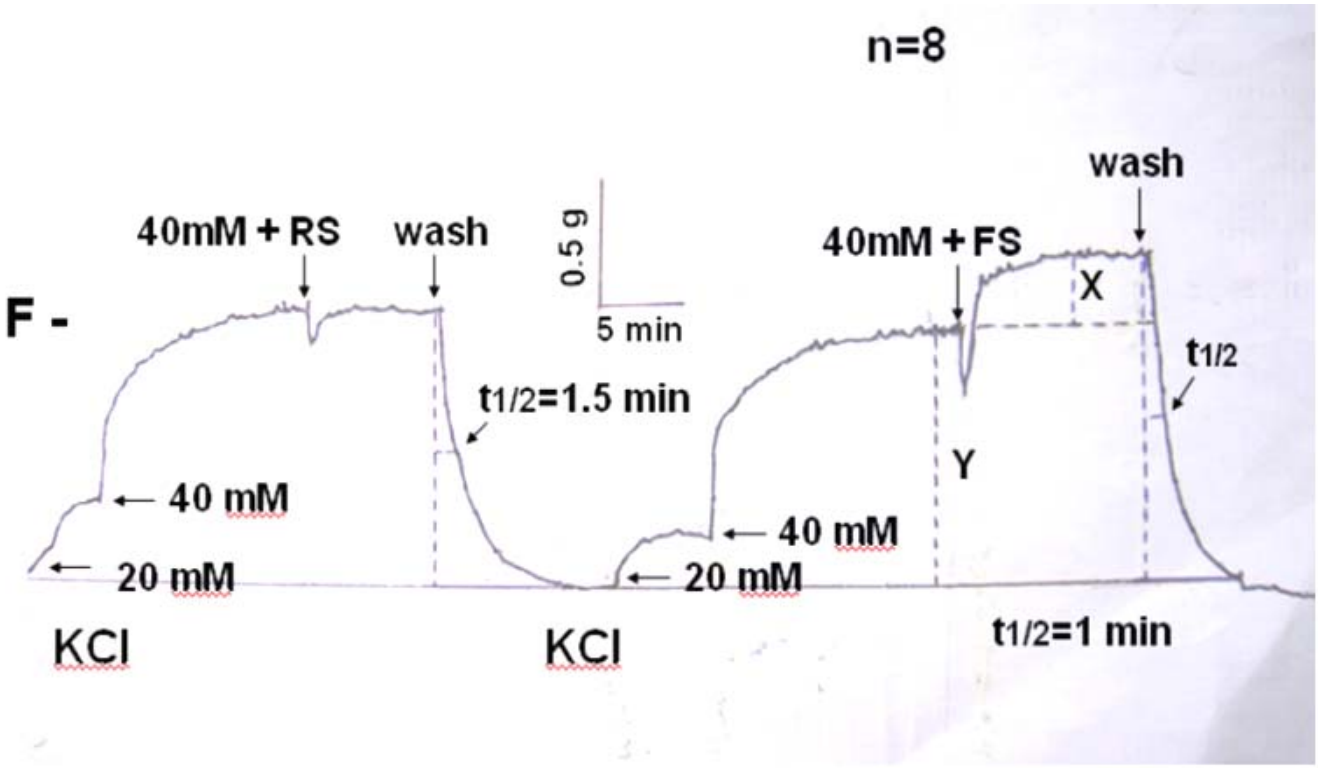


**Figure 22** : After the transfer of the F solution to the fat denuded aortic rings (F-) in response to KCl (40 mM), it showed a significant increased contractile response comparable to the control groups.(in before transfer F solution or upon addition of Ringer solution)

The percentage of rise up baseline is 34% ( X/Y=3.4/10=34% )

RS:Ringer's solution FS:Fat solution

Y: the magnitude in transfer of F solution (control)

X: the increment about the control

t1/2=half time maximal relaxation in minute

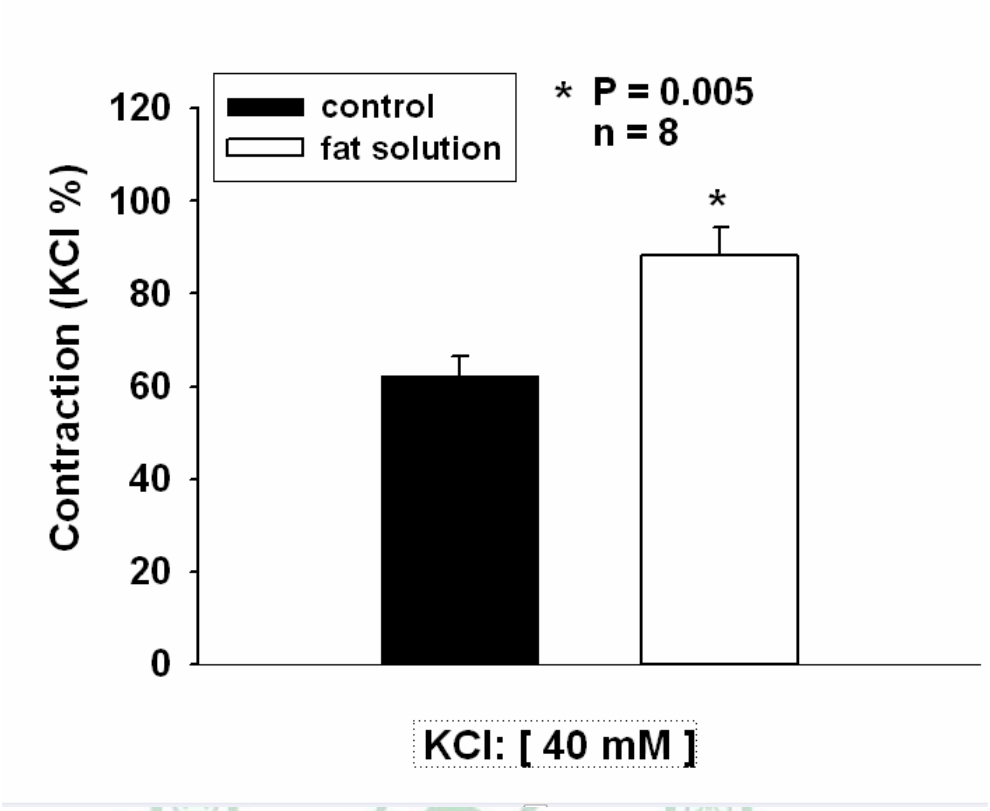


**Figure 23**：Transfer of F solution with the 40mM KCl to fat denuded aortic rings caused a significant enhancement of contractile response compared to the control group. * p = 0.005

### 3-3 Effects of different concentration fat solution on contraction to PE and KCl

To examine whether the effect of F solution is dependent upon the concentration of the substances released from the fat cells, and thus, the amount of fat tissues, we measured the weight of fat tissue incubated with a fixed volume (usually 20 ml) of Ringer's solution in each separate experiment. The concentrations of fat solution in the groups of 100 nM of PE were between 0.34 to 1.82 mg/ml, and in the groups of 20 mM of KCl were between 0.88 to 21.8 mg/ml. In both groups, there is a positive correlation between the enhanced vascular contraction and the wet weight of fat tissues (figure 24, 25).

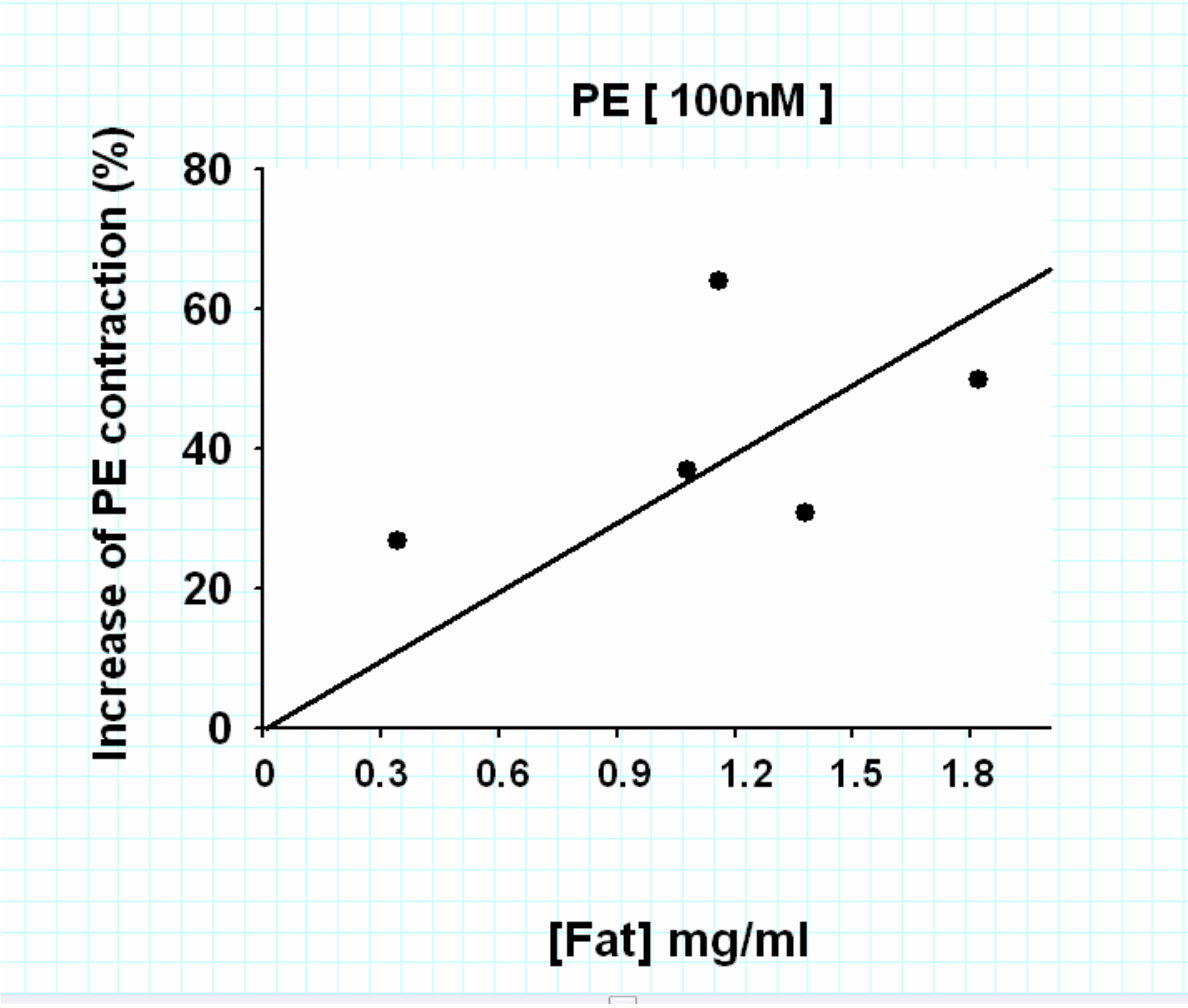


**Figure 24:** A positive correlation with an increase of vessel contraction relative to increasing concentration (from 0.34 to 1.82 mg/ml) of fat tissue in response to 100 nM PE.

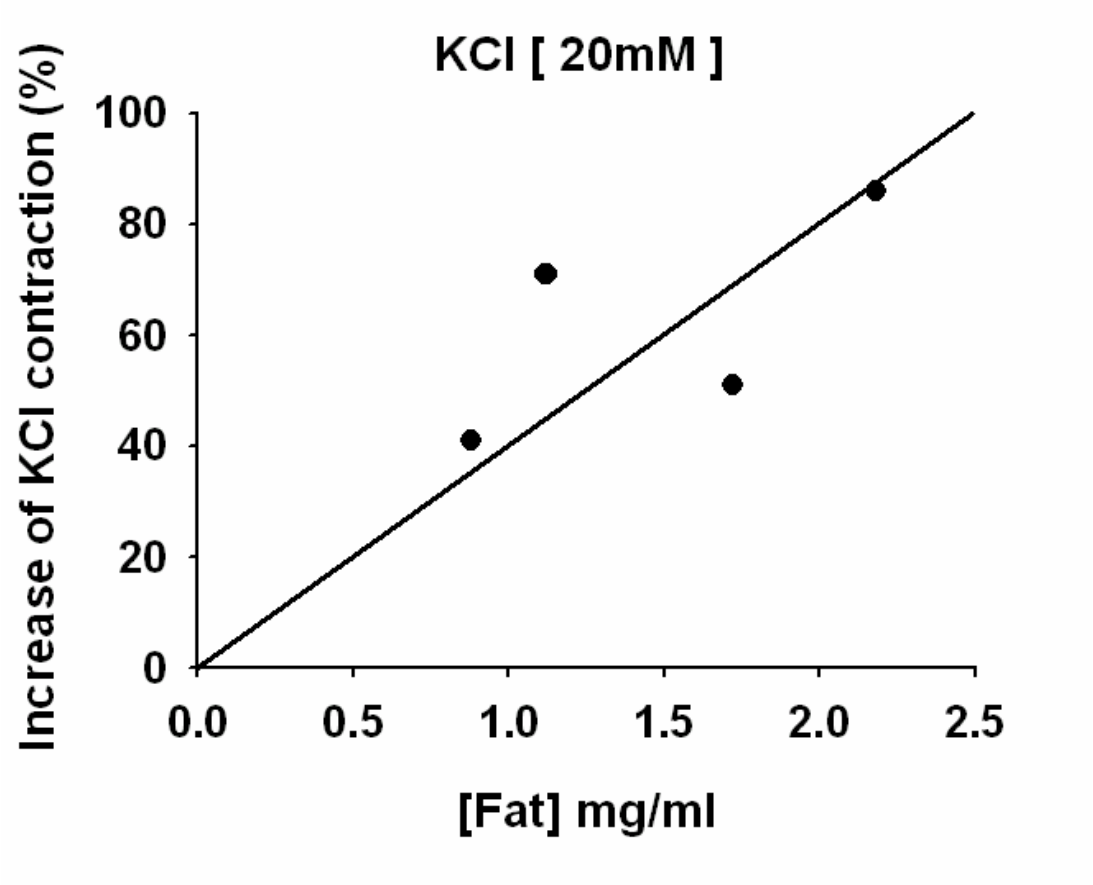


**Figure 25**: A positive correlation with an increase of vessel contraction relative to the increasing of the concentration (from 0.88 to 21.8 mg/ml) of fat tissue in response to 20 mM KCl.

## 3-4 Effect of boiled fat solution on contraction to PE and KCl

In order to learn whether the F solution contains any heat-sensitive diffusible active substances, we studied the effect of F solution under the similar experimental conditions as described above after the F solution was brought to boil and cooled down to room temperature before use. We did not find any significant difference in contractile responses before and after the transfer of boiled F solution to the fat denuded aortic rings using either KCl or PE as the stimulant. This result shows that the diffusible active substance in the F solution is heat-sensitive (figure 26).

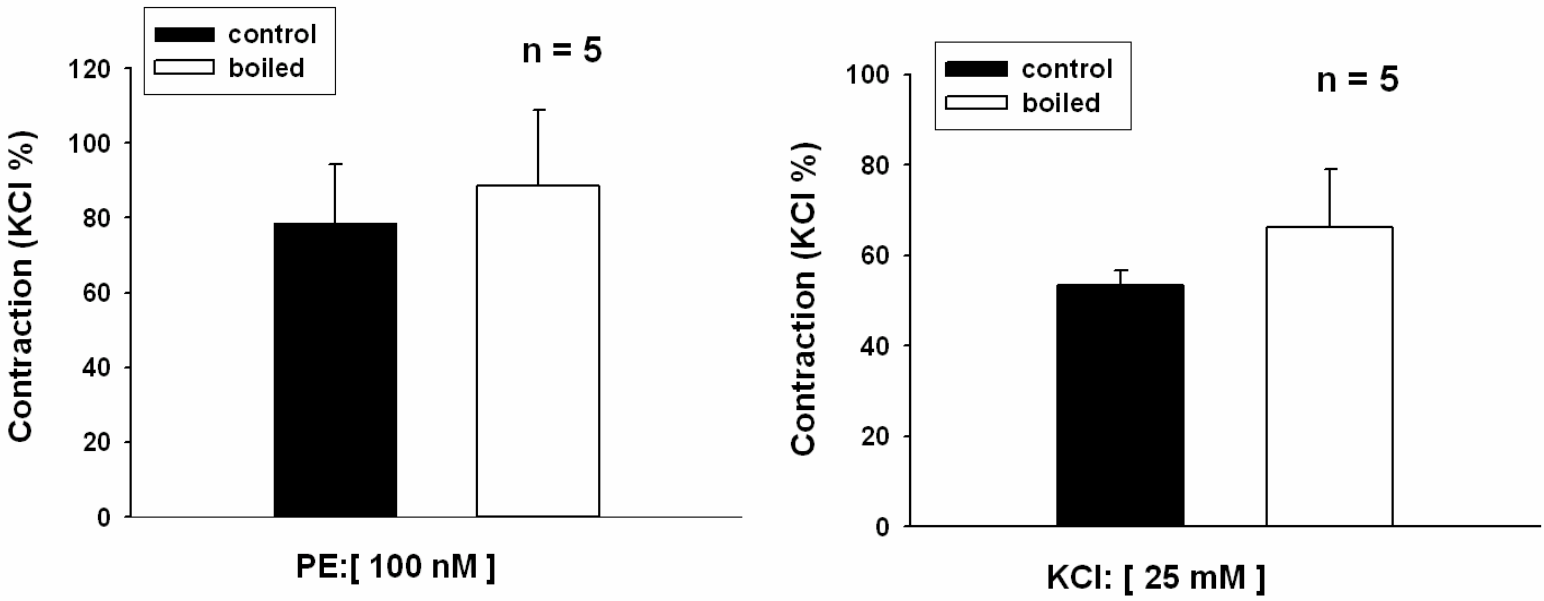


**Figure 26:** The effect of transfer of boiled F- solution to the fat denuded aortic rings that were contracted with PE and KCl. A small, but statistically not significant difference was found for PE and KCl induced contraction in compared to the control groups. ( control group: F solution not boiled )

## 3-5 The role of endothelium-nitric oxide formation on the effect of F-solution on contraction to PE

To learn whether the endothelial layer plays a role in the contractile effect by PVAT in rat's aorta to PE. We divided into two groups. The first group was endothelial intact, other group was inhibited the endothelial relaxant effect (inhibit nitric oxide formation) by L-NAME. All groups were stimulated by PE. Both groups have the increase vascular contractile effect to the PE. In the group of nitric oxide (NO) synthesis inhibition, it seems a more contractile effect compared to the group of endothelial intact. It is because loss of NO relaxation effect. This can be said that the vascular contraction by ADF were not affect by the nitric oxide (NO) (figure 27, 28).

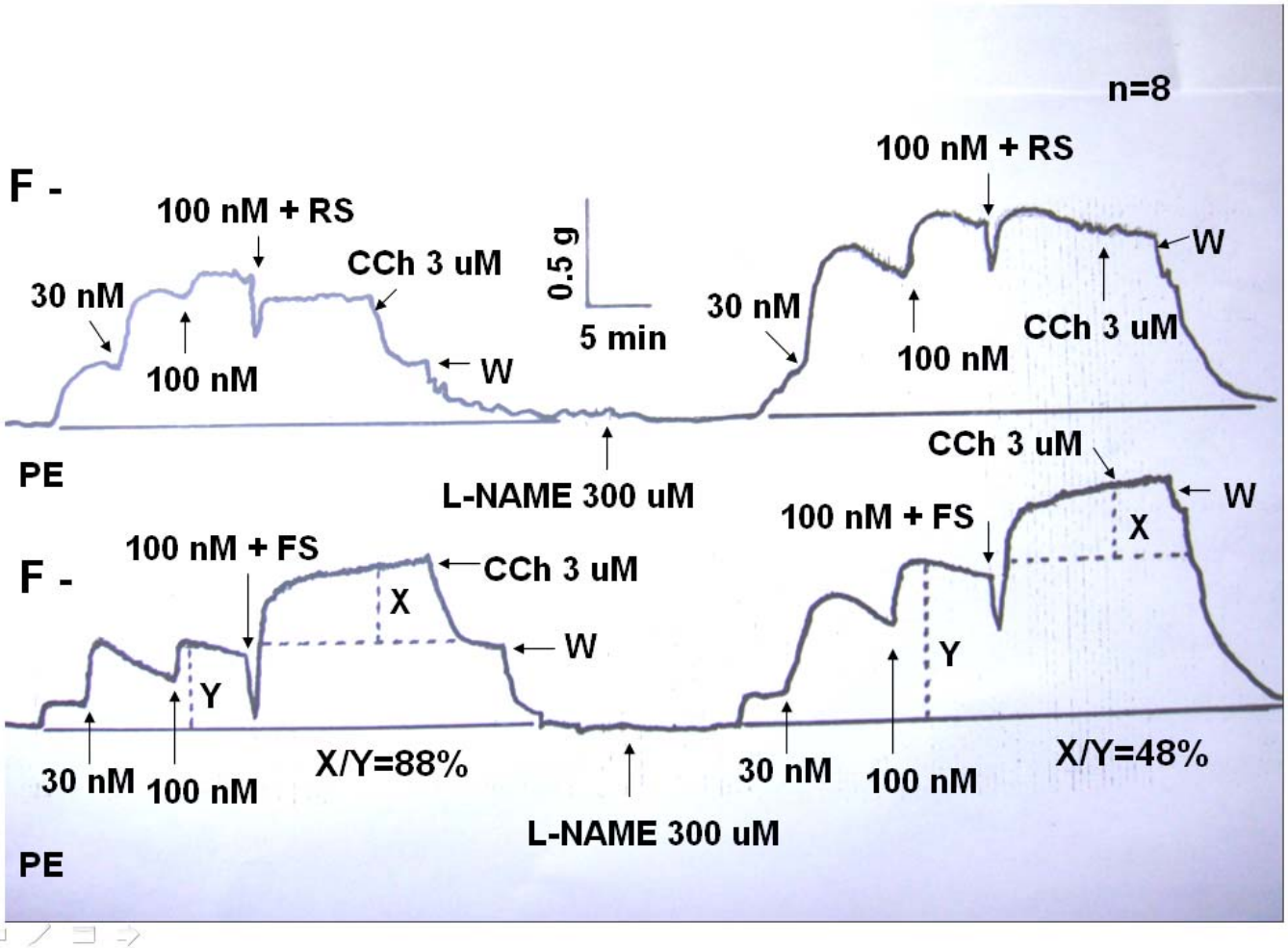


**Figure 27**: In F – aortic rings, transfer of Ringer's solution with the same concentration of 100 nM PE, it showed no significant change compared to the before transferring or adding L-NAME to in-active the nitric oxide (NO). In transfer of F solution with the same concentration of 100 nM PE, it showed a significant enhance of vessel contraction similar to the before transferring of F solution or adding of L-NAME.

RS:Ringer's solution FS: fat solution CCh: carbachol

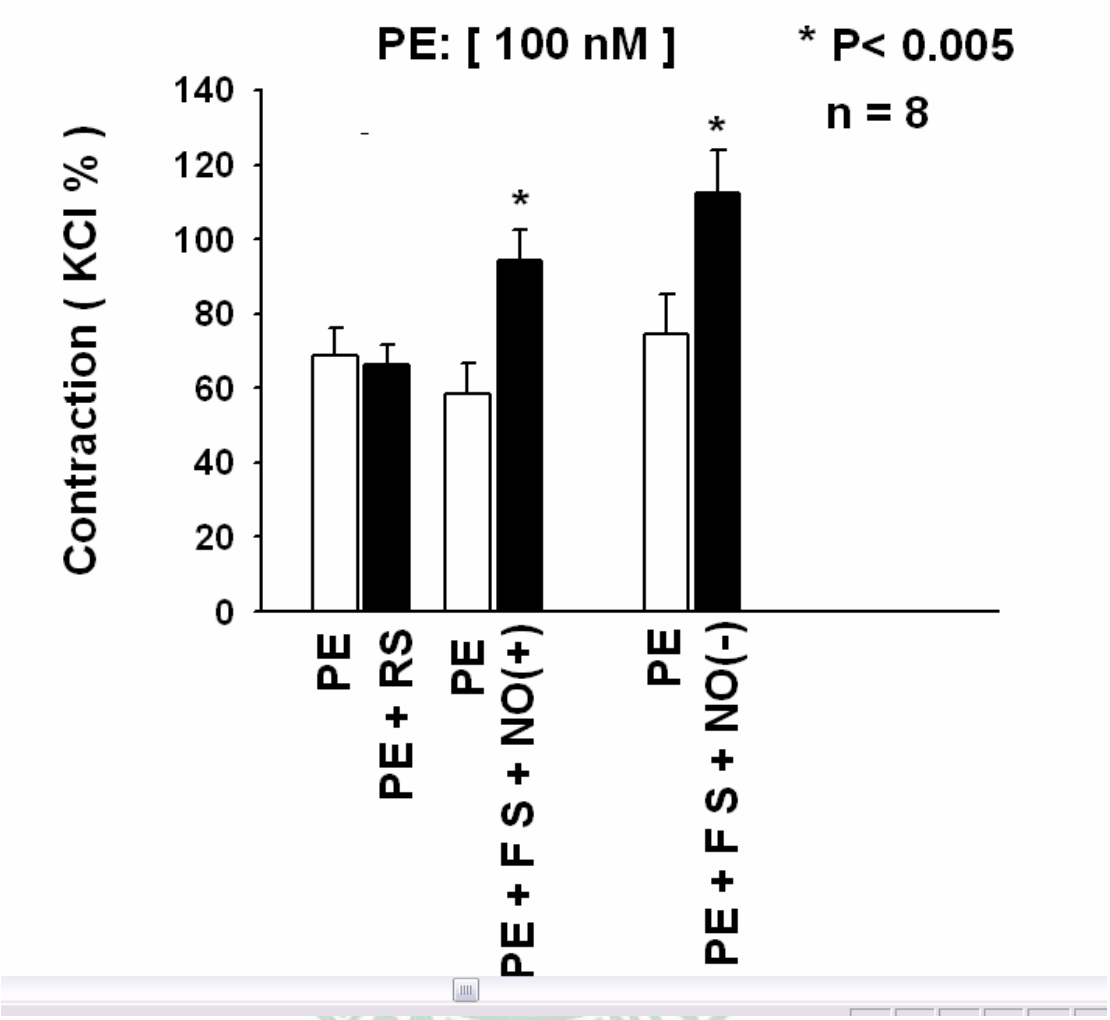


**Figure 28**: Fat denuded aortic rings with transfer F-solution in either nitric oxide intact NO(+) or nitric oxide inhibition NO(-). Both groups have a significant increase in vascular contractile response to PE (0.1 uM) but more contractile effect in group (NO-). No change in group of transfer Ringer's solution. * $p < 0.005$

RS: Ringer's solution FS: fat solution

NO(+): with nitric oxide NO(-): without nitric oxide

### 3-6 Effect of F- solution on Carbachol (CCh) induced aortic relaxation

In order to learn whether the ADF has an effect on the endothelium dependent relaxing response to CCh (3 uM). We studied F- solution on the fat denuded aortic rings. First we found there has a significant enhance sensitivity of low dose PE (10 nM) in F-solution group compared to the control group. Second, impeded the relaxing response to CCh was also observed in F-solution group. This phenomenon can be reversed after washout of the F-solution (figure 29, 30).

### 3-7 Effect of Angiotensin II (Ang.II) on F- aortic rings with/without transfer F solution

To learn whether the transfer F solution has an effect to Ang.II in vessel contraction. We did in 2 ways. In all the F denuded aortic rings, one is to transfer F solution into the organ bath, incubated for at least 30 minutes, then stimulated the aortic rings with 200 or 375 nM Ang.II and compared to the group of non-transfer F solution. It showed a significantly enhanced vessel contraction in the group of transfer F solution compared to the group of non-transfer F solution ( figure31). Another way is to stimulate the aortic rings by adding 200 nM Ang.II. When the vessels contractile reach to the plateau, transfers the F solution or Ringer's solution which containing the same concentration of Ang.II that was introduced. It showed no significant change of the vessel contraction in either before or after transfer fat solution or Ringer's solution with the same concentration of Ang.II ( figure 32).

We also tested whether the Ang.II can stimulate the vessels repeatedly. We stimulated the vessels by adding 100 or 200 nM Ang. II, after the vessels contraction, washed out and stimulated with the same concentration of Ang.II. It showed only a low or even absent of vessels' contraction compared to the first stimulation ( figure 33).

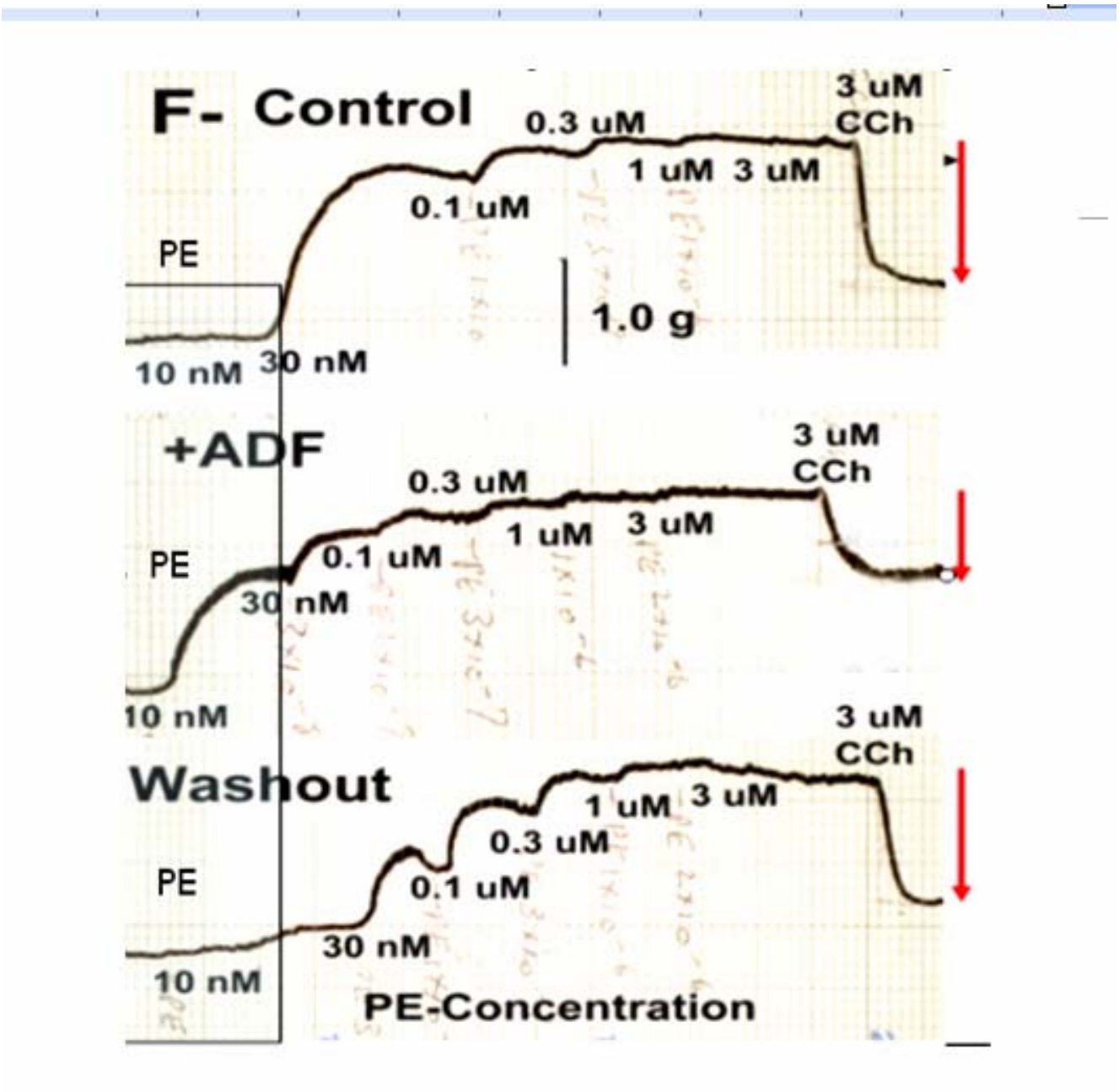


**Figure 29** : In F – control, a significant vessel contraction to the response of 30 nM PE stimulation, also a good relaxing response to the 3 uM CCh. After incubated with fat solution (+ADF), it showed a significant enhance vessel contraction in 10 nM PE, and reduced the relaxing response to the 3 uM CCh. After wash out of the ADF, the vessel contractile response to PE and relaxing response to CCh were reverse as same as the control.

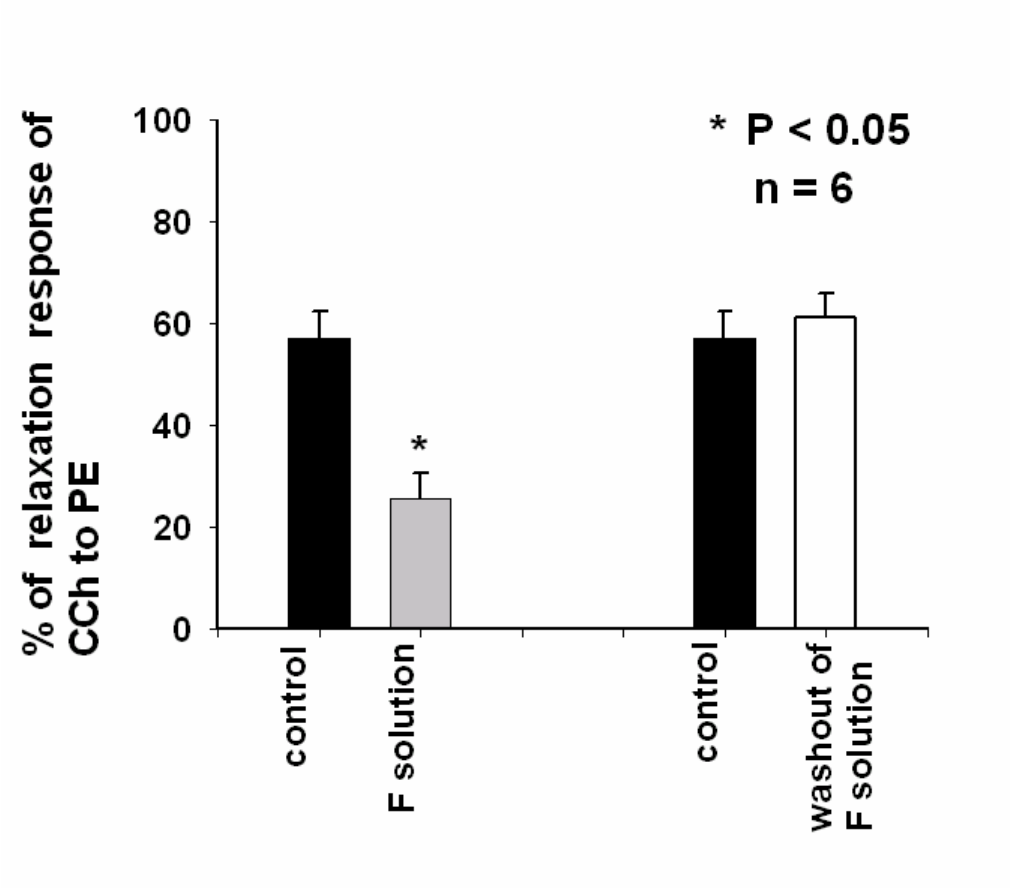


**Figure 30 :** The relaxation induced by 3 uM of Carbachol (CCh) in aortic rings pre-contracted with 1 uM PE. After introduction of the F-solution, the magnitude of the relaxation to CCh was significantly reduced. This phenomenon can be reversed after washout of the F-solution

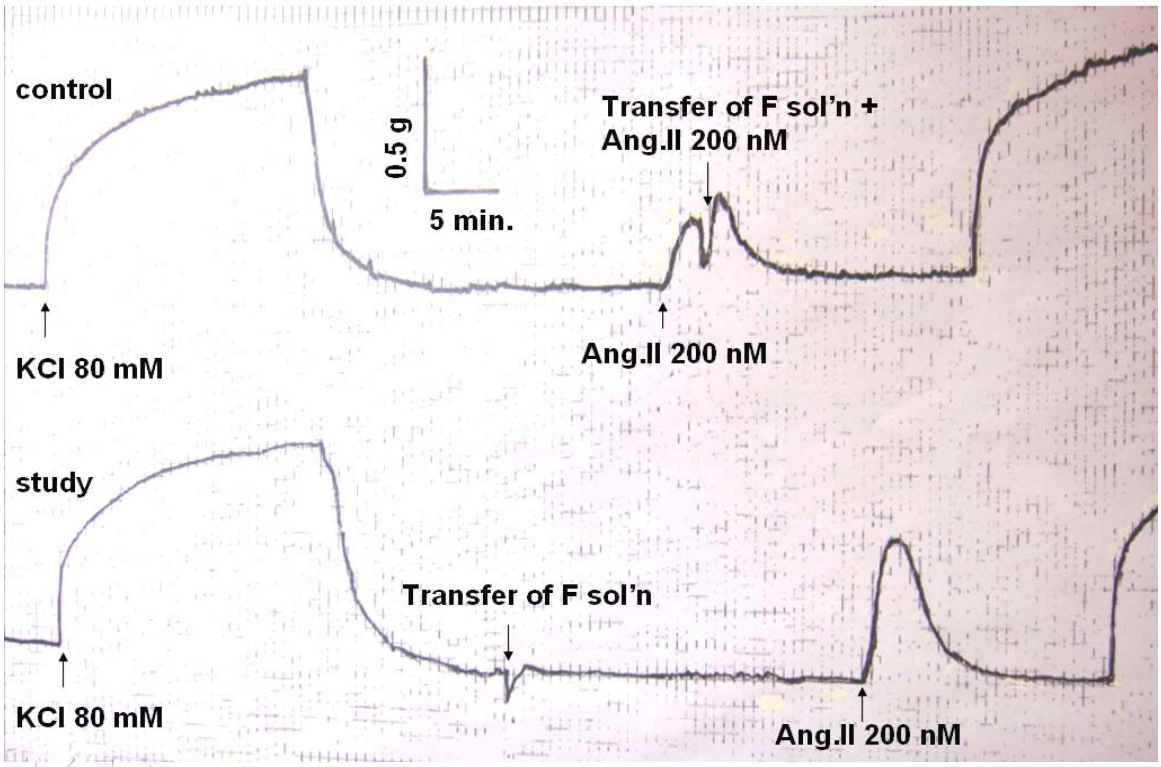


**Figure 31**：In groups of before transfer F solution and stimulated by 200 or 375 nM Ang.II, It showed a significant vessel contractile effect much more than control gropus. The percentage of contractile response to the 80 mM of KCl in study groups is 62%, and 25% in control groups. In control groups, after stimulated by Ang.II and transfer of F solution by adding the same concentration of Ang.II, it also showed a contractile response similar to the first stimulation ( the stimulation by 375 nM Ang.II is not shown)

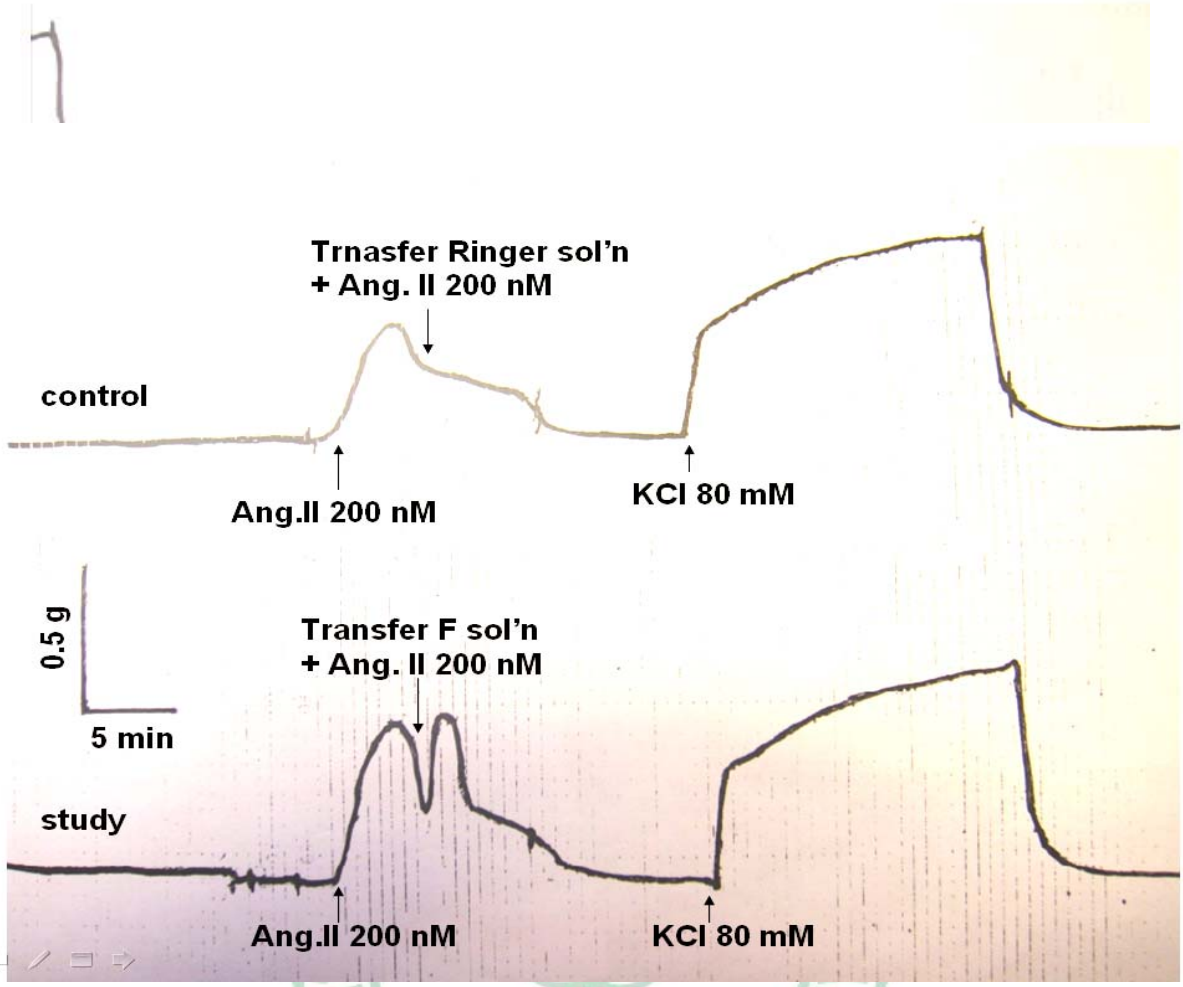


**Figure 32**: After the vessels were stimulated by 200 nM Ang.II, then transfer F solution or Ringer's solution with the same concentration of Ang.II. Both showed no significant change or even reduced in vessel contraction compared to the first stimulation. In study group, the percentage of contractile response to KCl is 77% in the first Ang.II stimulation, and 79% in the second transfer fat solution added with the same concentration of Ang.II. In control group, the percentage of contractile response to KCl is 61% in the first stimulation, and 31% in the second transfer Ringer's solution added with the same concentration of Ang.II.

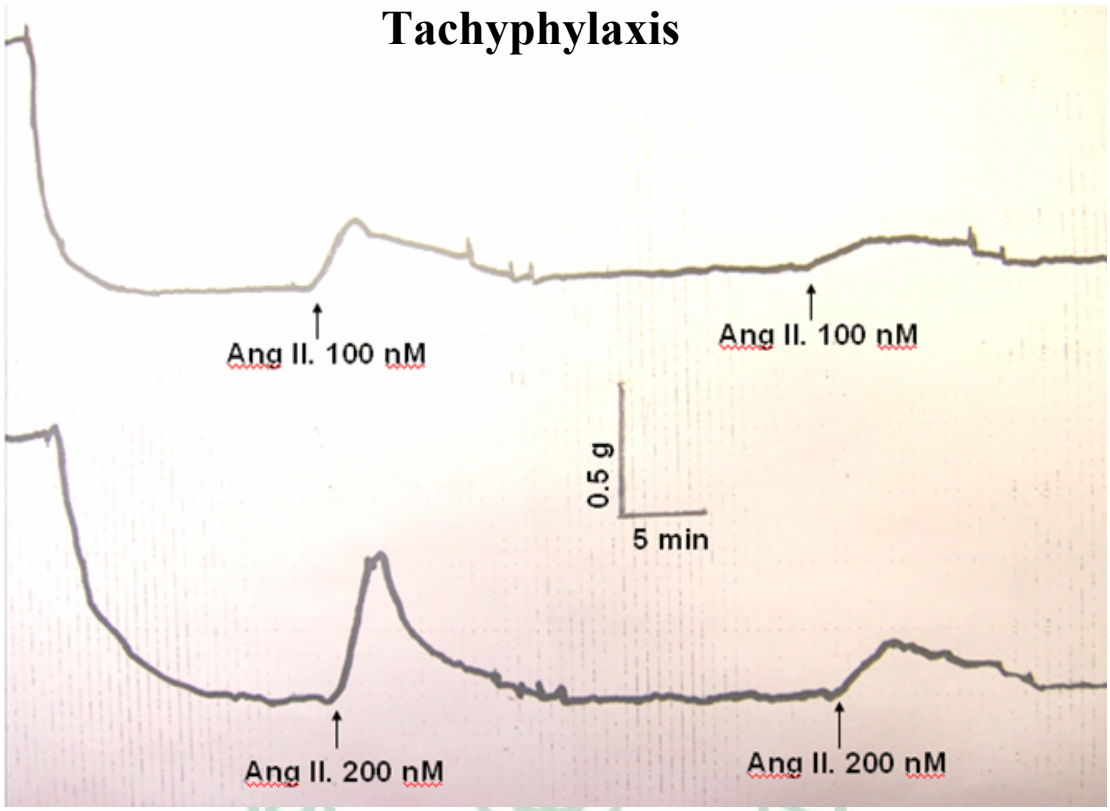


**Figure33**: After the aortic rings were stimulated by 100 or 200 nM Ang.II, a significant vessel contraction occurred. However, when repeat stimulated with the same concentration of Ang.II, it showed only a low or even absent of vessel contractile response.

**3-8 Effect of fat solution (containing adipose derived factor) on the resting tension**

In some groups of fat denuded aortic rings, when transfer of ADF- solution, it shows an increase of vessel excitation and tonic contraction with rhythmic and spike activity. This phenomenon can last for more than 40 minutes and return to normal after washout of the ADF- solution (figure 34).

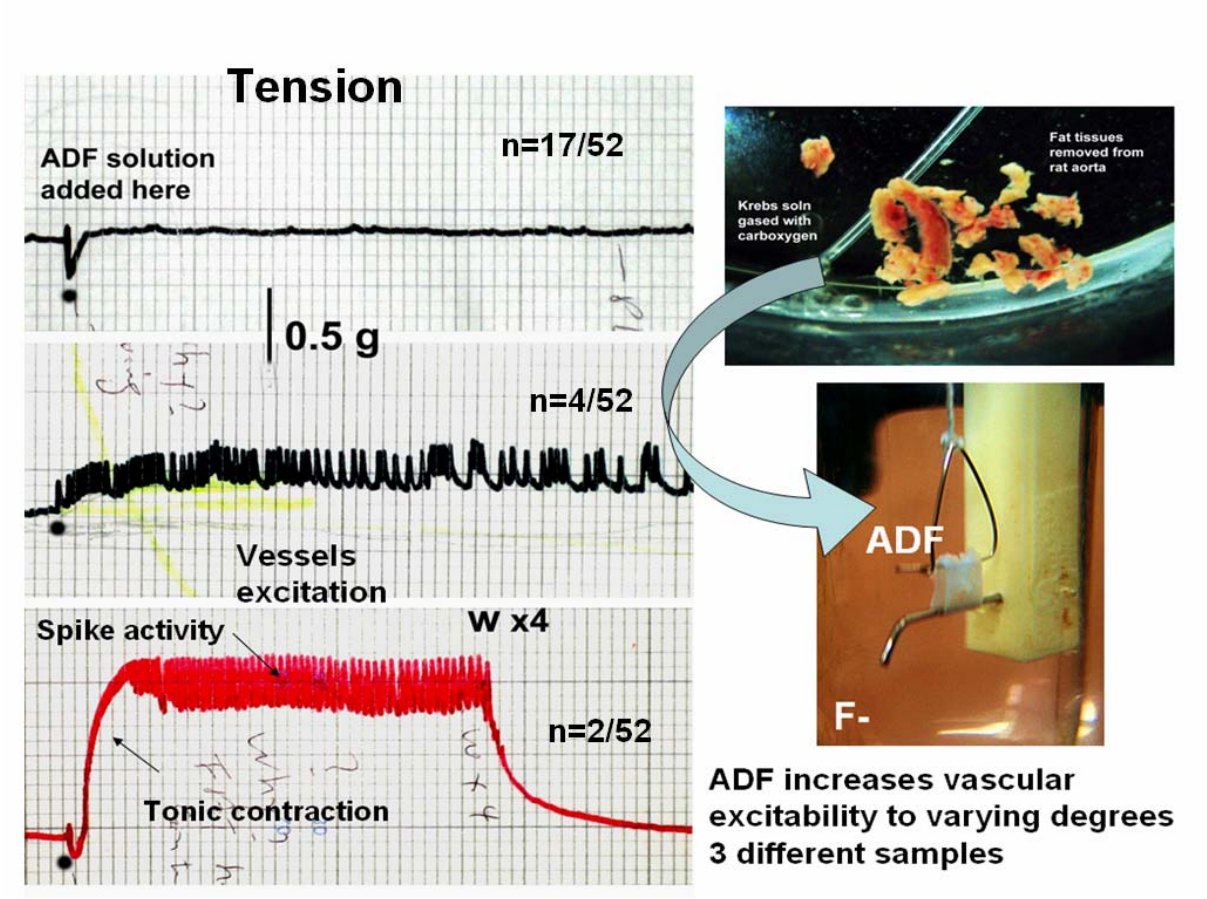


**Figure 34**: Effect of resting tension after transfer of ADF-solution, a rise up of baseline with vessel excitation and tonic contraction.. This phenomenon can last for more than 40 minutes. The baseline return to normal level after the ADF-solution has been washed out ADF-solution: fat solution containing adipose derived factor.

# Chapter 4

## Discussion:

It is well known that the fat tissue plays may different functions on our body. Obesity, manifest itself as a high body mass index (BMI), defined as the fat tissue accumulated much more than the normal.(82) Obesity is associated with a major pathological change in human, the so called metabolic syndrome. It includes high blood pressure. In prior studies, investigators reported the perivascular adipose tissue (PVAT) plays a vascular relaxation function due to the release a factor "adipoose derived relaxing factor" (ADRF) (76,77,78) Means if more adipose tissue surrounding the vessels will cause more relaxation to the vessels. Under this circumstance, people who are obese will have lower blood pressure than the non-obese one. It is clearly different from the clinical findings which indicate that obese people tend to have higher blood pressure. . We assume that the PVAT has some functions which can influence the blood vessel tone, can enhance vascular contraction instead of relaxation. We used the rat thoracic aortic rings, with either fat intact (F+) aortic rings, fat denuded (F-) aortic rings or F- aortic rings but with an extra transfer fat solution which stimulated by PE, KCl, Ang.II or relaxed by CCh. Our novel finding demonstrated that PVAT caused a significant enhance in sensitivity to PE and KCl and so increase vascular contractile response in either F+ aortic rings, F- aortic rings exposed to solution incubated with fat solution compared to F- aortic rings. Or in some groups of F- aortic rings exposed to fat solution they showed an elevation of resting tension soon after transferring fat solution to the F- aortic rings even in the absence of stimulant without PE or KCl, i.e. it changed the resting tension. They appeared with a vessel excitation and tonic contraction. This phenomenon can last for more than 40 minutes and returned to the baseline till the solution has been thorough washout. When those F- aortic rings had add with PE and up to an optimal level, we used CCh to test the endothelial-dependent relaxing function, it showed a good relaxation response. But when we transfer the fat solution to the F- aortic rings before reacted with PE, it showed an enhance sensitive to PE and, reduced the relaxation response to CCh. This phenomenon can reverse after washout of the fat solution.

The above findings are consistent with our hypothesis that the PVAT has an excitatory function, which can increase the vascular contractile response. We propose

that the PVAT can release a factor, so called “Adipose derived factor” (ADF) to cause elevated vascular tone, where the contractile response is proportional to the fatty mass. That means, the more concentration of F solution, the more vessel contractile response. These experimental results were different to the reported earlier. In those groups of F+ aortic rings, F-aortic rings with transfer fat solution (ADF), they all showed an enhance sensitive as elevated contractile response at low concentration of PE and KCl as compared to the F-aortic rings. In those rings after the transfer ADF to the F- aortic rings after stimulated with PE and KCl, an enhance contractile response to the vessel was also observed. However, in those groups of F+ aortic rings, after PE or KCl were washout, they usually shown a transit contraction and a slow relaxation

(a long half time of maximal relaxation). This phenomenon were not seen in those F- aortic rings or F- aortic ring with incubation of ADF, so it is possible to explain that the transient contraction and slow relaxation is due to the fatty barriers, causing a delay washout response to PE and KCl.

We used different methods to test the characters of PVAT. Matthias Lohn et al (77) reported that transfer of fat solution to the F- aortic rings, greatly eliminated the contractile response and dose response-relationship to incremental doses of Ang.II over a wide range ( from 1 to 300 nM). We used Ang.II at the concentration 100, 200 or 375 nM to test whether the ADF has a vaso-dilate response or not. We transfer ADF to F- aortic rings and found that transfer of ADF to the F- aortic rings either before or after stimulation by Ang.II. None of them showed vaso-dilating effect. Some of them even showed an increase contractile response as compared to the control groups. We were not able to construct a dose-response curve in the same aortic rings. It is because the phenomenon of tachyphylaxis (83,84), It is suggested that tachyphylaxis depends on a slowly reversible alteration of a calcium translocation step in the stimulus-response coupling. This can diminished response to later increments in a sequence of activation by Ang.II to the vessels (85). Gao et al (80) found that PVAT caused an increase in electrically stimulated vascular contractile response in mesenteric artery due to the formation of superoxide, and the contractile response can be diminished by superoxide dismutase (SOD). We used PE, KCl and Ang.II to stimulate the aortic rings in either F+, F- or F- following the transfer of F solution. Enhanced vessel contraction was observed in F+ and F-

following the transfer of F-solution. The enhanced vascular contraction in this groups is apparently due to a diffusible factor (or factors) released from the fat tissue.

To determine whether the ADF is a kind of heat sensitivity or not. We first boiled the fat solution (ADF) up to 100 C, then cooled down to the room temperature, and transferred to the F- aortic rings. The boiled ADF did not show an significant enhance sensitive to PE or KCl that increase vascular contraction in the F – aortic rings. We consider that ADF may be a heat sensitive protein or peptide, which can be de-natured by heat and thus loss its function (86,87).

Conclusion: According to our experiments, we can know that the fat tissue can release a factor ”adipose derived factor” that play a vessel contractile effect. Although the real mechanism is still unknown, it is quite consistent that in obese people who have a high percentage of high blood pressure or vascular disease than those who are non obese. It give us a further new concept to understand the relationship between obesity and systemic disease. The physiological significance of perivascular fat is at best speculatory and requires more rigid future investigation.

## References:


1. National institutes of Health, National Heart, Lung and Blood institute. Clinical guidelines on the identification, evalustion and treatment of overweight and obesity in adults, the evidence report. Obesity Research 1998,6 (suppl 2):51s-209s
2. Troiano RP, Frongillo EA Jr, Sobal J, Levitsky DA. The relationship between body weight and mortality: a quantitative analysis of combined information from existing studies. Int. J Obes. 1996, 20: 63-75
3. Rosenbaum M, Lwivwl RL, Hirsch J. Obesity . N Engl J Med 1997,337:396-408
4. Hirsch J, Knittle JL. Cellularity of obese and non-obese human adipose tissue. Fed.Proc. 1970, 29:1516-1521
5. Seidell JC, Flegal KM. Assessing obesity: classification and epidemiology. Br Med Bull. 1997,53(2):238-52
6. Poirier P, Alpert MA, Flaisher LA, Thompson PD, Suqerman HJ. Cardiovascular evaluation and management of severely obese patients undergoing surgery: a science advisory from the American Heart Association. Circulation. 2009, 120(1):86-95.
7. Verma S, Todd J. Anderson. Fundamentals of endothelial function for the clinical cardiologist. Circulation, 2002, 105: 546-549
8. Skilton MR, Celermajer DS. Endothelial dysfunction and arterial abnormalities in childhood obesity.Int J Obes (Lond). 2006, Jul:30(7):1041-9.
9. Moncada S, Higgo A. The L-arginine-nitric oxide pathway. N.Engl J Med. 1993, 329:2002-2012
10. Yanagisawa M, Kwrihara H, Kimura S. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 1998, 332:411-415
11. van Breemen C, Skarsgard P, Laher I,. McManus B, Wang XD. Endothelium smooth muscle interactions in blood vessels. Clin. Expt Pharmacol Physiol. 2007, 24: 989-992
12. Webb RC. Smooth muscle contraction and relaxation. Advan. Physiol. Edu. 2003, 27, 201-206
13. Laflamme K, Roberge CJ, Grenier G, Rémy-Zolghadri M, Pouliot S, Baker K, Labbé R, D'Orléans-Juste P, Auger FA, Germain L. Adventitia contribution in

vascular tone: insights from adventitia derived cells in a tissue engineered human blood vessel. FASEB J. 2006, 20:1245-1247

14. Stenmark KR, Pavie N, Frid M, Gerasimovskava E, Das M. Role of the adventitia in pulmonary vascular remodeling. Physiology (Bethasda) 2006, 21:134-145
15. Halligan KE, Jourd heuil FL, Jourd heuil D. Cytoglobin Is Expressed in the Vasculature and Regulates Cell Respiration and Proliferation via Nitric Oxide Dioxygenation. J.Biol.Chem. 2009, 284: 8539-8547
16. Rajagopalan S, kurz S, Munzel T. Angiotensin II mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation: contribution to alteration of vasomotor tone. J. Clin.Invest 1996,97:1916-1923
17. Ntambi J M, Kim Y C, Adipocyte differentiation and gene expression. J Nutrition. 2000, 130: 3122s-3126s
18. Tilg H,. Moschen AR. Adipocytokines mediates linking adipose tissue, inflammatory and immunity. Nature review immunology 2006, 6: 772-783
19. Nomiyama T, Perez-Tilve D, Ogawa D, Gizard F, Zhao Y, Heywood EB, Jones KL, Kawamori R, Cassis LA, Tschöp MH, Osteopontin DB. Mediates obesity-induced adipose tissue macrophage infiltration and insulin resistance in mice J. Clin. Invest. 2007, 117: 2877-2888 .
20. Smith S R, Zachwieja JJ. Visceral adipose tissue: a critical review of intervention strategies. Int J Obes Relat Metab Disord 1999, 23: 329-335
21. Berg JM, Tymoczko JL, Stryer L. Biochemistry 2007, 6 edition. P.219
22. Johnson PR, Greenwood MRC. The adipose tissue in cell and tissue biology. Textbook of Histology. 1998, 6 edition, 191-196
23. Nedergaard J, Bengtsson T, Cannon B. Unexpected evidence for active brown tissue in adult humans. Am. J Physiol. Endocrinol Metab. 2006, 293 (2) E444-452
24. Ronti T, Lupattelli G, Mannarino E. The endocrine function of adipose tissue: an update. Clinical endocrinology. 2006, 64: 355-65
25. Haffner S, Taegtmeyer H. Epidemic Obesity and the Metabolic Syndrome. Circulation. 2003,108:1541-1545
26. Wilson PW, Kannel WB. Obesity, diabetes and risk of cardiovascular disease in the elderly. Am J Geriatr Cardiol. 2002,11: 119-123

27. Eroqul S, Sude LE, Bozbus H, Haberal A, Ozbicer S, Demir O, Muderrisqlu H. Association of serum adiponectin levels and coronary flow reserve in women with normal coronary angiography _Eur.J Cardiovasc Prev Rehabil. 2009,16(3):290-296
28. Reilly MP, Rader DJ. The Metabolic Syndrome, more than the sum of its parts? Circulation 2003,108:1540-1551
29. Pi-Sunner FX. Pathophysiology and long-term management of the metabolic syndrome. Obesity research 2004, 12 suppl:174s-179s
30. Hansson GK. Inflammation, Atherosclerosis and Coronary Artery Disease. N Engl J Med. 2005,352:1685-1695
31. Miller YL, Chang MK, Binder CJ, Shaw PX, Witztum JL. Oxidized low density lipoprotein and innate immune receptors. Curr Opin Lipidol. 2003,14:437-445
32. Kovanen PK, Kaartinen M, Paavonen T. Infiltrates of activated mast cells at the site of coronary atheromatous erosion or rupture in myocardial infarction. Circulation 1995, 92:1084-1088
33. Chatzinikalaou A, Fatouros L, Petridou A, Jamurtas A. Adipose tissue lipolysis is upregulated in lean and obese men during acute resistance exercise. Diabetes care. 2008,31:1397-1399
34. Van Harmelen V, Eriksson A, Astrom G, Wahlem K, Naslund E. Vascular peptide endothelin-1 links fat accumulation with alterations of visceral adipocytes lipolysis. Diabetes 2008,57:378-386
35. Kerm PA, Ranganthan S, Li CL, Wood L, Ranganathan G. Adipose tissue tumor necrosis factors and interleukin-6 expression in human obesity and insulin resistance. Am. J Physiol Endocrin Metab. 2001,280:E745-751
36. Heart Research Centre. Update. November 2007
37. Brown-Ayers H. Adopting low risk dietary and lifestyle factors related to lower incidence of high blood pressure. JAMA. 2009,302(4):401-411
38. Hanna NN, Kenndy DE. Hypertension in patient with pheochromocytoma 1999, vol.1, No.6: 540-545
39. Agroyannis B, Mourikis D, Kopelias I, Tzanatos H, Soubassi L, Fourtounas C. Ammonia concentration in the renal veins in unilateral renal artery stenosis with hypertension. QJ med.1998, 91: 367-369
40. Juncas LI. Intrarenal mechanisms of salt and water retention in the nephritic syndrome. Kidney international. 2002, 61: 1182-1195

41. Umpierrez GE, Smiley D, Robulino G. Free fatty acid induced blood pressure elevation and endothelial dysfunction in obese african americans with type 2 diabetes. J Clin. Endocrin Metab. 2008,11:1-17
42. Steinberg H. Tarshoby M. Monestel R. Hook G. Gronin J. Johnson A. Elevated circulating free fatty acid level impaired endothelium-dependent vasodilation. J Clin Invest. 1997,100:1230-1239
43. Paolisso G, Manzella D, Rizzo MR, Razno E. Elevated plasma fatty acid concentrations stimulate the cardiac automatic nervous system in healthy subjects. Am J of Clin Nutri. 2000,72: No.3, 723-730
44. Blaske T. Smooth Muscle. Wikipedia. 29 May, 2008
45. Murph RA. Signal transduction in smooth muscle. Review of physiology, biochemical and pharmacology. J physiol. 1999,519: 520-540
46. Lohman W, Galinska Rakoczy A, Hatch Y, Tobacan LS, Craig R. Structural basis for the activation of muscle contraction by troponin and tropomyosin J Mol. Biol. 2009,388(4): 673-81
47. Walcott S, Faqnant PM, Trybus KM, Warshaw DM. Smooth muscle heavy meromyosin phosphorylated on one of its two heads supports force and motion. J Biol Chem. 2009,284(27):18244-18251
48. Craig R, Greene LE, Eisenberg E. Structure of the actin-myosin comples in the presence of ATP. PNAS 1985,52:3247-3251
49. Rayment I, Holder HM, Whittaker M, Yohn CB, Loreny M. Structure of the actin myosin complex and its implication for muscle contraction. Science 1993,.261: 58-65
50. Barritt G J. Receptor activated Calcium inflow in animal cells:a variety of pathways tailored to meet different intracellular calcium signalling requirements. Biochem J 1999,337:153-169
51. Dolphin AC. A short history of voltage gated Calcium channel. Br. J Pharmacol 2006,147:(Suppl 1) S56-62
52. Tikhonov DB, Zhorov BS. Structural Model for Dihydropyridine Binding to L-type Calcium Channels. J Biol Chem. 2009,284(28):19006-19017
53. Abernethy DR, Soldatov NM. Structure-Functional Diversity of Human L-Type Calcium Channel. Perspectives for New Pharmacological Targets J Pharmacol Exp Ther. 2002,300(3):724-728.

54. Santana LF. Relation between sarcolemmal calcium current and calcium sparks and local theories for cardiac excitation-contraction coupling. Circ.Res. 1996,78: 166-171
55. Burgess DE, Crawford D, Delisle BP, Santi J. Mechanism of Inactivation Gating of Human T-Type (Low-Voltage Activated) Calcium Channels. Biophysical Journal. 2002,82:issue 4,1894-1906.
56. Blaustein MP, Lederer WJ. Sodium/Calcium exchange: Its physiological implication. Physiological review 1999,79:no.3,763-854
57. Insel PA. Adrenergic receptors – evolving concepts and clinical implications New Eng J of Med. 1996,334: no 9,580-585
58. Chen Q, Konopka JB. Regulation of the G protein coupled alpha factor pheromone receptor by phosphorylation . Mol cell Biology. 1996,16: 247-257
59. J Struct Funct Genomics. Calmodulin target database. 2000,1(1):8-14
60. Danser AH, Schalekamp MA, Bax WA, van den Brink AM, Saxena PR, Riegger GA, Schunkert H.. Angiotensin converting enzyme in the human heart. Effect of the deletion / insertion polymorphism. Circulation, 1995,92:1387-1388
61. Wolf G. 'The road not taken': role of angiotensin II type 2 receptor in pathophysiology. Nephrol Dial Transplant. 2002,17:195-198
62. Kim D, Aizawa T, Wei H, Pi X, Rybalkin SD, Berk BC, Yan C. Angiotensin II increases phosphodiesterase 5A expression in vascular smooth muscle cells: a mechanism by which angiotensin II antagonizes cGMP signaling. J Mol.Cell Cardiol. 2005, 38(1):175-184
63. Kim D, Rybalkin SD, Pi XC, Wang YI, Zhang CX, Munzel T, Beavo JA, Berk BC, Chen Y. Upregulation of Phosphodiesterase 1A1 Expression Is Associated With the Development of Nitrate Tolerance Circulation. 2001,104:2338-2343
64. Sachse A. Wolf G. Angiotensin II-induced reactive oxygen species and the kidney. J Am.Soc.Nephro. 2007, 18(9):2439-2446
65. Klabunde RE, Cardiovascular physiology concepts. 2009, 2 August
66. Brooks DP. Experimental Biology 1995 Symposium On Endothelin Receptors: Role In Renal Function And Dysfunction. Clin.and Exp.Pharm.and Physiol. 2007, 23: issue 4. 325-325
67. Konczalla J, Vatter HL, Weidauer S, Raabe A, Seifert V. Alteration of the Cerebrovascular Function of Endothelin B Receptor After Subarachnoidal

Hemorrhage in the Rat. Experimental Biology and Medicine. 2006,231:1064-1068

68. Liu M, Simon MI. Regulation by cAMP-dependent protein kinease of a G-protein-mediated phospholipase C. Nature 1996,382:83-87
69. Zhou L,. Siegelbaum SA. Pathway and Endpoint Free Energy Calculations for Cyclic Nucleotide Binding to HCN Channels.Biophysical Journal. 2008,.94: issue 12, L90-L92.
70. Moncada S. Nitric oxide: discovery and impact on clinical medicine. J R Soc Med. 1999,92(4):164–169.
71. Alderton WK, Cooper CE, Knowles RG. Nitric oxide synthases: structure, function and inhibition. Biochem J. 2001,357 (Pt 3):593-615
72. Inagaki N, Seino S. ATP-sensitive potassium channels: Structure, Functions and Pathophysiology. Japanese J.of Physiology. 1998,48:397-412
73. Lang SB, Campbell EB, Mackinnen R. Crystal structure of a mammalian voltage-dependent shaker family potassium channel. Science. 2005,309:897-903
74. Lee SY, Baneriee A, Innon MR. Two separate interfaces between the voltage sensor and pore are required for the function of voltage-dependent K(+) channels. Plos Biol. 2009,7(3):e47
75. Havase M, Hashitani H, Kuhri k, Suzuki H. Role of K+ channels in regulating spontaneous activity in detrusor smooth muscle in situ in the mouse bladder. J Urol. 2009,181(5):2355-2365.
76. Soltis EE, Cassis LA. Influence of perivascular adipose tissue on rats aortic smooth muscle responsiveness. Clin Exp Hypertens A. 1991,13 (2):277-296.
77. Lohn M, Dubrovska G, Lauterbach B, Luft FC, Goliasch M, Sharma AM. Periadventitial fat releases a vascular relaxing factor. FASEB.2002,16:1057-1063
78. Verlohren S, Dubrovska G, Tsang SY, Essin K, Luft FC, Huang Y, Gollasch M. Visceral periadventitial adipose tissue regulates arterial tone of mesenteric arteries. Hypertension. 2004,44:271-276
79. Gao YJ, Zeng ZH, Teoh K, Sharma AM, Abouzahr L, Cybulsky I, lamy A, Semelhago L, Lee RMKW. Perivascular adipose tissue modulates vascular function in the human internal thoracic artery. J. of thoracic and cardiovascular surgery. 2005,130:no.4,1130-35

80. Gao YJ, Takemori K, Su LY, An WS, Lu C, Sharma AM, Lee RMKW.. Perivascular adipose tissue promotes vasoconstriction: The role of superoxide anion. Cardiovascular Research. 2006,71:363-373
81. Morrow JD. Is Oxidant Stress a Connection Between Obesity and Atherosclerosis ? Arteriosclerosis, Thrombosis, and Vascular Biology. 2003,23: 368-370.
82. Lara-Castro C, Weinsier R, Hunter G. What impacts visceral adipose tissue accumulation over time? – Obesity Nutrition Research Newsletter. Nov. 2002.
83. Robertson MJ, Wragg A, Clark KL. Modulation of tachyphylaxis to angiotensin II in rabbit isolated aorta by the angiotensin $AT_1$ receptor antagonist, losartan Regulatory peptides. 1994,50: issue 2,137-145
84. Sambhi MP, Barrett JD. Development of Tachyphylaxis to Aspartyl and Not to Asparginyl Angiotensin II in the Rat. Circulation Research. 1967,21:327-335
85. Paiva TB, Aboulafia J, Nouaihetas VL, Paiva AC. Tachyphylaxis and angiotensin receptors. General pharmacology. 1976,7(2-3):185-188
86. Heymann E. Dilatometric investigations on the heat-denaturation of proteins. Sir William Ramsay Laboratories of Inorganic and Physical Chemistry, University College, London. 1935, Nov.20.
87. Kazmirski SL, Wong KB, Freund SMV, Tan YJ, Fersht AR, Daggett V. Protein folding from a highly disordered denatured state: The folding pathway of chymotrypsin inhibitor 2 at atomic resolution. PNAS. 2001, 98:no.8, 4349–4354