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Departament de Fisiologia

VASCULAR REACTIVITY OF CAROTID AND RENAL ARTERIES TO NATRIURETIC PEPTIDES: ALTERATIONS DUE TO DIABETES

Diploma thesis

Supervisor: Doc. Ing. Barbora Szotáková, Ph.D.

Specialized supervisor: Dr. José M. Centeno Guil

Hradec Králové 2014

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UNIVERZITA KARLOVA V PRAZE

FARMACEUTICKÁ FAKULTA V HRADCI KRÁLOVÉ

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REAKTIVITA KAROTID A RENÁLNÍCH ARTERIÍ NA NATRIURETICKÉ PEPTIDY: ZMĚNY V DŮSLEDKU DIABETU

Diplomová práce

Vedoucí diplomové práce: Doc. Ing. Barbora Szotáková, Ph.D.

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Hradec Králové 2014 Kristýna Čáňová

I hereby declare that this thesis is my original author work. All literaure and other sources, which I used, are properly cited.

Prohlašuji, že tato práce je mým původním autorským dílem. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci řádně citovány. Práce nebyla využita k získání jiného nebo stejného titulu.

V Hradci Králové, 1. 3. 2014 ..……...

Kristýna Čáňová

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ABSTRACT

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Title of diploma thesis: **Vasclular reactivity of carotid and renal arteries to natriuretic peptides: alterations due to diabetes**

Diabetes is associated with increased prevalence of hypertension, cardiovascular and renal disease. Atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) play an important task in cardiovascular pathophysiology and are considered to have cardioprotective and renoprotective effect in patients with diabetes.

The aim of this work was to study the response of rabbit carotid and renal arteries to atrial and brain natriuretic peptides and whether this response is altered in diabetes.

Six weeks after diabetes induction by alloxan, the renal and carotid arteries were isolated from the body and each segment was tested for isometric tension in an organ bath. All segments were preconcentrated with phenylephrine and then with the cumulative addition of doses of ANP and BNP (10^{-12}-10^{-7} M) to the organ bath, the concentration-response curves to ANP and BNP were measured.

In all cases, natriurtic peptides produced a relaxation of the carotid and renal arteries and showed a hyporreactivity in carotid and renal arteries of diabetic rabbits. Although this hypoactivity was not present in all cases, it would be clearly observed in the case of an increase in sample size.

ABSTRAKT

Univerzita Karlova v Praze, Farmaceutická fakulta v Hradci Králové

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Název diplomové práce: **Reaktivita karotid a renálních arterií na natriuretické peptidy: změny v důsledku diabetu**

Diabetes souvisí se zvyšující se prevalencí hypertenze, kardiovaskulních a renálních onemocnění. Atriální natriuretický peptid (ANP) a B-typ natriuretického peptidu (BNP) hrají významnou roli v kardiovaskulární patofyziologii a předpokládá se, že mají ochranný vliv na srdce a ledviny u pacientů s diabetem.

Cílem této práce bylo studovat vliv atriálního a mozkového natriuretického peptidu na králičí karotidy a renální arterie a zjistit, jestli je tato reakce změněna u diabetu.

Šest týdnů po navození diabetu alloxanem, byly renální arterie a karotidy vypreparovány z těla a každý segment byl vložen do lázně pro izolované orgány, kde bylo měřeno izometrické napětí. Všechny segmenty byly nejprve kontrahovány fenylefrinem a do lázně byly přidávány dávky ANP a BNP (10^{-12}-10^{-7} M), z naměřěných dat byly vytvořeny křivky závistlosti relaxace na koncentraci přidaných NP (natriuretických peptidů).

Ve všech případech, natriuretické peptidy způsobují relaxaci karotid a renálních arterií a ukazují hyporeaktivitu u karotid a renálních arterií z diabetických králíků. Ačkoliv tato hyporeaktivita nebyla pozorována u všech případů, byla by pravděpodobně zjištěna v případě většího počtu vzorků.

CONTENTS

1 INTRODUCTION

Diabetes mellitus, or simply diabetes, is a chronic disease that occurs when the pancreas is no longer able to make insulin, or when the body cannot utilize the insulin produced by pancreas. Insulin is a hormone produced by pancreatic beta cells, that reduce amount of sugar in the blood. Diabetes is divided to three types: when the pancreas doesn't make insulin it's type 1 diabetes, when the pancreas doesn't make enough insulin, or the insulin cannot be utilized it's called diabetes mellitus type 2 and when the insulin is less effective during pregnancy it's Gestational diabetes (web International Diabetes Federation).

Patients with diabetes suffer from a lot of various complications. The most serious are chronic vascular complications like macroangiopathy and microangiopathy. The macroangiopathy affects the heart and blood vessels and may result in fatal complications such as coronary heart disease and stroke. In microangiopathy we can find for example diabetic nephropathy, retinopathy and neuropathy which can lead to dialysis or kidney transplant (nephropathy), blindness (retinopathy) or amputation of the feet and lower limbs (neuropathy).

Diabetes mellitus is very serious disease, prevalence of diabetes is increasing, mostly due to increases in overweight and obesity, unhealthy diet and physical inactivity.

The diabetic state in rabbits we experimentally induced with chemical diabetogen called alloxan. Six weeks after diabetes induction, renal and carotid arteries were isolated and each segment was prepared for isometric tension recording in an organs bath. With a cumulative dose of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) added to organs bath we made the concentration-response curves and determined, how is the diference in relaxation between carotid and renal arteries from control and diabetis rabbit.

2 THEORETICAL PART

2.1 Diabetes mellitus

The World Health Organization (WHO) defines diabetes as a chronic disease, caused by a deficit, whether inherited or acquired of production of pancreatic insulin or due to a lack of response to this hormone. Insulin is a hormone that regulates the concentration of glucose in the blood, so diabetes is characterized by a chronic hyperglycemia, which eventually damages many organs and systems in a severe way.

Among others, it can damage heart, blood vessels, eyes, kidneys and nerves. It also affects the quality of life and the economic well-being of the patient, and of course affects the health system by the increased use of medical services and labor casualties caused by this disease.

Symptoms of this disease are polydipsia, polyphagia, polyuria, blurred vision and loss of weight. Severe forms can develop ketoacidosis or a non ketoacidotic hyperosmolar state that may lead the individual to a state of stupor and coma, leading to the death of the patient if adequate treatment is not provided. In any case, the symptoms described may not appear or may not be severe. In that case it is established a state of hyperglycemia which may cause functional and pathological changes long before they are diagnosed.

There are about 60 million people with diabetes in the European Region, or about 10.3 % of men and 9.6 % of women aged 25 years and over. Prevalence of diabetes is increasing among all ages in the European Region, mostly due to increases in overweight and obesity, unhealthy diet and physical inactivity.

Worldwide, high blood glucose kills about 3.4 million people annually. Almost 80 % of these deaths occur in low- and middle-income countries, and almost half are people aged under 70 years. WHO projects diabetes deaths will double between 2005 and 2030 (web WHO).

2.1.1 Types of diabetes

The diabetes includes a set of heterogeneous disorders, but with common elements like hyperglycemia and intolerance to the glucose, due to the shortcoming of insulin, the alteration of the effectiveness in the action of the insulin or both. According to its etiology, the Atlas of the Diabetes of the International Federation of Diabetes (2009) includes 3 types of diabetes mellitus, based on its etiology and clinical presentation of the disorder: diabetes type 1, diabetes type 2 and gestational diabetes mellitus.

2.1.1.1 Type 1 diabetes

It is called Insulin-dependent diabetes. It is caused by the destruction of insulin-producing beta-pancreatic cells, usually due to an autoimmune reaction, producing these little or no insulin. The reason why this happens is not fully understood.

The disease usually triggered in children or young adults, being more frequent in childhood. Patients with type 1 diabetes need to control their glucose levels in blood by daily injections of insulin, and could not survive without it. Its appearance is usually sudden and abrupt. Symptoms may include abnormal thirst and dryness of mouth, frequent urination, extreme tiredness or lack of energy, constant appetite, sudden weight loss, slow healing of wounds, recurrent infections and blurred vision. The incidence of type 1 diabetes is increasing, likely and mainly due to changes of environmental risk factors. The factors of environmental risk, increasing the height and weight development, increased maternal age at the time of delivery and, possibly, some aspects of the diet and exposure to some viral infections could start autoimmunity or accelerate destruction of the beta cells.

Diabetes mellitus has a high impact economic for the health system and society. This disease develops during its evolution numerous complications and supposes medical consultation, hospitalization, disability and death pensions. It is estimated that the total health expenditure arising from the prevention, control and treatment of diabetes is 5 to 10 % of the global health budget. In 2010, the costs derived from the concepts mentioned above and the complications arising from the disease, amounted to a total of

about 418 billion international dollars (dollar value corrected according to purchasing power parity). Towards 2030 is expected this figure to exceed the 561 billions of international dollars (International diabetes federation, 2009).

In Czech Republic, there is an increasing concern about diabetes. In the last years the number of diabetic patients has been increasing continuously, as well as associated alterations, as retinopathy (Pelikánová, 2013).

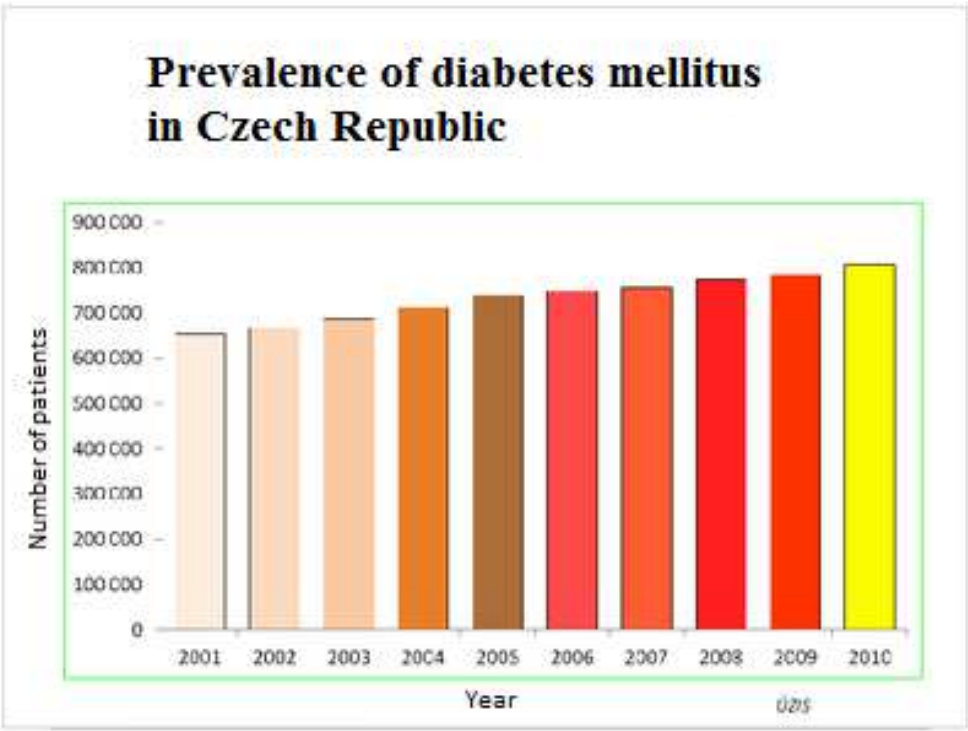

Figure 1 - Prevalence of diabetes mellitus - increasing number of patiens with diabetes mellitus in Czech Republic between years 2001-2010 (ÚZIS, Czech Republic)

And although it has been observed that childhood type 1 diabetes incidence is intermediate when compared to other European countries, and is very unlike to reach dramatically high figures, a continuous rise may be expected.

Diabetes in Spain, however, is situated after Russia, Italy and Turkey, in the fourth place from country in Europe with the largest number of diabetics (approximately 2.7 million). It is expected that by 2030 the figure of people affected will increase in a million. In 2002, spending associated to diabetes oscillated between 2400 and 2675

million euros, i.e. 6.3 - 7.4 % of the total expenditure of the national health system (Oliva et al., 2004). Approximately 60 % of the costs are due to hospitalization, being mostly due to cardiovascular complications (Hart et al., 1997).

2.1.1.2 Type 2 diabetes

Diabetes mellitus type 2, is called non-insulin dependent diabetes or adult-onset diabetes, and accounts for at least 90 % of all cases of diabetes. It is characterized by the resistance to insulin and a relative shortcoming of the above mentioned hormone. The diagnosis usually takes place around 40 years, although everyday is more frequent in children. Diabetes type 2 can remain asymptomatic for many years and the diagnosis usually is a consequence of associate complications or by means of a blood test or urine.

There is an association between diabetes type 2 and obesity, since the obesity for itself can cause resistance to insulin and increase the glycaemia. It is hereditary, although the main susceptibility genes have not been identified yet. There are several factors involved in the development of diabetes type 2, like the obesity, inadequate diet and lacking of physical activity, advanced age, resistance to insulin, and presence of diabetes in relatives.

Patients with diabetes type 2 do not depend on exogenous insulin, but they might need it to control the hyperglycemia if they do not obtain it by means of diet and oral glycaemia decreasing drugs.

The increase of the predominance in diabetes type 2 is associated with cultural and social changes, with the aging of the population, with diet changes, with the decrease of the physical activity and with slightly healthy patterns of life style and behavior.

Both type 1 and type 2 diabetes are serious. There is no such thing as mild diabetes.

2.1.1.3 Gestational diabetes

It is the intolerance to glucose (in different grades) that begins or is detected for the first time during the pregnancy. The definition is applied if insulin is used in its treatment and also if the problem persists after the pregnancy.

It is important to maintain under control the glycaemia levels, which reduces the risk for the fetus (big size, trauma during the childbearing, hypoglycemia and jaundice). The women with gestational diabetes have a major risk of developing diabetes type 2. The gestational diabetes is associated with an increase on the risk of obesity and abnormal metabolism of the glucose in the infancy and adult life of the children.

Other specific types of diabetes also exist (International diabetes federation, 2009).

In 2010 in Czech Republic, there were 92 % people with type 2 diabetes mellitus and only 7 % with diabetes mellitus type 1 (Figure 2).

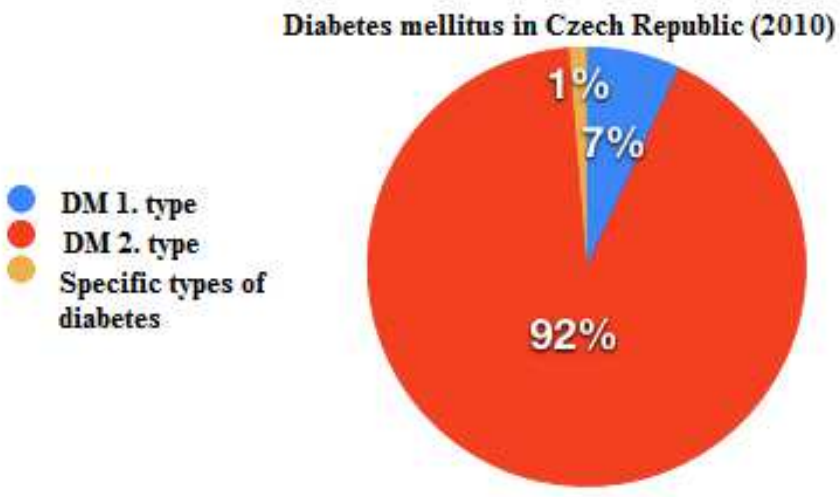

Figure 2 - Percentage of types of diabetes mellitus in Czech Republic in year 2010 (ÚZIS, Czech Republic, http://www.aidia.cz/4319/statistika-diabetu/)

2.1.2 Complications of diabetes

Today, the largest chronic complications produced by diabetes affect many organ systems. In virtually all developed countries, diabetes is one of the major causes of blindness, kidney failure, and amputation of the lower limbs. It is also one of the leading causes of death, in part due to a marked increase in the risk of cardiovascular disease. In addition, the disease involves large economic costs.

The sustained blood glucose, even without symptoms that alert the individual, will damage the tissues, often causing severe diseases. The kidneys, eyes, peripheral nerves and the vascular tree are the organs in which usually diabetic complications are more severe (Figure 4).

In Czech Republic in 2010, the rethinopathy and nephropathy were the most common chronic complications in diabetic population (Figure 3).

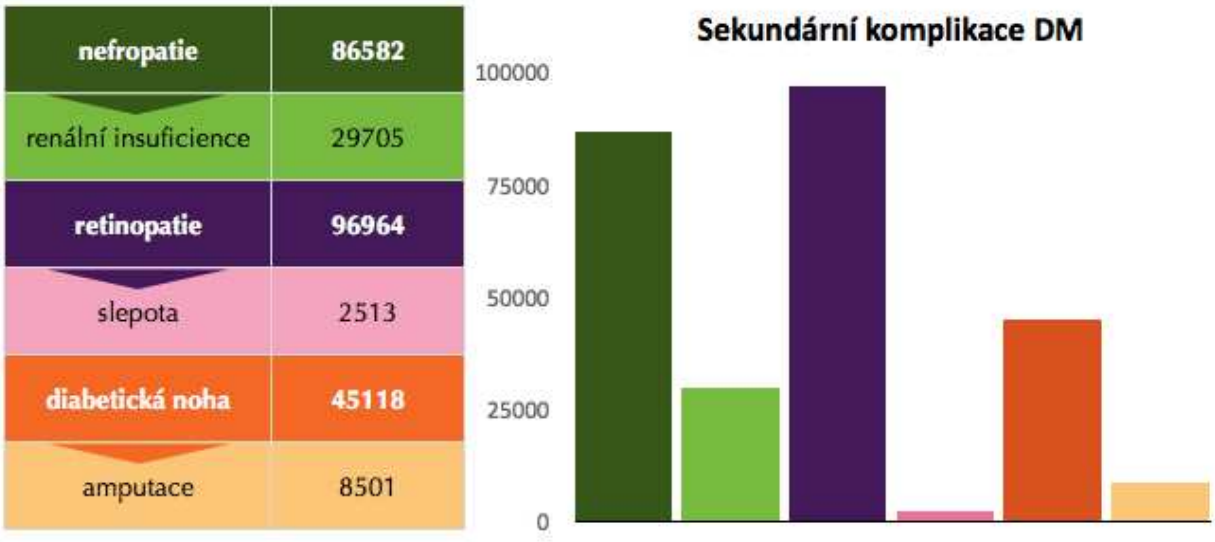

Figure 3 - Chronic complications of diabetes mellitus, Czech Republic, 2010 (ÚZIS, Czech Republic, http://www.aidia.cz/4319/statistika-diabetu/)

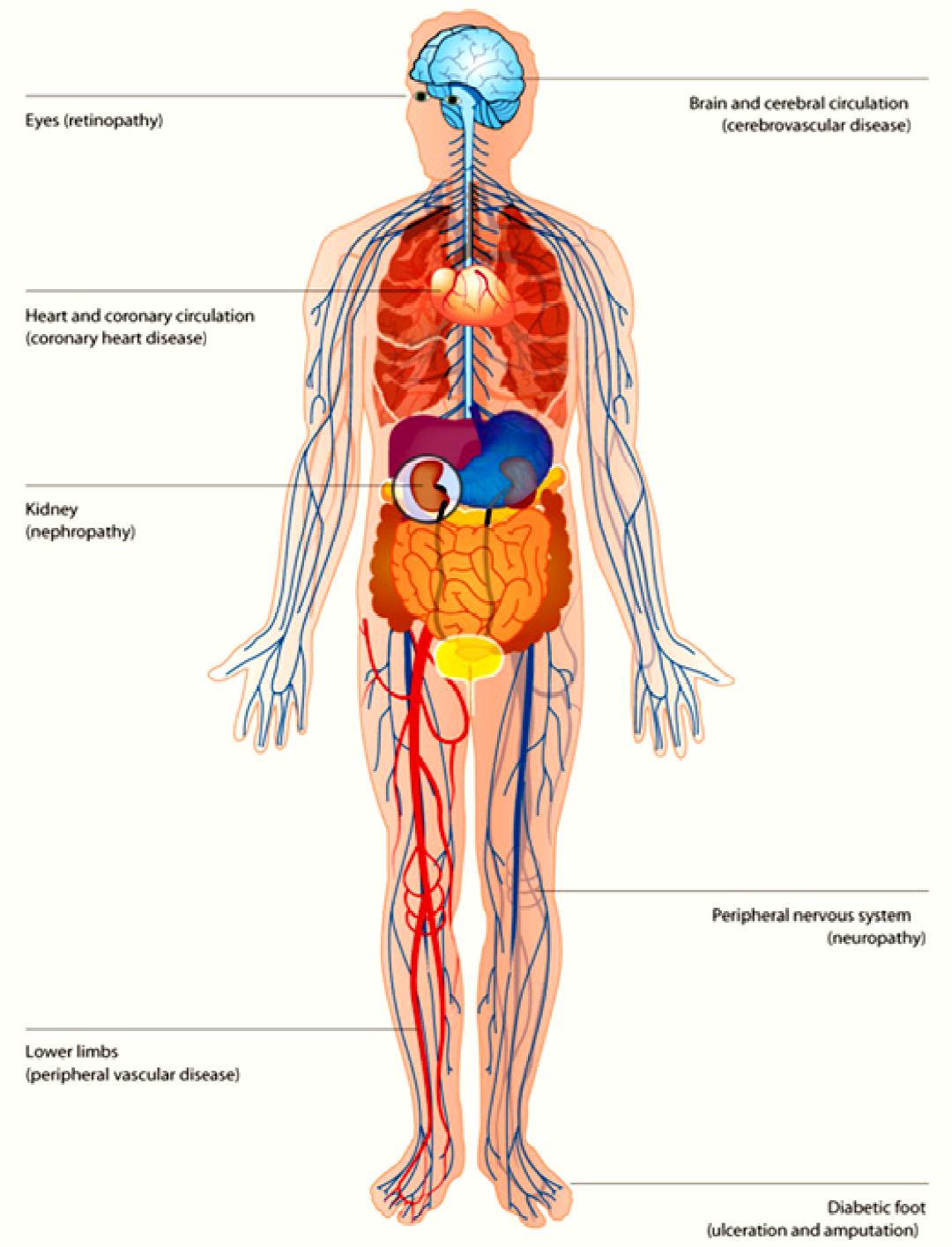

Figure 4 - Main complications of diabetes (International Diabetes Federation Atlas, 4th edition, 2009).

2.1.2.1 Vascular complications associated with diabetes mellitus

The vascular complications can be of two types depending on the caliber of the blood vessels that are affected (Plutzky and Orasanu, 2009): macroangiopathy (stroke, ischemic heart disease, myocardial infarction) and microangiopathy (retinopathy, nephropathy, neuropathy, ischemia of the lower limbs). There is a clear relationship between the chronic complications and the magnitude and duration of hyperglycemia, which affects both the endothelium and vascular smooth muscle (Plutzky and Orasanu, 2009). Half of the diabetic patients die of cardiovascular disease (primarily heart disease and stroke) (Roger et al., 2012). The macroangiopathies fundamentally affect the big vessels (coronary, carotid arteries of the lower limbs) that show abnormalities similar to those of the arteriosclerosis. Macroangiopathies are the main cause of myocardial infarction, ischemia and cerebral infarction, and ischemic gangrene of the lower limbs, in subjects with diabetes. But the main diabetic complications appear in the microcirculation (microangiopathy). One of the most characteristic manifestations is the thickening of the basal vascular membrane. This reduces the delivery of nutrients to the tissues and the elimination of waste products, which leads to irreparable damage to the tissues. This fact is one of the most frequent causes of non-traumatic amputations of the lower limbs. Diabetic retinopathy and nephropathy are the main manifestations of microangiopathy, with blindness and kidney failure as final consequences.

2.1.2.2 Diabetes and renal impairment

Diabetic nephropathy is one of the main causes of kidney failure in developed countries. In patients with type 1 diabetes, nephropathy develops from the first 10 years of the disease. However, as the start of the type 2 diabetes is uncertain, since it is often diagnosed late, it is not uncommon to find patients with abnormal kidney function at the time of diagnosis. It is considered a progressive disease characterized in a first phase of the microalbuminuria (urinary excretion of albumin between 30 to 299 mg/dl), followed by progression to macroalbuminuria (> 300 mg/24 h), increase in serum creatinine concentration, hypertension and, finally, renal failure (Seaquist and Ibrahim, 2010; Figure 5).

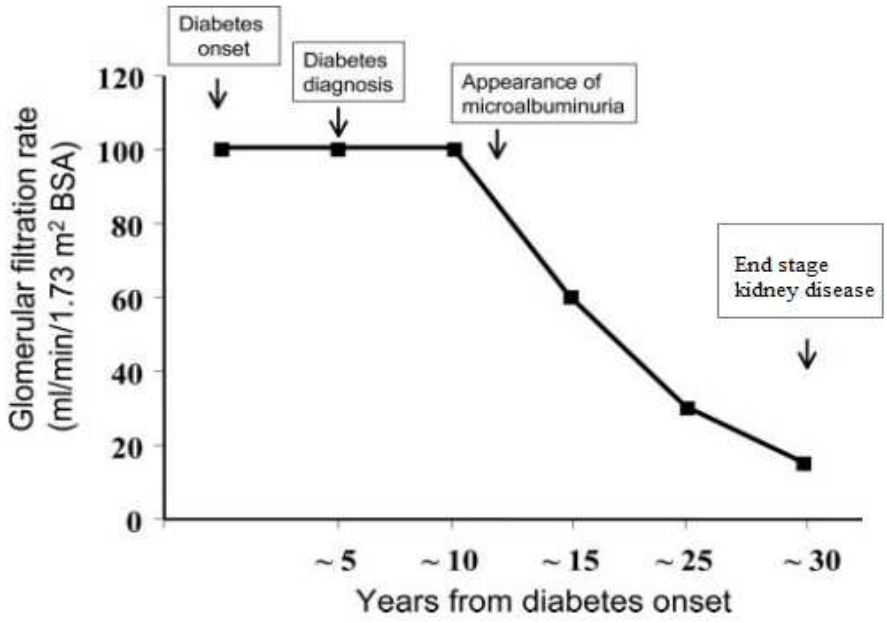

Figure 5 - Typical progression of kidney disease of the patient diabetic (Seaquist and Ibrahim, 2010)

2.1.3 Diabetes risk factors

There is nothing that you can do to prevent type 1 diabetes. Type 2 diabetes is a little more complex – it's a combination of our genes and our lifestyle.

<u>You are more at risk of developing type 2 diabetes if:</u>

- You are over 40, however, type 2 diabetes is increasing in all age classes and now it occus also at children and young people.
- You have someone with diabetes in family history.
- You are overweight with BMI over 25 kg/m^2. Overweight and obesity have been estimated to account for about 65–80% of new cases of type 2 diabetes.
- You don't have enough physical activity. Studies have shown that just 30 minutes of moderate exercise a day, five days a week is enough to promote good health and reduce the chance of developing type 2 diabetes.
- You have ever had high blood pressure, a heart attack or a stroke.

- If you’re a woman and you’ve had gestational diabetes. These women have an increased risk of developing type 2 diabetes in later years (web Diabetes; web WHO).

2.1.4 Prevention

Increasing body weight cause a lot of health complications and first appear in children and young people. To help prevent type 2 diabetes and its complications, people of all ages should do sports and be physically active, eat a healthy food, watch their body weight and avoid smoking cigarette, because it increases the risk of cardiovascular diseases.

Individuals with impaired glucose tolerance (IGT) or impaired fasting glycaemia (IFG) are in the intermediate stage between normality and diabetes and are at high risk of developing type 2 diabetes. This risk can be reduced by change of the lifestyle and pharmacological treatment.

2.1.5 Diagnosis and treatment

Type 2 diabetes can be diagnosed at an early stage through relatively inexpensive blood testing. However, 50 % of people with diabetes may be undiagnosed.

Diabetes is treated by lowering blood glucose and observing the levels of other known risk factors that damage blood vessels. Patients with type 1 diabetes need insulin; people with type 2 diabetes can be treated with oral medication, but may also require insulin. Other important measures include control of blood pressure and cholesterol level, foot care, and regular screening for retinopathy and early signs of diabetes-related kidney disease. Treatment should be supported by a healthy diet, regular physical activity, maintaining a healthy body weight and avoiding tobacco use.

Training for self-management strategies in people with type 2 diabetes is effective in improving fasting blood glucose levels, glycated haemoglobin and diabetes knowledge

and in reducing systolic blood pressure levels, body weight and the requirement for diabetes medication (web WHO).

2.2 Experimental models of diabetes

The experimental models of diabetes mellitus show many features of clinical diabetes, such as hyperglycemia. For this reason they have been used to understand the etiology of the disease and also to investigate the mechanisms involved in the diabetes complications. These models use animals that spontaneously suffer a syndrome similar to diabetes, or animals in which the diabetic state is experimentally induced with chemicals, viruses or excising the pancreas. None of these models, however, is fully equal to the human disease.

Intact organisms are highly complex and does not allow a detailed study of the molecular mechanisms and the interaction or agonists/antagonists of their receptors. To overcome these limitations *in vitro* models are used. In these models the contribution of each component of the blood is removed and the temperature of the tissue, the extracellular medium and even the bioavailability of nutrients and ions are controlled. The species used are determined by different factors. In general, the size of the animal is proportional to the experimental cost, and greater size also makes it less manageable. One of the fundamental principles is to use the fewest number of animals as possible, avoiding procedures that produce pain, suffering, or prolonged unnecessary injury.

Among the different models the most commonly used is chemical diabetes. Diabetogens widely used and very effective are alloxan and streptozotocin. In 1943 it was discovered the ability of the alloxan to destroy the insulin-producing beta cells of the pancreas of the rabbit, and since then has been used commonly in laboratory (Lenzen, 2008).

Alloxan is an oxygenated pyrimidine derivative (Figure 6). It is a hydrophilic compound that neutral pH drops quickly to dialuric acid (Figure 7), which is the toxic form of the compound. This compound generates reactive oxygen species and seems to be that these latter are involved in the beginning of the changes that are toxic to the pancreatic beta cells.

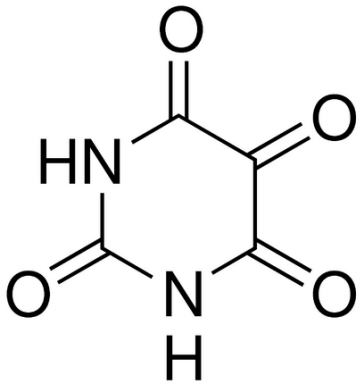

Figure 6 - Structure of Alloxan (web Sigma Aldrich)

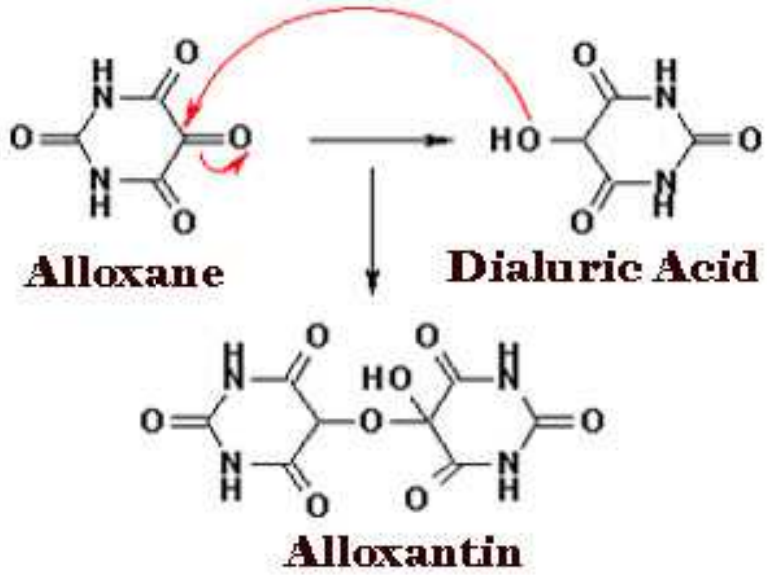

Figure 7 - Conversion of Alloxan to Dialuric Acid (web Alloxan)

After intravenous injection, the animals show a transient hypoglycemia that revert back to hyperglycemia after 24-48 hours. The alloxan-injected animals suffer a total lack of insulin from their pancreatic beta cells and will require the administration of insulin to live. However, despite their insignificant levels of endogenous insulin, they can survive for months without treatment. The administration of streptozotocin or alloxan to an adult animal cause in the animal a state similar to that seen in patients with type 1

diabetes: hyperglycemia, polydipsia, polyphagia, polyuria, as well as most of the complications associated with diabetes (cardiomyopathy, neuropathies, coronary dysfunction, liver abnormalities, etc.).

2.3 Regulatory mechanism of vascular reactivity

The interaction between various vasoconstrictor and vasodilator substances released by the endothelium and smooth muscle of the vascular wall, the perivascular nerve endings, and blood cells, is responsible for establishing the vasomotor response. The alteration of the release of different vasoactive agents can cause endothelial dysfunction, vascular remodeling and vascular inflammation, and may be a key in the pathophysiology of cardiovascular diseases such as arteriosclerosis, high blood pressure or diabetic vasculopathy (Grover-Páez and Zavalza-Gomez, 2009).

Blood vessels are formed by an outer layer or adventitious and a middle layer formed by smooth muscle cells. In addition, at the innermost part is located the intimate layer, formed by the endothelium and a basal membrane. Endothelial cells form a continuous layer that lines the luminal face of the arteries, veins, capillaries, the lymph vessels and the heart of the mammals. In the endothelial cell we can find two zones, the apical or luminal, and the basal, that interacts with extracellular matrix proteins of the basal lamina which is attached at, setting the cells to the subendothelial layer.

Initially it was thought that the endothelium was a simple barrier between the blood and the vascular wall. From the decade of the 80s, the endothelium is seen as a genuine organ that consumes a large amount of energy, due to its intense metabolism, and that plays an important role. The endothelium is a clear regulator of vascular contraction, of the leukocyte adhesion, growth of vascular smooth muscle cells and platelet aggregation through the production of a series of biologically active molecules (Amezcua, Palmer et al., 1989). It also maintains a balance between vasodilator and vasoconstrictor (Félétou and Vanhoutte 2009; Figure 8), among other functions.

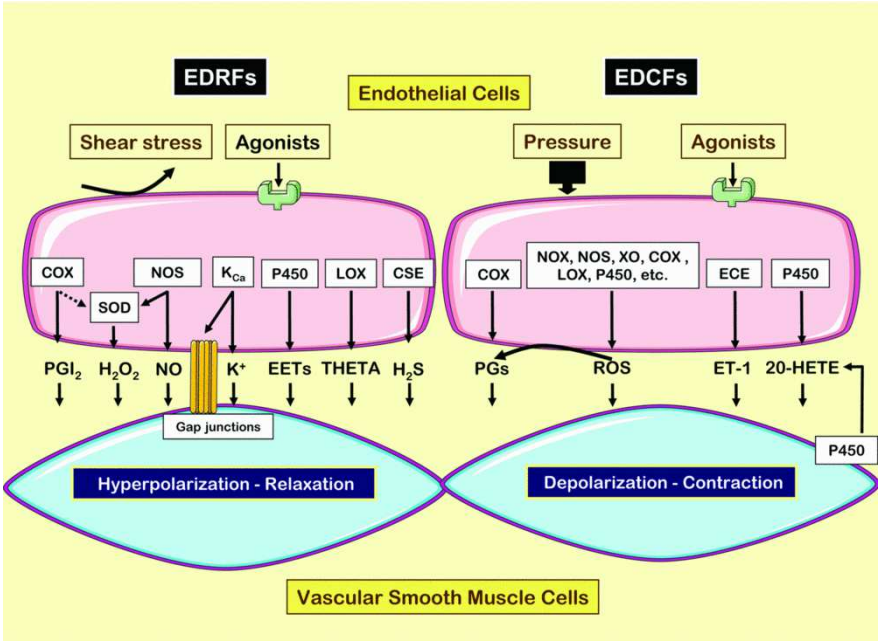

Figure 8 - Relaxations and contractions dependent of the endothelium (Félétou and Vanhoutte, 2009)

The membrane of the endothelial cells has a great variety of receptors to numerous vasoactive substances, which makes them partakers of numerous physiological activities. Therefore, the endothelial dysfunction is involved in numerous pathologies.

Endothelial cells produce three main substances with vasodilating action: nitric oxide (NO), endothelium-derived hyperpolarizing factor (EDHF) and prostanoids such as prostacyclin (prostaglandin I2). But the tone and structure of the vascular system are also controlled through endothelial vasoconstrictor substances, maintaining a delicate balance with the vasodilating substances. The most interesting representatives are endothelin, angiotensin II and vasoconstrictor prostanoids (thromboxane A2, prostaglandin F2α, etc.). NO and prostanoids are involved in a multitude of pathophysiological processes, including inflammation and atherosclerosis. NO modulates the activity of the cyclooxygenase (COX) and alters the production of prostanoids (Upmacis et al., 2006).

2.4 Endothelial dysfunction in diabetes

As explained, the endothelium plays an important role in regulating the tone and blood flow through the release of vasoconstrictor substances (endothelin, angiotensin II, thromboxane A2, etc.), in balance with vasodilating substances (prostacyclin, NO, EDHF, etc.). The loss of the ability of the endothelium to maintain vascular homeostasis is called endothelial dysfunction (Grover-Páez and Zavalza-Gomez, 2009; Ding and Triggle, 2010; Tabit et al., 2010). This term is often used as a lower bioavailability of NO, but endothelial dysfunction also includes an increase in the production of vasoconstrictor and an alteration in the regulation of inflammation, thrombosis, and cell growth in the vascular wall that may end up in atherosclerosis, characterized by a thickening of the tunica intima with plates containing macrophages filled with lipids and fats, especially cholesterol. In endothelial dysfunction there is loss of vasodilators, with the consequent increase in vascular tone. In these conditions there is usually an increase in the production of endothelin-1 and other endothelial vasoconstrictor, and occurs vasospasm and increased arterial stiffness.

As shown in Figure 9, the risk factors as smoking, hypertension, hypercholesterolemia, hyperglycemia and family history of some aterosclerotic diseases are connected with the loss of the ability of the endothelium. Newly discovered risk factors as infection, physical inactivity and obesity are connected with the endothelial dysfunction too (Widlansky et al. 2003).

Endothelial dysfunction has been described in various metabolic and cardiovascular abnormalities (hypertension, coronary heart disease, dyslipidemia and diabetes type 1 and 2), therefore it is logical that, taking into account that diabetes is a very important cardiovascular risk factor, numerous clinical studies have established a relationship between diabetes and endothelial dysfunction (Grover-Páez and Zavalza-Gomez, 2009; Tabit et al., 2010).

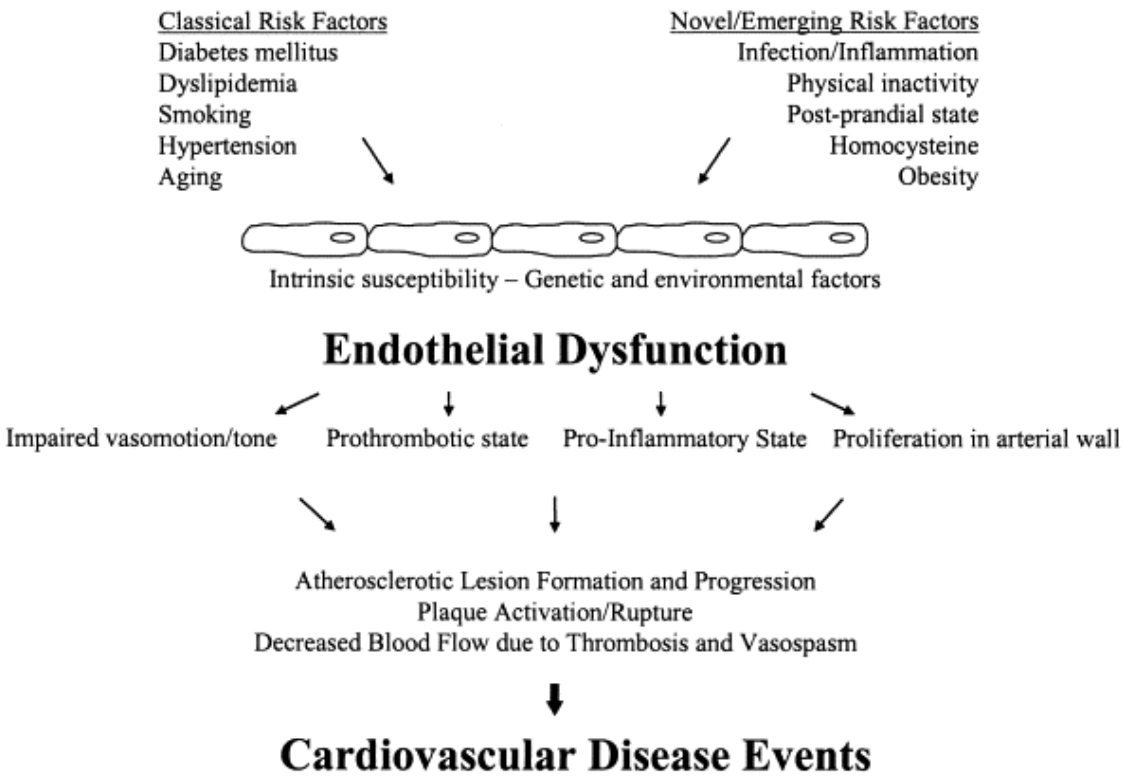

Figure 9 - The role of endothelial dysfunction in the pathogenesis of cardiovascular disease events, (Widlansky et al. 2003)

2.5 Natriuretic peptides

Natriuretic peptides are a family of structurally related but genetically distinct hormones that regulate blood volume, blood pressure, ventricular hypertrophy, pulmonary hypertension, fat metabolism, inhibition of the activity of sympathetic and relaxation of the smooth muscle (Figure 10).

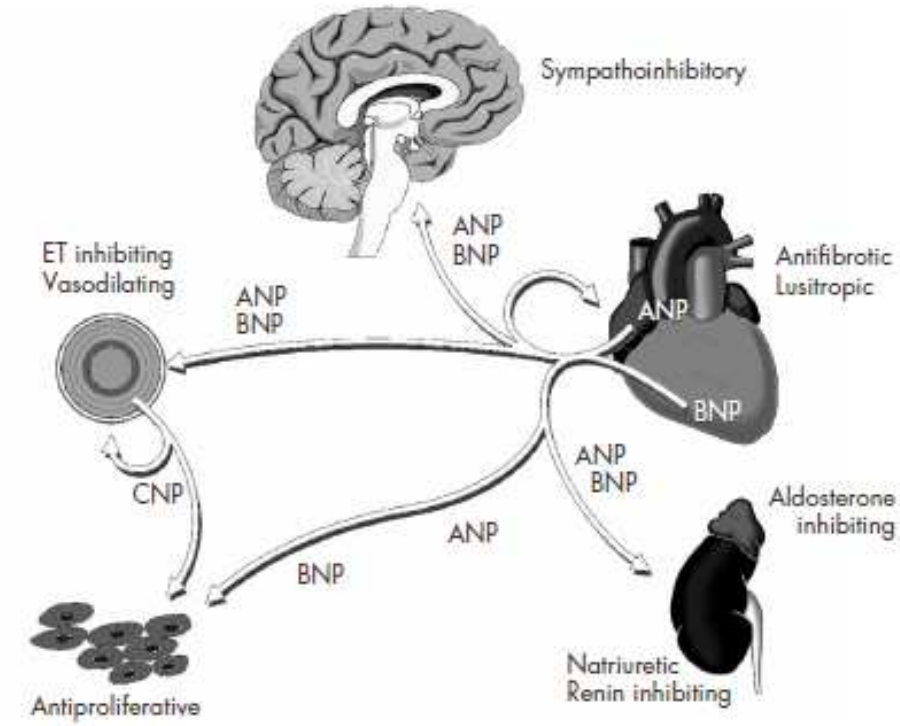

Figure 10 - Function of natriuretic peptides. ET= endothelin, ANP= atrial natriuretic peptide, BNP= B-type natriuretic peptide (Bhalla et al. 2004).

In mammals, there are generally three natriuretic peptides (NP): ANP, BNP, and CNP, although CNP does not stimulate "natriuresis" at physiological concentrations. All three main NP share common 17-amino-acid ring structure (Figure 11). These peptides were given a number of different names such as atrial natriuretic factor, cardionatrin, cardiodilatin, atriopeptin, and atrial natriuretic peptide (ANP); the latter description is most often used today. B-type natriuretic peptide (BNP), which was originally called brain natriuretic peptide, and C-type natriuretic peptide (CNP) were subsequently purified from porcine brain extracts based on their ability to relax smooth muscle (Potter et al., 2006).

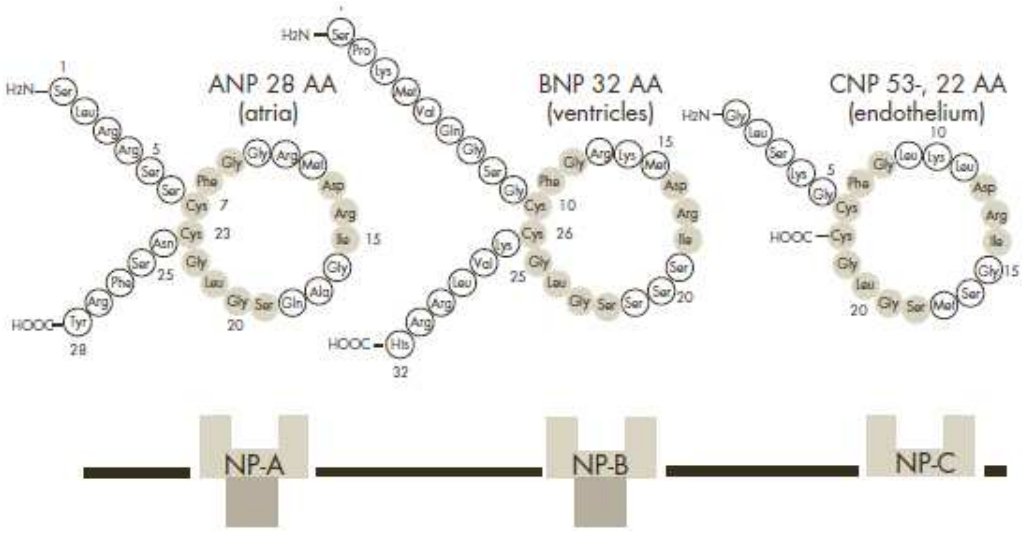

Figure 11 - The common structure of natriuretic peptides, (Bhalla et al. 2004).

The increased plasma levels of ANP in various cardiovascular diseases is a fact (Potter et al., 2006). This increase would have a protective effect in these patients, because would help to reduce the cardiac overload. The expression of ANP also is increased in hypertensive patients with myocardial hypertrophy and heart failure, but it is not known the meaning of this data (Pandey, 2008).

Very high plasma levels of BNP are associated with increased mortality and an increase in the need of hospitalization in serious cardiovascular complications (heart failure, stroke, atrial fibrillation), although the predictive value of myocardial infarction is less. It has also been suggested that increased plasma levels of ANP and BNP in the acute phase of stroke are associated with an increase of mortality in the years following the ischemic episode (Mäkikallio et al., 2005; Jensen et al., 2009; Shibazaki et al., 2009).

A study shows that diabetic patients with heart failure have higher plasma values of NTproBNP than nondiabetics, while other neurohormones have similar values, constituting a good prognostic marker in the above-mentioned diabetic patients (Van der Horst et al., 2010).

It has been shown that the ANP prevents endothelial dysfunction induced by diabetes, which could be usefulness in the prevention of vascular complications of diabetes (Woodman et al., 2008).

Previous studies from laboratory of Physiology, University of Valencia, have shown that diabetes modifies the mechanisms that regulate the reactivity of the carotid artery of rabbit to vasoconstrictors such as 5-hydroxytryptamine (Miranda et al., 2000a) and endothelin-1 (Llorens et al., 2004) and vasodilators such as the acetylcholine (Miranda et al., 2000b) and testosterone (Marrachelli et al., 2010).

3 AIMS

The general aim of this preliminary study is to determine the response of rabbit carotid and renal arteries to atrial and brain natriuretic peptides (ANP, BNP) and whether this response is altered in diabetes, as well as compare and observe differences in the vascular reactivity to the peptides as a result of the disease.

4 MATERIALS AND METHODS

Fourteen male New Zealand white rabbits were used in the present study. Animals were randomly divided into two experimental groups: 8 in the control group and 6 destined for induction of diabetes. Housing conditions and experimental procedures were in accordance with the European Union regulations on the use of animals for scientific purposes (86/609/EEC, Article 5, Appendix II) and as promulgated by Spanish legislation (RD 1201/2005).

4.1 Induction of diabetes

Rabbits weighing 2.64 ± 0.05 kg were sedated with intramuscular injection of ketamine (40 mg/kg; Ketolar) after fasting 24 h. Diabetes was induced by injecting i.v. alloxan (100 mg/kg) into the lateral ear vein. To prevent hypoglycemia, 10 ml of glucose 5% was injected intravenously after alloxan, and drinking water was supplemented with 10% glucose for the first 24 h after the alloxan injection. Thereafter, the animals were maintained on tap water, regular food *ad libitum*, and no insulin treatment for 6 weeks. Control rabbits (2.63 ± 0.06 kg) did not get any injection and were maintained under the same conditions for the same time period. Control and diabetic animals were well compared with no significant differences at baseline for body weight and glycaemia measures. Plasma glucose concentrations were weekly measured by the glucose oxidase method with a glucose analyser (Glucometer Elite, Bayer).

4.2 Isometric tension recording

Six weeks after diabetes induction, rabbits were anaesthetized and killed with 2% i.v. sodium pentothal (150 mg/kg of sodium thiopental, Tiobarbital Braun) in the marginal ear vein. Death occurs by intoxication as a result of the excess of anesthesia.

To isolate the renal arteries the animal is situated in supine position and a longitudinal incision is practiced in the abdominal region media. After the visceral package is moved apart, both kidneys are isolated and both renal arteries are extracted by blunt dissection (Figure 12).

To isolate the carotid arteries a longitudinal incision is practised in the middle region of the neck and both carotids are isolated by blunt dissection (Figure 13).

Immediately the renal and carotid arteries are submerged in a physiological saline solution cold (4°C) and then with the aid of a stereomicroscope (Wild M3B clicking) and using a cold light source (Euromex EK-1) in order not to damage the tissue, the traces of blood on the inside are removed. Both common carotid and renal arteries were dissected free and cut into cylindrical segments measuring 3-4 mm in length. Each segment was prepared for isometric tension recording in an organ bath.

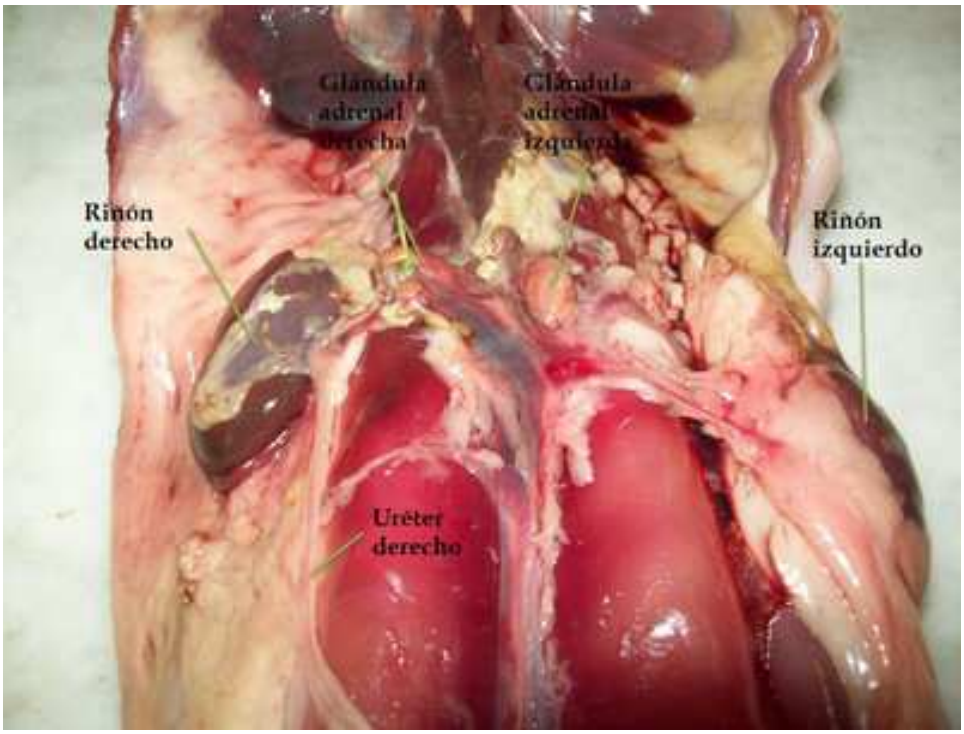

Figure 12 -Removal of the renal artery in the rabbit (web Bioterio**)**

Glándula adrenal derecha - Right adrenal gland

Glándula adrenal izquierda - Left adrenal gland

Riñón derecho - Right kidney

Riñón izquierdo - Left kidney

Uréter derecho - Right ureter

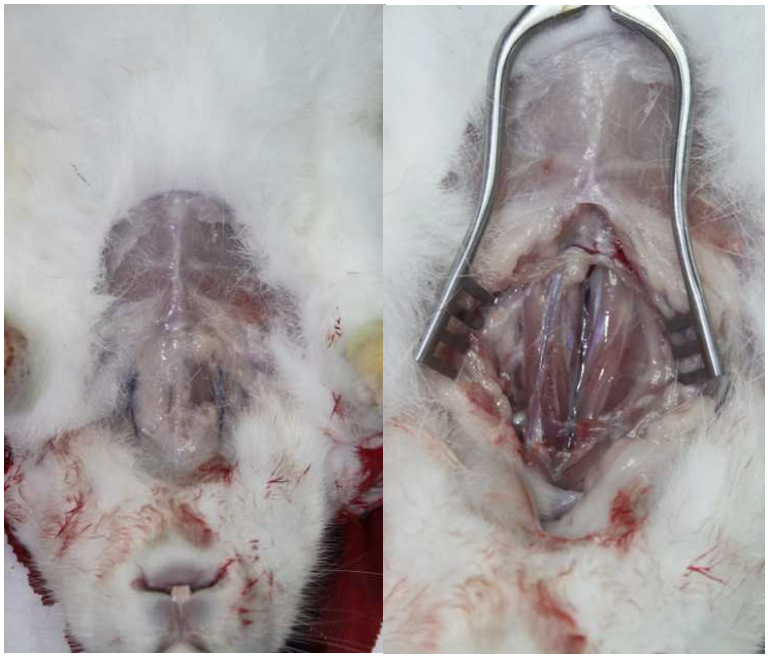

Figure 13 - Removal of the cariotid arteries in the rabbit

For the registration of the isometric tension developed by the arterial segments, the fine ends of two rigid stainless steel wires (207 µm in diameter) are introduced through the vessel lumen. One of the wire is attached to a fixed support, in such a way that the artery is in horizontal position. The other is connected to a transducer of isometric tension, that can be towed vertically, in a direction perpendicular to the major axis of the arterial segment, in response to voltage changes that occur in the vessel wall. The isometric tension is conveniently amplified, digitized, recorded and stored for later analysis.

Each vascular segment, with the wires in the interior of his lumen, it is housed in an organ bath containing 5 ml of a solution that simulates the physiological conditions (Ringer-Locke solution). This solution is continuously bubbled with carbogen gas (95% O_2 and 5% CO_2), which gives it a pH of 7,3 -7,4. A hot water circuit, which surrounds the glasses of the baths, allows to maintain the solutions with the vascular tissues at a constant temperature of 37 ºC. There is a reserve of the solution, in conditions identical to those described above, which is used to renew the solution in which are immersed the arterial segments (Figure 14).

Figure 14 - Organ bath for isometric tension recording

The system of measuring and recording for each one of the arterial segments consists on a transducer of isometric tension (Panlab Mod. UF-1/ Letica TRI 201), a micrometric screw adapted to the transducer, capable of moving the transducer assembly-wire, a voltage amplifier (Amplifier Panlab 40154/Letica ISO 506), and a computerized system for digitalizing and recording (PowerLab 8sp and software Chart 5, ADInstruments).

Changes in isometric tension produced by different stimuli applied on the arterial segments are quantified by measuring, from the line indicating the active tone, the displacement occurred up to the maximum effect developed by each dose.

Once the arterial segments are placed in the organ baths, after calibration of the appliance, a basal tension of 2 g. is applied to them by rotation of the screw micrometer. The arterial segments tend immediate to relax, so the tension has to be adjusted periodically until they stabilize at the basal tension. During this period the nutritive fluid is renewed every 20 minutes. Once a stable baseline tension is reached, the functional integrity of endothelium was checked by examining the relaxant action of acetylcholine (10^{-5} M) in arteries precontracted with phenylephrine (10^{-7} M).

Finally, after several washings of the arteries with the nutrient solution over a period of 45-60 minutes, the concentration-response curves to ANP and BNP are done. Each dose is added when the immediately preceding has developed its maximum effect, being the concentration of the drug in the bath when you apply a dose, the sum of the latter with the doses administered prior to it. In each arterial segment was obtained a single curve concentration-response to the ANP and BNP. Each concentration–response curve to ANP and BNP was expressed as a percentage of the active tone.

4.3 Drugs and solutions

Alloxan, phenylephrine, acetylcholine, ANP and BNP were purchased from RBI-Sigma-Aldrich Química (Madrid, Spain). Alloxan and phenylephrine were dissolved and diluted in saline solution. ANP and BNP were dissolved in 0,5% aqueous acetic acid and diluted in a mixture of phosphate-buffered saline solution. The composition of the Ringer–Locke solution was (mM): NaCl, 120; KCl, 5.4; $CaCl_2$, 2.2; $MgCl_2$, 1.0; $NaHCO_3$, 25; and glucose, 5.6.

4.4 Statistical analysis

In the concentration-response curves, relaxation values are expressed as a percentage of the active tone. Statistical comparisons were performed using either two-way ANOVA with Bonferroni's multiple comparisons post hoc test, to determine significant differences among the means of the data groups, or unpaired Student's t-test. $P < 0.05$ was considered significantly different.

5 RESULTS

In arteries isolated from control and diabetic rabbits, precontracted with phenylephrine (10^{-7} M), the addition to the organ bath of cumulative doses of ANP (10^{-12}-10^{-7} M) induced a relaxation response depending on the concentration used.

In carotid artery, the relaxation produced by the ANP was different between control and diabetic rabbits (Figure 15).

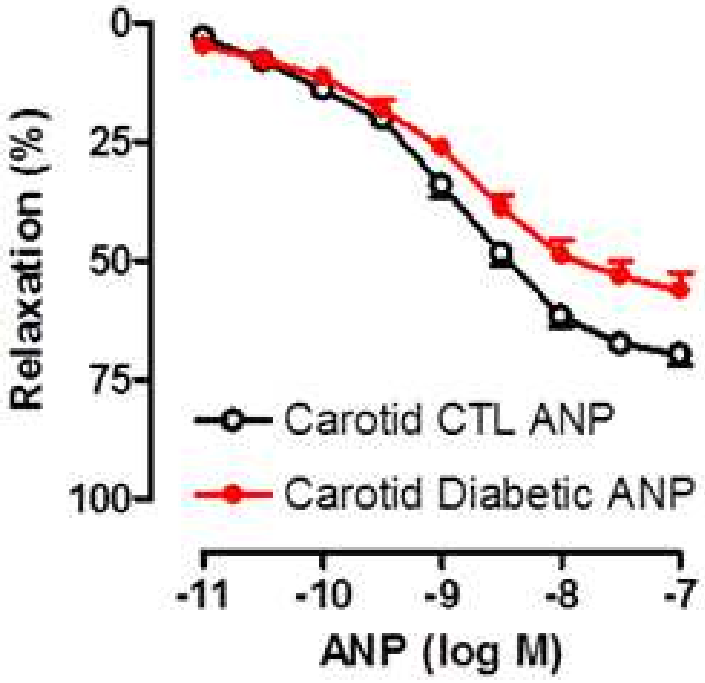

Figure 15 – Concentration–response curves for ANP in phenylephrine precontracted carotid arteries from control (carotid control: n=12) and diabetic (carotid diabetic: n=10) rabbits. Values are expressed as a percentage of the active tone and represent means ± SEM of "n" arterial segments from at least 4 animals. ***P<0.001 significantly different from corresponding control value.

While in the renal artery, the addition of cumulative doses of ANP produced a relaxation that was significantly lower in diabetic animals (Figure 16).

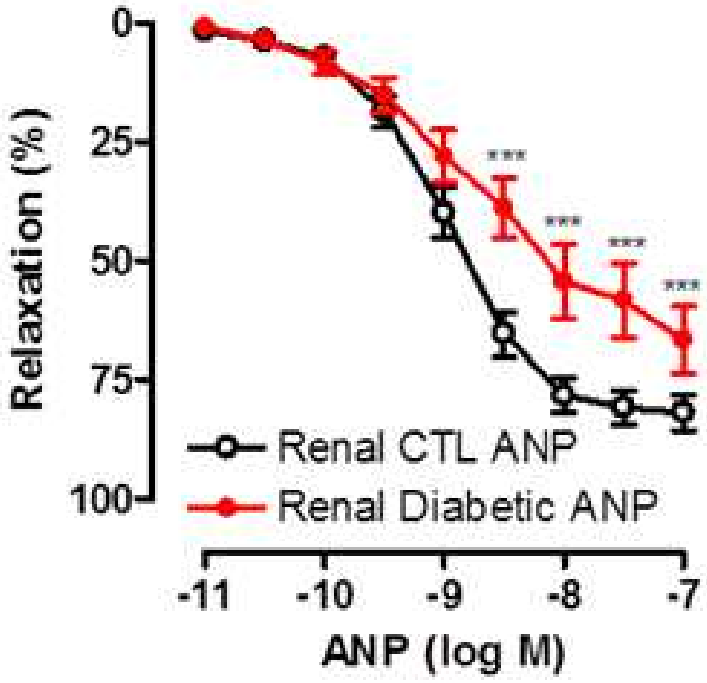

Figure 16 - Concentration–response curves for ANP in phenylephrine precontracted renal arteries from control (renal control: n=13) and diabetic (renal diabetic: n=9) rabbits. Values are expressed as a percentage of the active tone and represent means ± SEM of "n" arterial segments from at least 4 animals. ***P<0.001 significantly different from corresponding control value.

In carotid artery, the addition to the organs bath of cumulative doses of BNP (10^{-12}-10^{-7} M) also produced a relaxing response of magnitude dependent on the concentration used. This relaxation was significantly lower in arteries of diabetic animals (Figure 17).

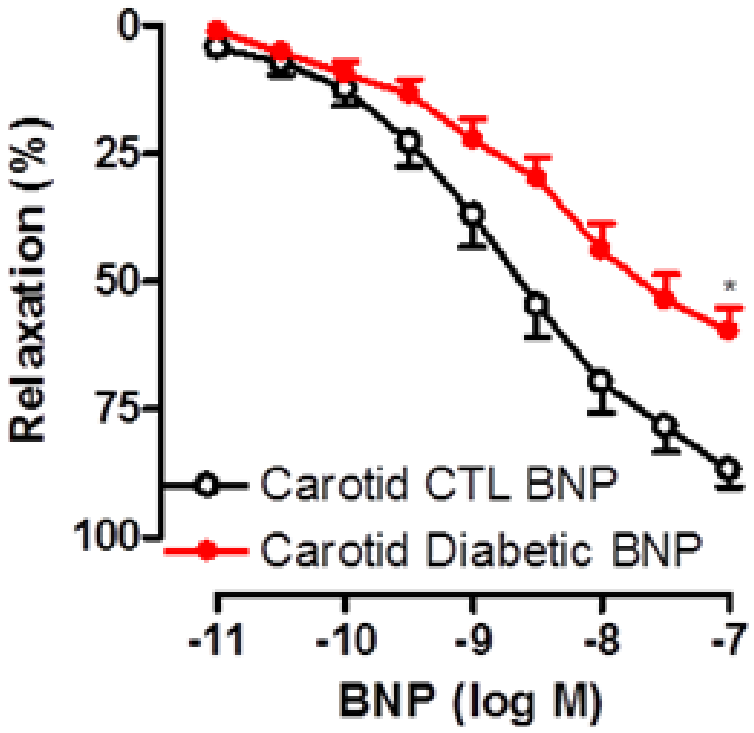

Figure 17 – Concentration–response curves for BNP in phenylephrine precontracted carotid arteries from control (carotid control: n=13) and diabetic (carotid diabetic: n=9) rabbits. Values are expressed as a percentage of the active tone and represent means ± SEM of "n" arterial segments from at least 4 animals. *P<0.05 significantly different from corresponding control value.

On the contrary, in renal arteries, the relaxation produced by BNP resulted significantly higher in arteries of diabetic animal than in arteries of control animal (Figure 18).

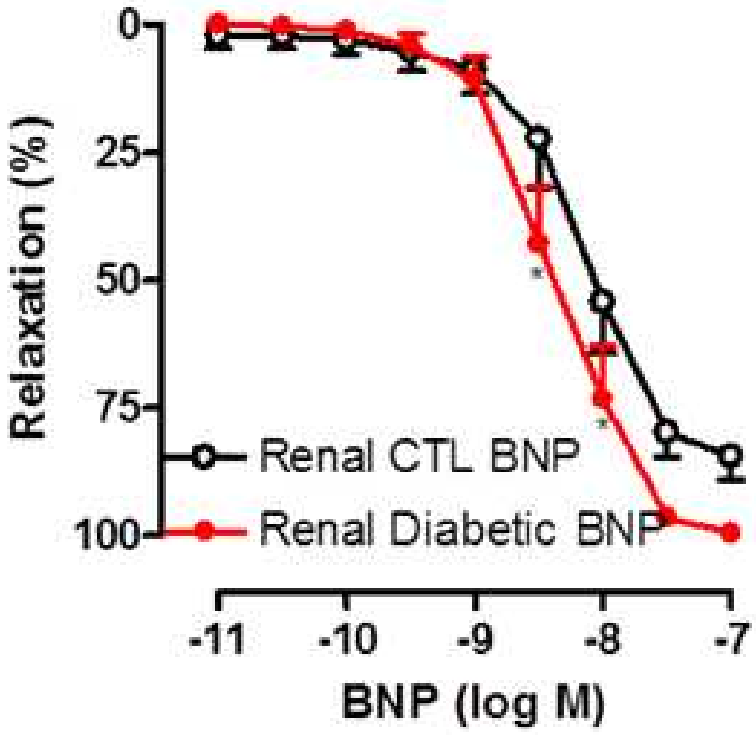

Figure 18 - Concentration–response curves for BNP in phenylephrine precontracted renal arteries from control (renal control: n=12) and diabetic (renal diabetic: n=9) rabbits. Values are expressed as a percentage of the active tone and represent means ± SEM of "n" arterial segments from at least 4 animals. *P<0.05 significantly different from corresponding control value.

When we compare the effects of both natriuretic peptides in each experimental situation, it can be observed that, in carotid artery in control animal, the BNP seems to show a higher maximum effect (Figure 19).

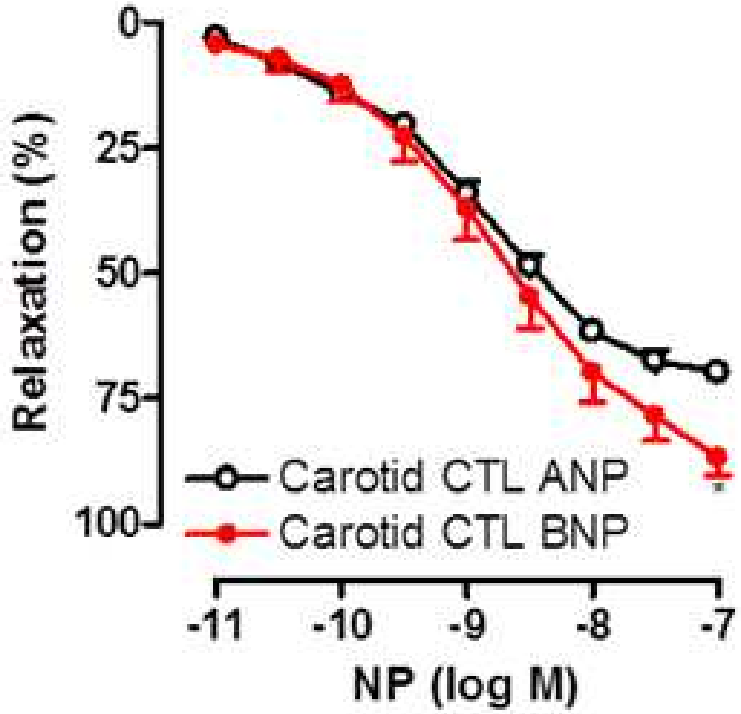

Figure 19 – Concentration–response curves for ANP and BNP in phenylephrine precontracted carotid arteries from control (control ANP: n=12; control BNP: n=13) rabbits. Values are expressed as a percentage of the active tone and represent means ± SEM of "n" arterial segments from at least 4 animals. *P<0.05 significantly different from corresponding control value.

In carotid artery taken from diabetic rabbit there are no differences in the relaxation produced by both natriuretic peptides (Figure 20).

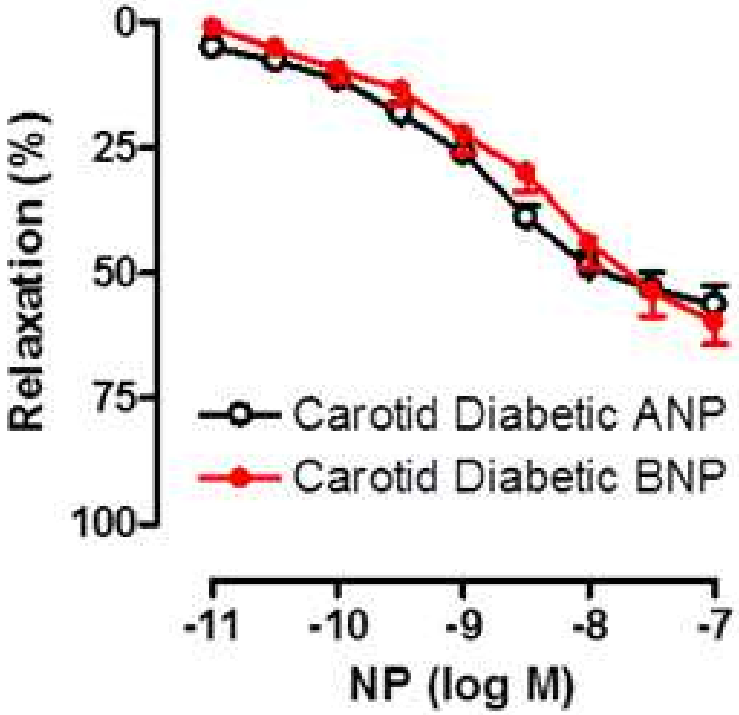

Figure 20 - Concentration-response curves for ANP and BNP in phenylephrine precontracted carotid arteries from diabetic (diabetic ANP: n=10; diabetic BNP: n=9) rabbits. Values are expressed as a percentage of the active tone and represent means ± SEM of "n" arterial segments from at least 4 animals. *P<0.05 significantly different from corresponding control value.

If we study the effects of both natriuretic peptides in renal artery it can be observed that in control animals, BNP shows a lower potency than ANP (Figure 21).

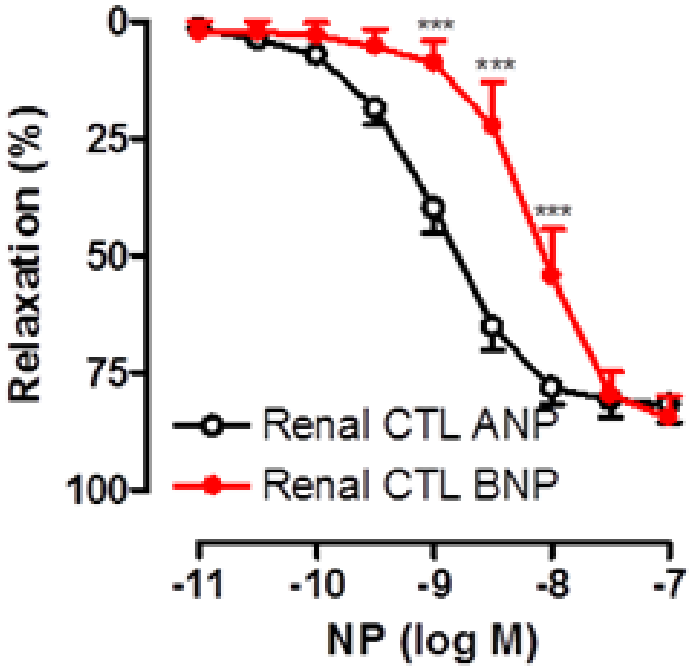

Figure 21 – Concentration–response curves for ANP and BNP in phenylephrine precontracted renal arteries from control (control ANP: n=13; control BNP: n=12) rabbits. Values are expressed as a percentage of the active tone and represent means ± SEM of "n" arterial segments from at least 4 animals. *P<0.05 and ***P<0.001 significantly different from corresponding control value.

However, in diabetic animals, BNP presents a higher maximum effect than ANP (Figure 22).

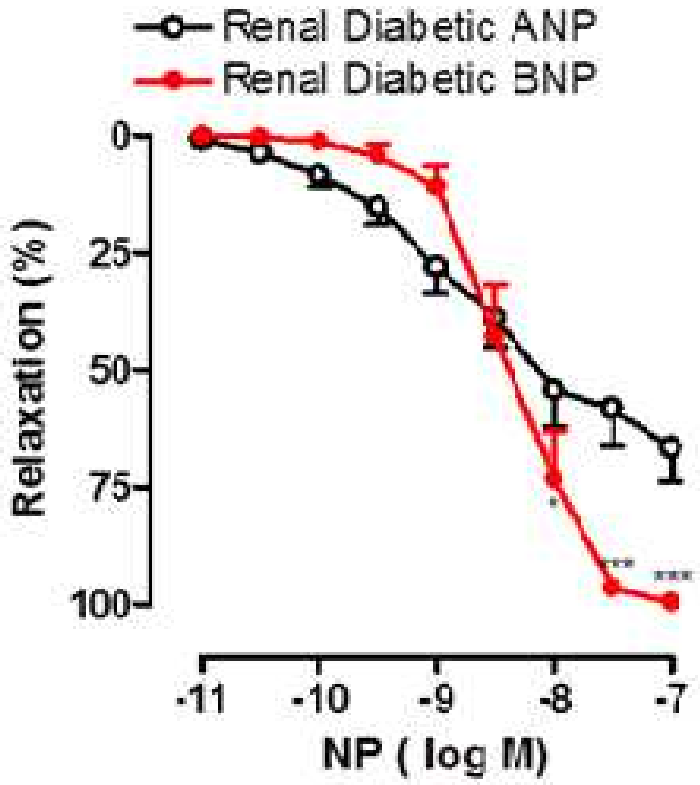

Figure 22 - Concentration-response curves for ANP and BNP in phenylephrine precontracted renal arteries from diabetic (diabetic ANP: n=9; diabetic BNP: n=9) rabbits. Values are expressed as a percentage of the active tone and represent means ± SEM of "n" arterial segments from at least 4 animals. *P<0.05 and ***P<0.001 significantly different from corresponding control value.

6 DISCUSSION

The experimental model of diabetes which is used in animals, generates similar disorder that occur in humans. This model uses the toxic agent alloxan, which causes the destruction of the pancreatic cells producing insulin, thus generating in the animal a situation of insulin-dependent diabetes (similar to diabetes type 1), in which appear the characteristic signs of the desease (Wang et al., 2010). The model used, in addition, is appropriate taking into account the infrastructure available in laboratory of Physiology, University of Valencia, as we have an organ bath system appropriate to the diameter of rabbit arteries.

There is a clear relationship between diabetes and natriuretic peptides. It has been proven that in diabetic animals there is an alteration of the release of ANP, which is increased in their plasma, as for example in rat (Obineche et al., 2006) and rabbit (Yegin et al., 1995).

In this preliminary study we determined the response of the carotid and kidney arteries of rabbit to atrial and brain natriuretic peptides (ANP and BNP), in two experimental situations: control and diabetic animals.

In all cases, natriuretic peptides produced a relaxation of the renal and carotid arteries. Similar results in different vascular beds in animal and human models have been observed in previous studies (Sabrane et al., 2005; Sugamori et al., 2002). Other studies have shown a close relationship between natriuretic peptides and diabetes and insulin resistance (McKenna et al., 2005; Pfister et al., 2011).

In previous studies in laboratory of Physiology, University of Valencia, we have observed that diabetes causes an alteration of vascular reactivity to ANP and BNP. In both cases, the arteries of diabetic animals show hyporeactivity to natriuretic peptides (Marrachelli et al., 2012; Centeno et al., 2013).

In the present study, however, the results have been varied. We have observed that in renal artery of diabetic animals, ANP produces a minor relaxation when compared to arteries of control animal, thus coinciding with studies in which there have been less response to ANP in kidney of diabetic rats (Sechi et al., 1995; Bryan et al., 2007). But in carotid artery, although a minor relaxation can be seen in diabetes, this is not

significant. We think that an increase in the sample size could dispel doubts about this point, and probably a less relaxation to ANP in diabetes would be obtained.

As for BNP, in carotid artery we can see again a less relaxation in arteries from diabetic animals. However, in renal artery, this hyporeactivity does not exist and the arteries of diabetic animals show even more relaxation to BNP than the control animals. Again we must indicate that it is a work of very short duration. In particular, the sample size in the case of BNP is the smallest, which leads to the existence of a considerable mean standard error, which prevents statistics of having the adequate potenty. In general, the entire study should extend in time in order to increase all sample sizes and obtain more homogeneous data allowing more conclusive results.

When we compare both natriuretic peptides in both experimental situations we can observe that there are no differences in the magnitude of the relaxation produced by ANP and BNP in diabetic rabbit carotid artery, whereas in control, BNP shows a slightly higher relaxation than ANP. However, we think that these differences could be eliminated by increasing the sample size. In renal artery the situation observed is very different and, with the objections that the small sample size involved, shows that the vascular bed has an influence in the reactivity shown by both natriuretic peptides. Thus, we see that in arteries from control animals, BNP shows less relaxation to the ANP, while BNP-induced relaxation in diabetic animals is higher.

It would be necessary to realize further investigation to determine what is the exact effect of the natriuretic peptides studied. Previous results from laboratory of Physiology, University of Valencia, show some of these aspects as well as the mechanisms involved in the vascular response of both vascular beds to these peptides in diabetes. The implication of these peptides in the cardiovascular system and in the regulation of blood pressure is very well known. An alteration in its production and release as a result of diabetes could have serious consequences for health. It is necessary to continue studying the altered mechanisms in the release of the natriuretic peptides in diabetes to determine the contribution of the natriuretic peptides system to this disease and to the cardiorenal dysfunction.

7 CONCLUSION

1. The natriuretic peptides produce relaxation of the renal and carotid arteries in both control and diabetic rabbits.
2. The natriuretic peptides seem to show, as it has been observed in previous studies, a hyporeactivity in carotid and renal arteries of diabetic rabbits. This hyporeactivity, which has not been markedly shown in this study of small dimensions, would be observed in the case of an increase in sample size, as obtained in other works.
3. Vascular reactivity of the natriuretic peptides is different in carotid and renal artery, showing possible differences in their effect in different vascular beds.
4. The understanding of the mechanisms by which diabetes causes changes in vascular reactivity in these arteries and the existing relationship among the different regulatory systems, as the natriuretic peptides, could allow to contribute to the knowledge of the pathophysiology of diabetic vasculopathy. It is clear as well that there is a need to expand the sample size in our study to clarify the results, as well as doing other experiments to determine the role of the different mediators in the vascular response to the natriuretic peptides.

8 ZÁVĚR

1. Natriuretické peptidy způsobují relaxaci karotid a renálních arterií u kontrolních i diabetických králíků.
2. Stejně jak to bylo pozorováno i v předchozích studiích, natriuretické peptidy způsobují hyporeaktivitu u karotid a renálních arterií z diabetického králíka. Tato hyporeaktivita, která nebyla až tak zřetelně prokázána v této studii malého rozsahu, by byla pravděpodobně prokázána v případě většího počtu vzorků, jak bylo zřejmé z ostatních předešlých studií.
3. Reaktivita natriuretických peptidů v karotidě a renání arterii je různá, ukazuje možné rozdíly v účinku na různých místech cévního řečiště.
4. Pochopení mechanismů, kterými diabetes způsobuje změny v reaktivitě těchto arterií a porozumění vztahu mezi rozdílnými regulačními systémy, jako jsou natriuretické peptidy, by mohlo přispět k poznání patofyziologie diabetické vaskulopatie. Je zřejmé, že je potřeba zvýšit počet vzorků v této studii, abychom měli větší přesnost naměřených výsledků, a také dělat další experimenty kvůli určení role různých mediátorů v cevní odpovědi natriuretickými peptidy.

9 ABBREVIATIONS

ANP	Atrial natriuretic peptide
BMI	Body mass index
BNP	B-type natriuretic peptide
CNP	C-type natriuretic peptide
COX	Cyclooxygenase
CTL	Control
EDHF	Endothelium-derived hyperpolarizing factor
NO	Nitric oxide
NP	Natriuretic peptides
NTproBNP	N-terminal fraction of BNP
SEM	Standard error of the mean

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