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ANGIOTENSIN II AND MECHANISMS OF OXIDATIVE DAMAGE IN HUVECs

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A ***Mamma, Papà, Andrea e Claudio****:*

le quattro persone più importanti della mia vita...

A ***zio Gianni*** *e a* ***Nonno*** *che, da un mondo lontano insieme*

agli angeli del cielo, mi sono stati sempre vicino

nei momenti di difficoltà.

TABLE OF CONTENTS

ABSTRACT…….pag.6

INTRODUCTION……...…7-20

- *The Endothelium and vascular homeostasis*
- *Endothelium dysfunction, atherosclerosis and vascular inflammation*
- *Effects of Endothelium dysfunction*

OUTLINE OF THE THESIS.....21

ORIGINAL DATA

PART.1

1.1 Evaluation of Angiotensin II regulatory mechanisms involved in the production/degradation of Reactive Oxygen Species and Reactive Nitrogen Species.....22-45

- **CHAPTER 1.** *Renin- Angiotensin- System : from Angiotensin I to Angiotensin IV in Endothelial dysfunction.*
- **CHAPTER 2.** *Radical Oxidative Stress : Reactive Oxygen Species and Reactive Nitrogen Species in Human Endothelial Cells*
- **CHAPTER 3.** *Asymmetric Dimethyl Arginine and Oxidative Stress in Endothelial Dysfunction*

1.2 Monitoring of the protective effects of antioxidants flavonoids on membrane damage and radical species production.....46-59

- **CHAPTER 1.** *Antioxidant networks and redox signaling*
- **CHAPTER 2.** *Flavonoids : Epigallocatechin-gallate and its effects in vitro*
- **CHAPTHER 3.** *Therapeutic Opportunities for treatment of Endothelial Dysfunction*

PART.2 " *In vitro*"study on Angiotensin IV receptor and Angiotensin IV involved in stress pathway.....60-73

- **CHAPTER 1.** *Angiotensin IV and Renin-Angiotensin-System in vitro models.*
- **CHAPTER 2**. *Angiotensin IV receptor and IRAP receptor*
- **CHAPTER 3.** *Pharmacologic Profile of Angiotensin IV*

Materials and Methods.....74-88

Results and Discussion 89-120

CONCLUSIONS......120-122

PUBLICATIONS AND ABSTRACTS.....123-124

ACKNOWLEDGEMENTS.....125

LIST OF ABBREVIATIONS.....126

REFERENCES.....128-157

ABSTRACT

The endothelium is essential for the maintenance of vascular homeostasis. Central to this role is the production of endothelium – derived nitric oxide (EDNO), synthesized by endothelial isoform of nitric oxide synthase (eNOS). Endothelial dysfunction, manifested as impaired EDNO bioactivity, is an important early event in the development of various vascular disease, including hypertension, diabetes, genesis of tumors and atherosclerosis. Endothelial dysfunction is an early feature of atherosclerosis vascular disease, characterized by a decrease in nitric oxide (NO) bioavailability and a concomitant increase in vascular superoxide ($O_2^{\cdot -}$) formation. Loss of NO bioavailability precedes the development of overt atherosclerosis and is an independent predictor of adverse cardiovascular events. Indeed, decreased NO and enhanced production of reactive oxygen species (ROS) have been recognized as major determinants of age-associated endothelial dysfunction. The degree of impairment of EDNO bioactivity is a determinant of future vascular complications. Accordingly, growing interest exists in the pathologic mechanism involved. However it is clear that immunologic mechanisms operating in the context of common cardiovascular risk factors lead to impaired endothelial function, mainly as a consequence of decreased NO bioavailability and excessive oxidative stress.

The work submitted in this thesis describes on one side studies aimed to investigate cellular mechanisms underlying endothelial dysfunction and vascular damages driven by oxidative stress in the context of aging, hypertension and atherosclerosis using *in vitro* models. In addition, we desired to evaluate the efficacy of reducing agents such as flavonoid to monitor whether they actually have an action to recover from the cellular oxidative damage by these natural compounds and how real is their action at the level of microcirculation in vitro models. On the other side, we present studies focused on the pathophysiology of microcirculation as far as functional aspects are concerned in the context to better understand the functioning of the Renin- Angiotensin-System in particular if the Angiotensin IV is involved in mechanisms of oxidative stress and in Calcium intracellular levels.

Introduction

Modern Age has witnessed major developments in cardiovascular physiology thanks to scientists like E.H. Starling in the 1920s, who described the *"fundamental properties of the heart muscle itself and then found out how these are modified, protected, and controlled under the influence of the - nervous, chemical and mechanical- mechanisms which under normal conditions play on the heart and blood vessels",* quoting his remarkable studies [167,211]. The existence of pathological cardiovascular conditions has been recognized and described. The atherosclerotic vascular disease and its main clinical manifestation, *angina pectoris* were firstly reported in the 18th century. In parallel, the earliest descriptions of some attempts for a pharmacological treatment appeared. Nitroglycerin, for example, was initially prescribed by physicians in the late 19th century [172]. A number of new pharmacological agents found to be of benefit followed, leading to the constitution of the currently established cardiovascular pharmacotherapy. The atherosclerotic vascular disease was hypothesized to cause ischemia and infarction of the heart and other organs. Acute cardiovascular ischemic events, such as stroke, myocardial infarction or sudden death, were frequently associated with localized arterial thrombus formation. However for many decades, these conditions remained totally mysterious and unpredictable events. Moreover, hypertension was recognized to damage large blood vessels and the microcirculation of different target organs, even if the specific mechanisms involved were unknown.

Vascular biology, as the study of vascular cells under normal and pathological conditions, began in the 1970s. This novel research discipline, has since then enjoyed exponential growth, allowing the comprehension of common pathophysiologic processes of cardiovascular diseases (CVD). In 1980, the obligatory role of endothelial cells in relaxation of arterial smooth muscle by acetylcholine (Ach) was described [76]. A seminal event in the field of vascular biology, which was later awarded with the Nobel Prize in 1998 to Robert Furchgott, Louis Ignarro and Ferid Murad. By means of *in vitro* experiments in organ chambers, preconstricted arterial rings were demonstrated to relax in response to the muscarinic cholinergic agonist only if endothelial cells were present. Removing the endothelium by any means abolished the vasorelaxation, which was mediated by an undefined endothelium-derived substance that was named endothelium derived relaxing factor (EDRF). EDRF, subsequently, was shown to be, in large part, nitric oxide [153]. During the last three decades,

several studies have definitively proved that the endothelium is not only a cell monolayer covering the lumen surface of the vascular wall, but it is involved in many key regulatory functions for the homeostasis of cardiovascular system.

The endothelium and vascular homeostasis

In recent years the endothelium in the literature has been simply classified as a biologically inert barrier interposed between the blood and the vessel wall. It was considered a uniform layer of cells with the task of providing a protective surface that would prevent the adhesion of blood cells but that would be able to carry or convey liquids, solutes and various substances in the blood stream from this interstitium. Since a few years, detailed studies on anatomy and physiology of endothelium have been providing more information that make this definition unacceptable. In fact, currently the endothelial tissue is considered a real organ with the function to modulate vascular tone and extent of blood flow [190] and has a primary role in maintaining vascular homeostasis associated with features [178], in response to humoral, nervous and mechanical stimuli [190]. The endothelium is able to perform its functions only if intact, when it establishes a balanced equilibrium between factors related to the mechanism for inducing vasodilation and vasoconstriction factors generating mechanism. When the ability of endothelial cells is compromised to process the substances that are produced under physiological conditions the "endothelial dysfunction" is established [96]. The endothelium is the inner face of endoluminal arteries, veins, capillaries and lymphatic vessels lined by a continuous cell layer consisting of endothelial cells. The essential components of the walls, in endothelium, are classified as *(Fig. 1)*:

• *tunica intima:* inner layer, delimiting the lumen of the vessel, consisting of a monolayer of endothelial cells from the underlying basement membrane and the internal elastic lamina;

• *tunica media*: the middle layer consists of smooth circular and longitudinal fibers;

• *tunica adventitia*: outer skin consists of connective tissue and fibroblasts, separated from the tunica media by the external elastic lamina.

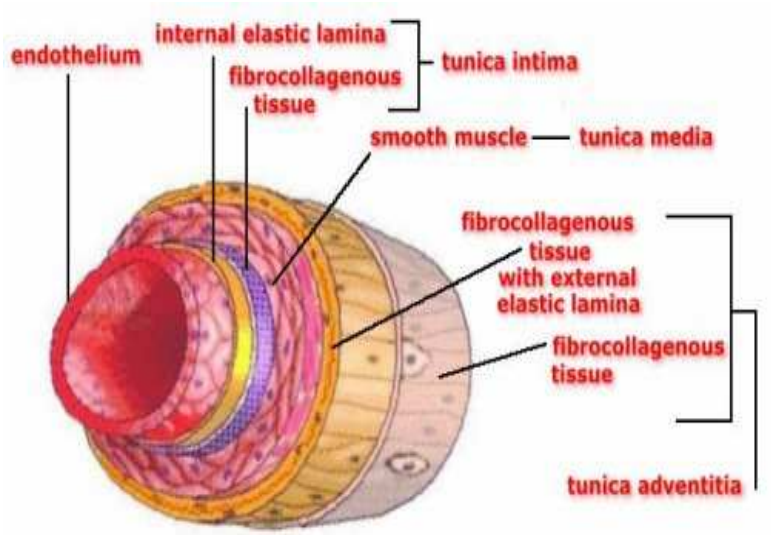


Figure 1- Endothelial wall- *Nature.com*

Endothelial cells are polygonal and flattened by microscopic study (30µm x 10µm). They contain numerous vesicles and form junctional complexes with other nearby cells. Cells are welded together by means of both tight junctions, through gap junctions. The only type of cell to possess Weibel-Palade bodies, structures surrounded by membranes with a diameter of 0.1 µm and a length of 3 mM, which are organelles of the deposit of von Willebrand factor (vWF) [178].

Endothelial function

Under physiological conditions, integrity of the endothelial cells is essential for normal function of blood vessels and maintenance of homeostasis through secretion or display on the membrane of different molecules that are responsible for the continuous regulation of vascular tone, control of blood pressure, the physiological regulation of leukocyte passage and maintenance of antithrombotic and anticoagulant effects [190,177]. Endothelial paracrine activity is directed both toward the lumen and towards the vessel wall. At the vessel lumen level, the endothelium regulates coagulation mechanisms and interactions with leukocytes and platelets [177,4]. At the vascular wall level, endothelium plays a central role in regulating blood pressure

and blood flow through a continuous modulation of vascular tone, which is under the control of local and systemic factors. Modulating vascular tone and the same structure, the endothelium plays a major role in vascular remodeling observed in hypertension [81], the stenosis after angioplasty [115] and atherosclerosis [25]. Central role in regulating endothelial cell homeostasis and thrombosis depends on the expression and release of many molecules, with autocrine and paracrine actions, such as prostacyclin PGI_2, nitric oxide (NO), platelet-activating factor (PAF), von Willebrand factor (vWF), thrombomodulin, tissue factor (thromboplastin) and its inhibitor (TFPI), factor, tissue plasminogen activator (t-PA) and its inhibitor (PAI-1) [4]. In addition to being involved in the mechanism of activation and aggregation of platelets, the PAF also increases the permeability of the endothelial barrier and, together with the platelet adhesion molecule (P-selectin), promotes leukocyte adhesion to the vessel wall [155]. The adenosine diphosphate (ADP) that is released by the activation of platelets, along with the thromboxane A2, promotes platelet activation and aggregation through a more positive feedback mechanism [49,93]. The vWF factor has essential functions in homeostasis: it mediates platelet adhesion to sites of vascular injury, mean plate-plate interaction, promotes platelet aggregation in vessels with high shear-stress due to the rapid blood flow and transport coagulation factor VIII in plasma [194,193]. Endothelial cells express on their surface a protein that is the intrinsic membrane thrombomodulin and is found exclusively on the endothelial luminal surface, surface that is not damaged. It has a strong affinity to thrombin, plasma protein involved in the mechanism of the coagulation cascade. In particular, it is involved in the conversion from fibrinogen to fibrin and also in the platelet activation and aggregation [45,21]. When thrombin binds to thrombomodulin, it causes conformational rearrangements of the second compound reducing its affinity for fibrinogen and activates circulating protein C which, together with protein S, inactivates factors V and VIII, inhibiting blood coagulation [65,77]. This suggests that thrombin has an anticoagulant action in the presence of thrombomodulin [52]. Thromboplastin is a main physiological activator of coagulation. In contrast to extravascular cells, under physiological conditions, endothelial cells do not express the thromboplastin in order to preserve the fluidity of the blood [50]. However, *in vitro* studies have shown that endothelial cells synthesize and express on the surface in response to thromboplastin thrombin, and cytokine-activated platelets. Endothelial cells also produce tissue factor inhibitor (Tissue Factor Pathway Inhibitor, TFPI), which is the most important physiological inhibitor of thromboplastin. This

protein is secreted by endothelial cells and is found in plasma or bound to the cell surface [197]. Under basal physiological conditions, endothelial cells do not express molecules that promote the adhesion of circulating leukocytes. However, the activation of endothelial cells by thrombin, endotoxin or inflammatory cytokines such as IL-1 and TNF-α, induces the surface expression of a series of molecules that are essential for adhesion and migration of leukocytes from the bloodstream into the damaged tissue [208]. This process is mediated by cell adhesion molecules (CAMs), glycoproteins expressed on the surface of activated cells that are involved in cell-cell and cell-matrix binding [61]. The binding of leukocytes to endothelium is mediated by immunoglobulin ICAM-1 (inter cellular adhesion molecule-1) and VCAM-1 (vascular adhesion molecule-1) that interact with integrins on the surface of circulating leukocytes and cause a stable relationship with the endothelium [19,230]. ICAM-1 is over-expressed in response to various stimuli such as inflammatory cytokines (IL-1, TNF-α, interferon γ). VCAM-1 is expressed by endothelial cells and smooth muscle cells of the vessel wall, promotes cell-cell adhesion and subsequent migration of inflammatory cells [62]. Locally, vascular tone is self-regulated mainly in response to mechanical stimuli by the power button and the shear stress affecting the vessel walls as a result of changes in blood flow [249]. This regulation is modulated by the synthesis and secretion of two potent vasodilators: prostacyclin (PGI_2) and nitric oxide (NO). PGI_2 is a prostaglandin deriving from arachidonic acid and is synthesized by the action of prostaglandin PGI_2 synthase from H_2 (PGH_2) which is produced by the hydrolysis of arachidonic acid through cyclooxygenase-2 (COX-2). Nitric oxide is a diatomic free radical generated by nitric oxide synthase hetero (NOS) through the oxidation of L-arginine to L-citrulline [84]. NO is produced in the endothelium and is released as a free radical and as a nitrosyl compound, pre-eminent importance in controlling the tone of the arteries and the microcirculation, both in basal conditions and after various kinds of stimulation [74,84]. Nitric oxide has been identified as a major anti-atherosclerosis factor [37] because of its protective action on blood vessels [159]. Nitric oxide induces vessel relaxation activating the guanylate cyclase enzyme. In addition, we found that nitric oxide inhibits the oxidation of low density lipoprotein (LDL). It is also an antagonist of platelet aggregation by inhibiting platelet activation [159]; moreover, it represses the expression of kB dependent nuclear factor of adhesion molecules that regulate the recruitment of leukocytes in the endothelium, in the early stages of atherosclerosis. Endothelial cells synthesize NO and PGI_2 in response to various substances to agonist action,

such as bradykinin, acetylcholine, serotonin, thrombin, adenosine triphosphate (ATP) and adenosine diphosphate (ADP) that increases the concentrations of cytoplasmic calcium, and also in response to mechanical stimuli, such as deformation of the plasma membrane due to shear stress [74,84]. The increased shear stress across the endothelium promotes the release of other agonists, vasodilators, such as ATP and phosphate leading to increased cytoplasmic calcium in adjacent cells, thus, stimulating the synthesis of other NO. These observations are in agreement with previous hypotheses that required calcium levels to activate the synthesis of NO, calcium levels are lower than those who need to activate the synthesis of PGI_2 [35]. The endothelium also acts in the process of modulation of the mechanism of vasoconstriction by means of certain substances and effector molecules such as angiotensin, endothelin, prostaglandin H2 and thromboxane A2 [47].

The Endothelium dysfunction, atherosclerosis and vascular inflammation

"Endothelium dysfunction" *(Fig.2)* refers to the condition in which the endothelium loses its physiological properties [179] and is determined by an imbalance in the production of mediators that regulate vascular tone, platelet aggregation, the coagulation and fibrinolysis .

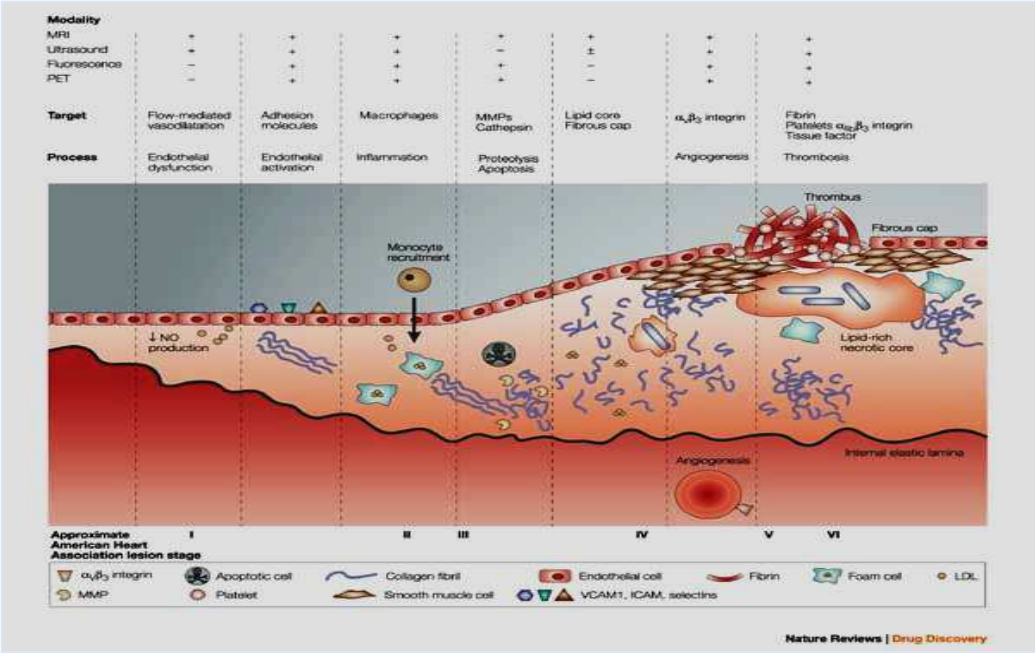


Figure 2- Mechanisms of Endothelial Dysfunction*(Nature,Review 2010)*

Endothelial dysfunction has been associated with a variety of processes including hypertension, atherosclerosis, aging, heart block and failure, coronary syndrome, obesity, infections, sepsis, arthritis rheumatoid diseases, thrombosis, cigarette smoking, and type 1 and 2 diabetes [72]. **Oxidative stress** plays a relevant role in atherosclerosis and cardiovascular diseases by promoting cellular dysfunction, inflammation and lipids and lipoproteins peroxidation, and is reducing the bioavailability of NO [259]. The mechanisms of endothelial dysfunction shows many unclear details, but what is definitely known is that the reduction in the bioavailability of NO is a relevant aspect of endothelial dysfunction [175]. This reduction of NO is followed by high levels of endothelial vasoconstrictive substances such as endothelin-1 [50]. The mechanisms by which NO bioavailability acts are essentially two: the first mechanism involves the down-regulation of the expression of eNOS (endothelial NO synthase) which synthesizes nitric oxide in the endothelium through the oxidation of nitrogen contained in the L-arginine, which is converted to L- citrulline. The reaction requires the presence of cofactors, such as nicotinamide adenine dinucleotide phosphate (NADPH), calcium / calmodulin (CaM), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and tetrahydrobiopterin (BH_4) [74,84] that are integrated in the structure of NOS, once the enzymes are expressed in the cell [249]. The other mechanism involves the up-regulation of vascular levels of ROS . It is known that high levels of ROS damage the vascular tissue, as is known to react with NO to form peroxynitrite ($ONOO^{\cdot-}$), thus removing NO. In fact, it was demonstrated that the increased expression of superoxide may lead to the "scavenging" of NO and its reduced bioavailability [114,236]. The shear stress signal and activity of eNOS are regulated by mechanisms of Ca^{2+} / calmodulin-dependent [29] and Ca^{2+} / calmodulin-independent [15]. The eNOS is a homodimer consisting of two monomers, each of which contains a reductase (C-terminal) domain that binds NADPH, FAD and FMN, and a domain which carries a heme oxygenase (N-terminal). These heme groups are required for dimerization of both monomers to form the active NOS dimer and electron transfer from flavin to heme of the opposite monomer. To become a fully functional enzyme complex, it binds to the oxygenase domain (6R) -5,6,7,8-tetrahydrobiopterin (BH_4), molecular oxygen and substrate L-arginine. Finally, a zinc ion binds at the interface of the dimer of NOS, and has mainly a structural function [15]. The eNOS catalyzes an electron transfer from NADPH reductase domain in the heme oxygenase

domain, by means of FAD and FMN. This electron transfer is favored by the binding of calmodulin induced by calcium, the domain reductase. The O_2 is reduced and L-arginine is oxidized by 2-step, through N-hydroxy-L-arginine to L-citrulline and NO. Once produced NO, a short half-life gas (6-7 seconds), readily crosses the plasma membrane of smooth muscle cells of the tunica media and activates the enzyme guanylate cyclase resulting in the production of intracellular cyclic GMP, which causes smooth muscle relaxation and vasodilation through a series of mechanisms that reduce the levels of intracellular free calcium . The activity of eNOS is regulated by the concentration of intracellular calcium and by phosphorylation of the reductase domain and the calmodulin binding domain. Depending on the stimulus, the specific kinase can phosphorylate eNOS, such as Akt / protein kinase B, protein kinase A, protein kinase 5'-adenosine monophosphate activated and calmodulin-dependent kinase II. If the electron flow is disturbed in eNOS (*in vitro*, this may be due to the absence of the cofactor BH4 or substrate L-arginine), it changes from a coupled eNOS (dimer), which leads to the formation of NO, to a decoupled state, which generates oxygen. the enzyme moves from an oxidant state to a reduced one [133]. During the normal catalytic function of eNOS, BH_4 acts as an electron donor. Under conditions of excessive production of oxidants, oxygen can react with NO, that formed $ONOO^{\cdot-}$ (peroxynitrite) [88]. It has been shown that $ONOO^{\cdot-}$ (peroxynitrite) oxidizes BH_4 in biologically inactive products that cannot be further recycled . The formation of peroxynitrite, through a process of nitration of protein affects the function of proteins and thus endothelial function; it also mediates the oxidation of LDL, contributing to the pro-atherogenic conditions [73]. Functions of nitric oxide include an important role in maintaining vascular endothelial integrity and stability, preventing platelet aggregation and leukocyte adhesion and maintenance of blood flow [31,179]. It is not so surprising that the damage to NO synthase, which is produced by the endothelium dysfunction, has deleterious effects on blood pressure and platelet count.Thus the damage to NO synthase allows changes to the vessel wall, that are known to be associated with vascular pathogenesis in general, and atherosclerosis in particular . The reduced concentration of NO causes an "up-regulation" of VCAM-1 in endothelial cells through the expression of NF-kB]. ROS, C-reactive protein (CRP) and oxidized LDL receptor 1 (LOX-1) increase expression of adhesion molecules (*Fig.3*), such as VCAM-1, ICAM-1 and E-selectins that are important in the early stages of the inflammatory process . VCAM-1 is responsible for ties with monocytes and T cells represent the first stage of

the invasion of the vessel wall by inflammatory cells . Endothelial dysfunction allows the infiltration and retention of LDL in the serum of the tunica intima of the vessel starting an inflammatory response. Within the intima, LDL particles are modified by oxidation or by promoting the enzymatic release of phospholipids that stimulate endothelial cells to express VCAM-1 and ICAM-1 and produce growth factors, such as granulocyte-macrophage colony stimulating factor (GM-CSF) [144]. The CAMs mediate the entry of specific leukocytes into the vessel wall, monocytes and lymphocytes at the site of endothelial damage [90,138]. Within the intima, GM-CSF stimulates monocytes to become macrophages, which plays an important role in local inflammatory response by producing inflammatory cytokines, chemokines and oxygen free radicals [138,191]. In addition, the secretion of extracellular matrix metalloproteases (MMPs) by macrophages contributes to the remodeling of the vessel wall and eventual plaque rupture. These molecules amplify the cellular response and begin promoting the low-intensity vascular inflammation, thrombosis, progressive thickening of intima and hence the formation and development of atherosclerotic plaque that may break possibly causing clinical manifestations [191].

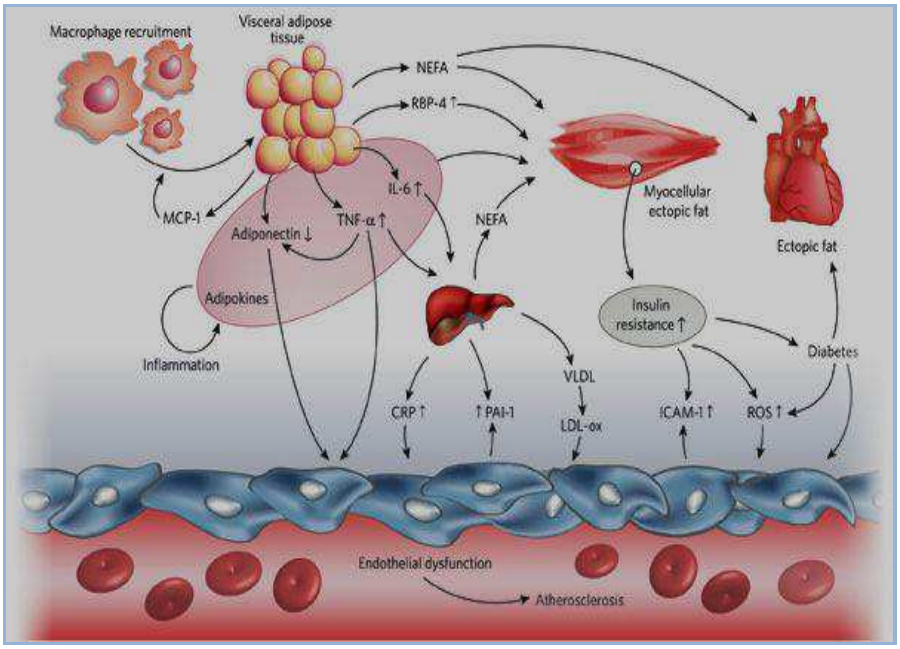


Figure 3- Different molecules involved in endothelial dysfunction- *Science.com*

In 1997, the isolation of putative endothelial progenitor cells (EPCs) from human peripheral blood has been reported [8]. Subsequently, evidence has accumulated documenting the presence of a population of endothelial precursor cells and/or adult stem cells, deriving from bone marrow, with a specific role in the maintenance of endothelial integrity against vascular injury [34].These cells are able to home areas of injury and ischemia-induced myocardial and peripheral neovascularization [109], healing endothelial integrity .

Effects of Endothelial Dysfunction

Endothelial function has largely been investigated through the assessment of endothelium dependent vasomotion. Indeed, an impaired endothelium-dependent relaxation reflects an extensive loss of endothelial function. Actually it was important to characterize how endothelial dysfunction relates to the pathogenesis of atherosclerosis. Studies focusing on these issues continue to provide remarkable evidence that the endothelial interface between the vascular wall and the circulation is the primary site to trigger off cardiovascular events [55].Under pathologic conditions, the endothelium has a reduced availability of vasodilating factors, in particular NO, and an augmented production of vasoconstricting factors, leading to impaired endothelium-dependent vasodilation. Furthermore, NO has demonstrated to exert a major anti-inflammatory effect and can therefore be considered the most important endogenous antiatherogenic molecule. Endothelial dysfunction promotes arterial inflammation and *vice versa*, chronic inflammation maintains a pro-inflammatory phenotype of the endothelium [55]. Therefore EC dysfunction seems to participate in atherosclerotic process from its inception onwards till ultimate complications with a complex and pleiotropic involvement of inflammation sustained by humoral and cellular inflammatory elements. Not only these effects are present in endothelial dysfunction but other factors are involved in its genesis.

Cardiovascular Disease and Endothelial Dysfunction

Endothelial dysfunction was first described in human hypertension in the forearm vasculature in 1990 [171]. Impaired vasodilation in hypertension was confirmed by many studies in different vascular beds, including small resistance vessels [173,201]. In stage I essential hypertension, we

showed that ~ 60% of patients exhibit impaired small artery vasodilatation when this is studied *in vitro* in vessels dissected from gluteal subcutaneous biopsies [174]. Impairment of vasodilation was also described in type 1 [14] and type 2 diabetes [189,154], coronary artery disease [122], congestive heart failure [24], and chronic renal failure [225,255]. Moreover, this manifestation of endothelial dysfunction not only is associated with cardiovascular disease but may also precede its development, as shown in a study on offspring of hypertensive patients. The study subjects displayed endothelial dysfunction despite being normotensive. Another study showed endothelial dysfunction in symptom-free children and young adults at high risk for atherosclerosis [12]. Also, in normotensive, normoglycemic, first-degree relatives of patients with type 2 diabetes, endothelial dysfunction was correlated with insulin resistance [64]. Endothelial dysfunction has been demonstrated in the metabolic syndrome and in dyslipidemia and may be associated with obesity [247], hyperhomocysteinemia , sedentary lifestyle, and smoking, in the absence of overt cardiovascular disease.

The ***pathophysiology of endothelial dysfunction*** is complex and involves multiple mechanisms.

NO

One of the most important vasodilating substances released by the endothelium is NO, which acts as a vasodilator, inhibits growth and inflammation, and has anti-aggregant effects on platelets. Reduced NO has often been reported in the presence of impaired endothelial function. It may result from reduced activity of endothelial NO synthase (eNOS; as a result of endogenous or exogenous inhibitors or reduction in the availability of its substrate, L-arginine) and from decreased bioavailability of NO. ROS are known to quench NO with formation of peroxynitrite [119], which is a cytotoxic oxidant and through nitration of proteins, they will affect protein function and therefore endothelial function. Peroxynitrite is an important mediator of oxidation of LDL, emphasizing its proatherogenic role . Moreover, peroxynitrite leads to degradation of the eNOS cofactor tetrahydrobiopterin (BH_4) [148], leading to "uncoupling" of eNOS. Using a novel peroxynitrite decomposition catalyst, FP15, endothelial and cardiac dysfunction could be prevented in diabetic mice . Oxidant excess will also result in reduction of BH_4 with increase in BH_2. When this occurs, formation

of the active dimer of eNOS with oxygenase activity and production of NO is curtailed (uncoupling of eNOS). The reductase function of eNOS is activated and more ROSs are formed, so NO synthase goes from its oxygenase function producing NO to its reductase function producing ROS, with the consequent exaggeration of oxidant excess and deleterious effect on endothelial and vascular function [222]. Oxidative excess is linked to a proinflammatory state of the vessel wall. ROS upregulate adhesion (VCAM-1 and ICAM-1) and chemotactic molecules (macrophage chemoattractant peptide-1 [MCP-1]) [87]. Inflammation decreases NO bioavailability. Indeed, C-reactive protein (CRP) has been shown to decrease eNOS activity [244]. The main source for oxidative excess in the vasculature is NAD(P)H oxidase [245,89]. Other sources include xanthine oxidase [88], the mitochondria [234] and uncoupled NOS.

Asymmetric Dimethylarginine

A relatively new and attractive mechanism that leads to reduced NO is asymmetric dimethylarginine (ADMA), an endogenous competitive inhibitor of eNOS, that has been linked to endothelial dysfunction. In human endothelial cells, which were stimulated with plasma from patients with chronic renal disease, inhibition of eNOS correlated with plasma ADMA levels [60]. ADMA levels were inversely related to endothelium-dependent vasodilation [254] in subjects with hypercholesterolemia, and infusion of L-arginine, the substrate of eNOS and competitor of ADMA, normalized endothelial function. It has been suggested that accumulation of this endogenous eNOS inhibitor leads to reduced effective renal plasma flow and increased renovascular resistance and BP [20]. Intravenous low-dose ADMA reduced heart rate and cardiac output and increased mean BP . ADMA is a product of protein turnover and is eliminated by excretion through the kidneys or metabolism to citrulline by the enzyme dimethylarginine dimethylaminohydrolase (DDAH). Recently, overexpression of DDAH was shown in transgenic mice to decrease ADMA, increase eNOS activity, and reduce BP [2], underlining the pathophysiologic importance of ADMA. Because ADMA is eliminated through renal excretion and degradation by DDAH, it is not surprising that it is increased in patients not only with chronic renal failure [51,111] but also with other diseases such as hepatic dysfunction [240]. New interest is focusing not only on the elimination but also on the generation of ADMA. Protein-arginine methyltransferases, which

produce methylated arginines, namely protein-arginine methyltransferase-1, were shown to be upregulated by shear stress, and this upregulation was associated with enhanced ADMA generation . Hypercholesterolemia is a risk factor for atherosclerosis, associated with endothelial dysfunction [168], and there is now also evidence that elevated ADMA levels are associated with hypercholesterolemia . Plasma ADMA levels were also increased in elderly hypertensive patients [20] and correlated with age and BP [110]. ADMA levels have been associated with increased cardiovascular risk factors in renal failure, such as CRP, carotid intima-media thickness, concentric left ventricular hypertrophy, and left ventricular dysfunction [110,150]. Moreover, it was found to be a predictor of acute coronary events [262], overall mortality of patients with chronic renal failure[239], and mortality of critical ill patients [240].

Oxidative Excess

In animal models of hypertension, oxidative excess leads to endothelial dysfunction as evidenced by improvement of the impaired endothelium-dependent relaxation after use of antioxidants [260]. Oxidative excess in hypertensive patients leads to diminished NO [57] and correlates with the degree of impairment of endothelium-dependent vasodilation and with cardiovascular events [226]. In patients with chronic renal failure, markers of oxidative excess also correlated with endothelial dysfunction [94]. Findings in animal models of chronic renal failure suggest that enhanced generation of ROS leads to decreased NO bioavailability and endothelial dysfunction, which may be improved by antioxidant pretreatment [5,92]. In humans with chronic renal failure, the administration of vitamin C improved endothelial dysfunction of resistance arteries but not of conduit arteries [243]. In animal models of diabetes, increased oxidative excess also led to endothelial dysfunction [46]. ROS also seem to be involved in the mediation of endothelial injury leading to programmed cell death or apoptosis and to a form of apoptosis characterized by detachment of endothelial cells called anoikis [76]. Apoptosis is induced by the loss of cell-matrix interactions, but its exact mechanisms and pathophysiological role in cardiovascular disease are not fully understood. Eicosapentaenoic acid, a polyunsaturated fatty acid contained in fish oil, was shown to protect endothelial cells from anoikis [113], which may contribute to the antiatherogenic and cardioprotective effects of fish oil.

Ang II

Ang II has been implicated in the pathophysiology of hypertension and chronic renal failure. Ang II infusion induces endothelial dysfunction in rats [227], increases ROS by stimulating NAD(P)H oxidase [89,182], and promotes vascular inflammation [233]. In hypertensive humans, interruption of the renin-angiotensin system with angiotensin-converting enzyme inhibitors or angiotensin receptor blockers restores endothelial function in contrast to a similar degree of BP lowering with a -blocker, which has no effect on endothelium-dependent vasodilation [201,233,203].

Hyperhomocysteinemia

A non traditional cardiovascular risk factor that leads to endothelial dysfunction is hyperhomocysteinemia. This has been evidenced by animal models of hyperhomocysteinemia [202]. Normotensive patients with hyperhomocysteinemia display endothelial dysfunction . Folic acid supplementation was able to reduce homocysteine levels and improved endothelial dysfunction in children with chronic renal failure . Cellular [248], animal [202], and human studies suggest that homocysteine reduces NO bioavailability by oxidative excess. There is now also evidence that homocysteine may cause ADMA accumulation by inhibition of DDAH . Experimental studies in humans have confirmed that hyperhomocysteinemia may lead to endothelial dysfunction via accumulation of ADMA [257,218]. However, not all studies support this link [219]. These mechanisms may explain the increased cardiovascular risk of patients with hyperhomocysteinemia. This is of special importance for patients with chronic renal failure, who often have increased homocysteine levels, which were shown to predict cardiovascular outcomes in a recent study .

Diabetes

In diabetes, additional mechanisms may trigger endothelial dysfunction. In states of insulin resistance, such as in type 2 diabetes, insulin signaling is altered, differently affecting the two major pathways emerging from the insulin receptor. The pathway leading via phosphoinositide 3-kinase, phosphoinositide-dependent kinase-1, and Akt/protein kinase B to phosphorylation and activation of eNOS is dramatically downregulated, whereas the pathway leading via mitogen activated protein kinases to mitogenic effects and growth is unaffected

[141,251]. Moreover, hyperglycemia leads to advanced glycation end products (AGE), which were shown to quench NO and impair endothelial function, as evidenced by inhibition of advanced glycosylation with aminoguanidine [48]. AGE induce ROS and promote vascular inflammation, with enhanced expression of interleukin-6, VCAM-1, and MCP-1. This turns into a vicious circle in diabetic nephropathy, because in renal failure, clearance of AGE is delayed, which further promotes vascular and renal injury [28]. Finally, acute hyperglycemia itself can reduce NO [258] and attenuate endothelium-dependent vasodilation in humans *in vivo*. Endothelial dysfunction has been proposed to be an early event of pathophysiologic importance in the atherosclerotic process and provides an important link between diseases such as hypertension, chronic renal failure, or diabetes and the high risk for cardiovascular events that patients exhibit with these conditions. Low NO bioavailability can upregulate VCAM-1 in the endothelial cell layer via induction of NF-κB expression. ROS, CRP, CD40 ligand, and lectin-like oxidized LDL receptor-1 (LOX-1) also upregulate endothelial expression of adhesion molecules. The expression of VCAM-1, ICAM-1, and E-selectin plays a role in the initiation of the inflammatory process. VCAM-1 binds monocytes and T lymphocytes, the first step of invasion of the vessel wall by inflammatory cells. NO inhibits leukocyte adhesion .Reduction in NO results in induction of MCP-1 expression, which recruits mononuclear phagocytes. Monocytes are transformed into lipid-loaded foam cells. Oxidized LDL, for example, is scavenged through LOX-1, which is highly expressed in blood vessels in hypertension, diabetes, and dyslipidemia. Oxidized LDL uptake by LOX-1 triggers a variety of actions: it reduces eNOS expression and further stimulates adhesion molecule expression. LOX-1 expression can be stimulated by Ang II and endothelin-1. As the atherosclerotic plaque progresses, growth factors secreted by macrophages in the plaque stimulate vascular smooth muscle cell growth and interstitial collagen synthesis. The event that initiates a majority of myocardial infarctions is the rupture of the fibrous cap of the plaque, inducing thrombus formation. Decreased NO and oxidative excess may activate matrix metalloproteinases (MMP) [82,140], namely MMP-2 and MMP-9, which weaken the fibrous cap. Because NO inhibits platelet aggregation , reduced NO contributes to thrombogenicity and to the severity of the event. Thus, endothelial dysfunction with reduced NO bioavailability, increased oxidant excess, and expression of adhesion molecules contributes not only to initiation but also to progression of atherosclerotic plaque formation and triggering of cardiovascular events [220].

Part.I: Evaluation of Angiotensin IIregulatory mechanisms involved in the production/degradation of Reactive Oxygen Species and Reactive Nitrogen Species

Experimental Protocols

Renin – Angiotensin- System : from Angiotensin I to Angiotensin IV concerning endothelial dysfunction.

1.1 Renin Angiotensin System (RAS)

New components and functions of the renin-angiotensin system (RAS) have already been completely revealed and described in full details. The classical RAS as it appeared in 1970 included renin, which acts on angiotensinogen for the production of Angiotensin I, which in turn is converted into Angiotensin II by Angiotensin converting enzyme (ACE) [78].

For decades, the Angiotensin II was regarded as the final product and the only bioactive peptide of the renin-angiotensin system (RAS) [224]. Angiotensin II was still considered the main effector of RAS, it was considered only as a circulating hormone that acted via angiotensin receptors, Angiotensin type 1 (AT_1) and Angiotensin type 2 (AT_2) receptors, on endothelial cells. Since then, a broad view of the RAS has gradually emerged. The RAS (local) tissue is identified in most organs. Recently an intracellular RAS was reported. Therefore, the RAS endocrine function, and paracrine, intacrine [78] were described. Other peptides of RAS denote those biological actions, the heptapeptide Angiotensin 2-8 [78] or heptapeptide Des Asp ^{1}-Ang II (Ang III) possess actions similar to those of Ang II. In addition, the hexapetide Angiotensin 3-8 [78], or hexapeptide Des Asp^{1}-Des Asp ^{2}-Arg Ang II (Ang IV), exerts its action through the receptor insulin-regulated aminopeptidase. Finally, Angiotensin 1-7 or hheptapeptide Des Phe ^{8}-Ang II (Ang 1-7), act through the receptor Mas [78]. Among these fragments of Angiotensin II, Ang IV has attracted more attention since it was unveiled to exercise a wide variety of effects, including, the ability to enhance learning and preserving the memory, anticonvulsant and anti-epileptogenic properties, protection against cerebral ischemia, vascular activity and involvement in atherogenesis. Some of these effects are mediated by AT_1 receptor but others are more likely by binding of Ang IV to the insulin-regulated aminopeptidase (IRAP), although the exact mechanism that mediates these actions is not yet well known [224]. In fact, three hypotheses have been proposed:

I. since Ang IV is an inhibitor of the catalytic activity of IRAP, its effects *in vivo* could result from an accumulation of the peptide substrates of IRAP;
II. IRAP is co-located with the glucose transporter *GLUT4* in several kinds of tissue and therefore, Ang IV may also interact with the uptake of glucose;
III. a final and more interesting hypothesis ascribes a receptor function to IRAP and thus an agonist role to Ang IV [224].

The discovery of ACE2 receptors and renin induced the perception of the RAS system as an unexpectedly complex one. The importance of this system in cardiovascular disease has been demonstrated by clinical benefit of ACE inhibitors and AT_1 receptor antagonists. Great expectations were created by the introduction of the inhibitors, currently renin. In fact, the RAS regulates many more functions than previously thought [78].

1.2 The "Classical RAS"

Many researches on the renin-angiotensin system paved the way for a better understanding of its physiology and pathophysiology. In early 1970, the main components of the "classical" RAS assets were identified and there was no convincing evidence for important functions in the systemic water balance and blood pressure homeostasis. At that time, however, there was widespread skepticism about the role that the RAS could play in cardiovascular diseases. Only after the discovery of ACE inhibitors acting by oral administration, among which the first one was captopril®, the fundamental importance of the RAS in cardiovascular homeostasis was understood. The introduction of Losartan, the first Angiotensin II type 1 receptor active and effective antagonist, further strengthened this concept [78].

1.3 Formation of Angiotensin ligands

The vision of the relatively simple "classical RAS" circulating with the angiotensinogen (AGT) synthesized by the liver, renin by the kidneys and the main effector peptide, Angiotensin II (Ang II) generated by the ACE in the vascular system, was completed with the cloning of AT_1 and AT_2 receptors [78, 121]. The Angiotensinogen protein serves as a precursor of Angiotensin peptides and is primarily formed and constitutively secreted by liver cells into the circulation. Following its release, plasma active renin [170] is an aspartil-protease [224] which hydrolizes Angiotensinogen [170] at the amino terminal to form the decapeptide, Ang I [9]. The circulating renin and its precursor, pro-renin, are released mainly by juxtaglomerular cells located in the afferent glomerular arterioles. However, other tissues secrete pro-renin into the bloodstream, and can be converted into pro-renin renin by limited proteolysis [170]. Ang I is a substrate for both the Angiotensin converting enzyme (ACE) (dipeptidyl carboxypeptidase) , a zinc metal-protease, the serine protease chymotrypsin-like chimase [58], that hydrolyzes the carboxy terminal dipeptide His-Leu away to form Ang II (*Fig.4*).

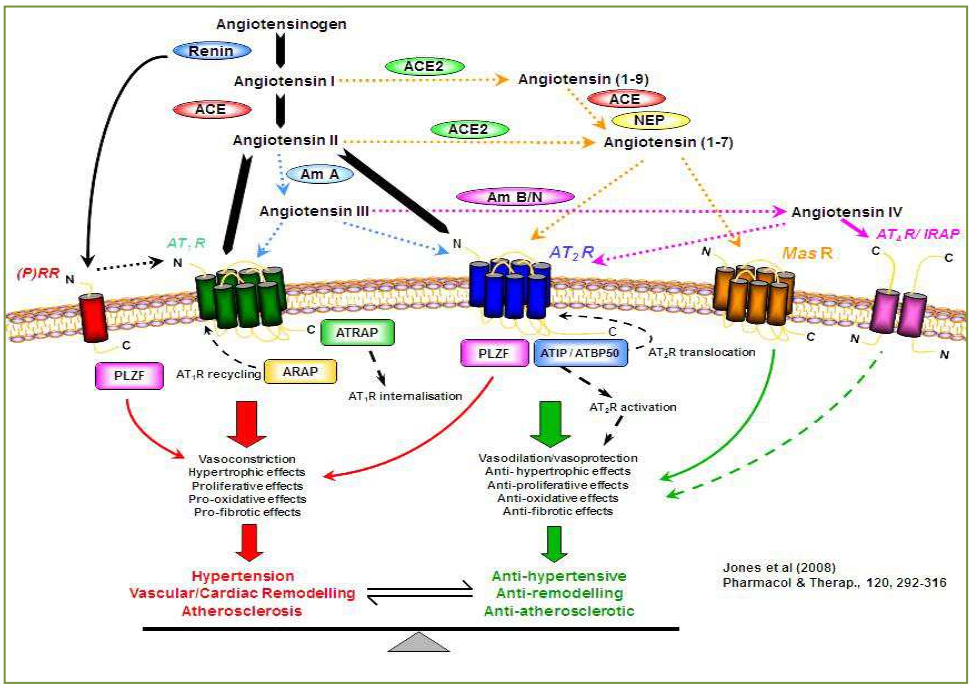


Figure 4 - Schematic synthesis of renin angiotensin system (Ras)-

The circulating Angiotensinogen is plentiful (500-600 nM), 1,000 times higher than Ang I (50-150 pM) and Ang II (50-100 pM) ones. Therefore, renin activity determines the rate of formation of Angiotensins from the plasma enormous concentration of Angiotensinogen reservoir. That is, even a small change in plasma renin activity can make a big difference in circulating levels of Ang I and Ang II [170]. Ang III is converted by glutamyl aminopeptidase A (AP-A) that cleaves the N-terminal Asp residue of Ang II. The alanyl aminopeptidase N membrane (AP-N) divides the terminal N-Arg of Ang III to form Ang IV .Although Ang III, Ang IV and Ang 1-7 have biological activities, their plasma levels are much lower than those of Ang II [170].

Ang IV may be further converted in Ang (3-7) by carboxypeptidase P (Carb-P) and propyl oligopeptidase (PO) that break the link Pro-Phe. Endopeptidases such as chymotrypsin are able to separate the residue of Val, Tyr, and together with the dipeptidyl carboxypeptidase that cuts the link His-Pro reduces the Ang IV and Ang (3-7) to inactive peptide fragments and individual amino acids (4,40). Ang II can also be converted to Ang (1-7) by cutting the Carb-P Phe, the mono-peptidase ACE2 recently discovered or ICA, which splits the dipeptide Phe-His from Ang (1-9), and can be further converted to Ang (2-7) by AP-A acting on the bond Arg-Asp. Over time it was revealed that in addition to the "RAS assets", there is a local "tissue RAS" in several organs and studied tissues. In fact, it has also been reported to generate intracellular Ang II [127]. In addition, Ang I is inactive , while both the heptapeptide Angiotensin 2-8 (Ang III) and the hexapeptide 3-8 (Ang IV) have been shown to be biologically active. In particular, the heptapeptide angiotensin 1-7 (Ang 1-7) seems to play an important role in counteracting many of the actions of Ang II . There are three recognized subtypes of receptors for Angiontensin, two structurally similar, and a third that is different. Subtypes AT_1 and AT_2 are coupled to protein G. Instead, since the subtype AT_4 is a much larger protein and is insensitive to the guanine nucleotide, it is not associated with a G protein. The actions of Ang II and III are mediated only by receptors AT_1 and AT_2 [78]. New findings indicate that Ang IV binds with low affinity AT_1 and AT_2 receptors, but with high affinity and specificity with AT_4 receptor. Nowadays there is a dispute about the identity of insulin-regulated amino peptidase of AT_4 (IRAP) or growth factor receptor c-Met. A specific binding site for Ang (1-7) was reported but not fully defined and even more surprisingly a receptor for renin / pro-renin. For all these properties, today our vision gives a quite articulate overview of the RAS [253].

1.4 Angiotensin II

Angiotensin II exerts its action through AT_1 and AT_2 receptors, which in principle but not always, exert opposing functions. AT_1 receptors mediate actions with potentially damaging consequences, if not adequately balanced. AT_2 receptors mediate protective action, whose clinical relevance has not yet been clearly established [78]. Most actions are attributed to hypertensive Ang II AT_1 receptor [121]. AT_1 receptor is widely expressed in various tissues of kidney and mediate cardiovascular diseases. On the other hand, AT_2 receptor is highly expressed in the mesenchyme during fetal life and decreases dramatically after birth [170]; in fact, in adulthood, an increase in receptor activity (up-regulation) may occur in most tissues after injury [224]. Angiotensin II is the main regulator of water balance and salt-hemodynamics, but also cell growth and cardiovascular remodeling. Thus, AT_1 receptors mediate vasoconstriction process, thirst and the release of vasopressin and aldosterone, growth and cell migration [78], the cycle of reproductive hormones and sexual behavior. Ang II causes the generation of **oxidant radicals** through the same receptor and is involved in the processes of inflammation, including atherosclerosis vascular aging [78]. The prevention of the hypertensive action and trophic Ang II, has proven to be among the most successful strategies for the treatment of hypertension and congestive heart failure. To this end, ACE inhibitors were administered to reduce plasma levels of Ang II and, at a later stage, non-peptide antagonists were developed to selectively block the AT_1 receptor [224]. AT_2 receptors are used for the vasodilatation, the release of nitric oxide (NO) and normally the vascular growth inhibition [78] as well as apoptosis and neuronal differentiation [224].

1.5 AT_1 and AT_2 Receptors

AT_1 receptor is a G protein-coupled receptor with the signaling pathway of phospholipase C and calcium. Thus, the ligand binds to the Angiotensin AT_1 receptor and induces a conformational change of the receptor protein that activates G proteins, and in turn, mediates signal transduction. This involves several transduction mechanisms associated with the plasma membrane such as phospholipase C, A_2 and D-adenylate cyclase, more channels as T and L-type voltage-sensitive calcium [53] ones. This receptor (designated AT_1A) is also coupled to intracellular signaling cascades that regulate gene transcription and expression of proteins involved in cell proliferation and growth in many target tissues. Cloning in

expression vectors was adopted to isolate the c-DNA encoding this receptor protein, and it was discovered that it has 7 trans-membrane domains and consists of 359 amino acids with a mass of about 41KDa. Subsequently, a second subtype of AT_1 was discovered and designated as AT_1B and has been cloned in rats, mice and humans. This subtype, has for about 92-95% homology with the amino acid sequence, is AT_1A subtype. Of these two isoforms, AT_1A subtype appears to be responsible for the classic features associated with the brain angiotensin system [241]. **AT_2 receptor** has been cloned and sequenced using an expression library made from fetal rat. In common with the AT_1 subtype, this receptor protein has seven trans-membrane domains characteristic of G protein-coupled receptors, however, it shows only 32-34% identity with the amino acid sequence of rat AT_1 receptor . The AT_2 receptor protein includes a sequence of 363 amino acids (40kDa) that about 99% is common in rats and mice, and 72% homologous in humans [53]. Although this AT_2 receptor has structural features in common with members of the family of receptors with 7 trans-membrane domains, it shows little or no functional similarities with this group, although it seems to be coupled to the G protein.

1.6 New functions mediated by the AT_1 and AT_2 receptors

Infusion of Angiotensin II, due to the decrease in plasma adiponectin, an insulin sensitizer, apparently acts via AT_1 receptors in rats [78]. The deletion of adiponectin may represent a mechanism by which Ang II causes impaired glucose tolerance [188]. Other metabolic actions of Ang II include the pro-inflammatory modulation of increased insulin secretion , apoptosis of β cells, reduced gluconeogenesis, hepatic glucose production [78] and increased plasma triglycerides [186]. Angiotensin type 2 receptor may also mediate neurotrophic effects in the central nervous system. In addition, the AT_2 receptor up-regulated in the ischemic brain, may exert protection against such damage [132]. The authors speculate that the latter effect is mediated by the AT_2 receptor and may in part explain the superior protection against stroke in patients treated with Losartan compared to those treated with atenolol (β-blocker) [78]. Another explanation may be that Losartan lowers blood pressure (BP) more effectively in the middle of atenolol [205]. In rat kidney, Ang III but not Ang II, has been reported to induce natriuresis, mediated by the AT_2 receptor [176]. The increased natriuresis blocks the aminopeptidase N, an enzyme that metabolizes Ang III to Ang IV. The authors speculated that amino-peptidase blockers were developed for the treatment of

diseases characterized by fluid retention and sodium, such as hypertension and heart failure. In theory, these inhibitors may also exert beneficial actions by reduced tissue levels of Ang IV. Interestingly, the renal interstitial fluid contains higher concentrations of about 1000 times the plasma Ang II and Ang III ones [78]. The effects of AT_2 receptor stimulation are slightly controversial [33]. Therefore, the beneficial effects include the effects of bradykinin-NO vasodilatory, natriuretic and antifibrotic effects. Potentially harmful effects are apoptosis, signal transduction of nuclear factor-kappa B (NF-kB) and the induction of chemokine [78]. Although many experimental results suggest positive actions of the AT_2 stimulation [33], the results at the clinical level are lacking. Although treatment with AT1 receptor blockers (ARBs) substantially increase plasma levels of Ang II and probably cause increased stimulation of AT_2 receptors [78].

1.7 Alternative generation of Ang II

Angiotensin II can be generated enzymatically by chimase in some pathological conditions. Chimase is stored in a macromolecular complex with heparin proteoglycan in secretory granules of mast cells. In order to become enzymatically active, complexed chimase must be released from the granules of mast cells following example to vascular damage. Therefore, the chimase is inactive in normal vascular tissue and can produce Ang II only in atherosclerotic or damaged arterial walls. Note that inhibitors of endogenous serine proteases present in the interstitial fluid, are potent inhibitors of chimase [78]. Inhibitors of chimase prevent injuries and later vein graft and arterial dilation in dogs, while ACE inhibitors are ineffective [160]. However, the effects of inhibitors may depend on the effects of other compounds, such as decreased synthesis by transforming growth factor-β (TGF-β), the stabilization of the granules of mast cells and not on decreasing the formation of Ang II. In addition, ARBs, block the actions of Ang II regardless of the enzyme that creates this hormone, but are not superior to ACE inhibitors, as demonstrated in several clinical trials [78]. Although the results of the experimental animal with inhibitors are attractive [160], the possible importance of the synthesis of Ang II through chimase is unclear and the inhibitors that are safe and useful for human experimentation were not yet developed .

1.8 Angiotensin III (2-8 heptapetide)

Angiotensin III is well known, since 1970, to cause vasoconstriction and release of aldosterone. It is generated from Ang II between amino-peptidase A. Ang III exerts actions similar to those of Ang II, AT_1 and AT_2 receptors pathways. While Ang II is considered the main effector of RAS, Ang III may be equally and even more important in some actions mediated by the AT_1 receptor, such as the release of vasopressin [78].The systemic infusion of Ang II or Ang III in conscious dogs at the same concentration, produced equipotent effects on Blood pressure (BP), on the secretion of aldosterone, sodium excretion and plasma renin activity, indeed all inhibited by Candesartan (AT_1 agonist) [106]. This study showed that Ang II plays a dominant role as an effector of the classical "RAS assets" .

1.9 Angiotensin IV (3-8 hexapeptide)

Angiotensin IV can be generated by aminopeptidase M by Ang III. This biologically active peptide, has attracted a growing interest in the discovery and cloning of the insulin receptor-regulated aminopeptidase (IRAP), a binding site and likely Ang IV receptor (AT_4) [78]. Many studies demonstrate that Ang IV produces functions in the central nervous system suggesting its role as a neuropeptide or neuromodulator. The initial interest of Ang IV arises from its ability to improve memory recall and learning [224] in rodents (role models). It even pours out the memory deficits caused by scopolamine, mecamylamine, alcohol abuse, or destruction of ischemia on hippocampal piercing. *In vitro* and *in vivo* improve long-term potentiation (LTP) . In addition to its role in favour of memory, Ang IV (depending on the dose) [224] attenuates seizures induced by pentilentetrazolium (PTZ) and pilocarpine [246] and plays an antiepileptic effect. It is also skilled in influencing dopaminergic neurotransmission in the striatum and in protecting brain ischemia caused by neurological damage. It also promotes cell survival in the hippocampus [224]. Ang IV increase of renal blood flow is blocked by the antagonist of AT_4, divalinal - Ang IV, and is not affected by AT_1 receptor antagonists. This effect is also accompanied by an increase in urinary excretion of sodium. In other conflicting findings, Ang IV decreases renal blood flow and increases blood pressure. These effects are AT_1 dependent and in fact are repressed by the antagonists of this receptor [246]. This peptide also regulates cell growth of cardiac fibroblasts, endothelial cells and vascular smooth muscle cells. It seems that the Ang IV is involved in vascular and inflammatory response so that it could play a role in cardiovascular pathophysiology [78]. Ang IV participates in various

stages of atherogenesis including the initial and later stages of plaque such as the plaque rupture and thrombus formation [195]. In atherosclerotic lesions, the average Ang IV binding increases in the neointima and in the layer of re-endothelialization cells, suggesting a role for the hexapeptide in vascular remodelling in response to damage [224]. In particular, the angiotensin 3-8 hexapeptide decreases superoxide interaction with AT_2, and therefore, plays an important role in reducing oxidative stress, it increases the expression of nitric oxide synthase by endothelial post-transcriptional modulation, it increases the release of NO, which in turn stimulates soluble guanylciclase by accumulation of cGMP (effector molecule). It also gives increments of the endothelium-dependent vasodilation. The phenomena just mentioned, derived from the interaction with the AT_2 receptor and AT_4. Consequently, the Ang IV evokes an obvious vasoprotective effect [253]. Ang IV up-regulates several pro-inflammatory factors. In vascular smooth muscle cells, Ang IV increases the production of chemotactic protein for the monocytes-1 (MCP-1), the main chemokine used in the recruitment of monocytes and the expression of ICAM-1 (intracellular adhesion molecule) responsible for attachment and transmigration of circulating cells into the damaged tissue. Ang IV also increased cytokines such as interleukin-6 and tumor necrosis factor-α and stimulates the production of the prothrombotic factor plasminogen activator inhibitor-1 (PAI-1) and could thus participate in the perpetuation of the inflammatory response and thrombus formation. Finally, Ang IV activates NF-kB, a transcription factor implicated in inflammatory diseases and central immune responses. The mechanism by which Ang IV produces these effects in vascular smooth muscle cells is unclear but the involvement of the AT_1 receptor was excluded, since the same effects occur in Knock-out mice for the AT_1 receptor [224].

Radical Oxidative Stress and cellular mechanisms of regulation

2.1 Oxidative stress and Endothelial Dysfunction

The endothelium is essential for the maintenance of vascular homeostasis. Central to this role is the production of endothelium-derived nitric oxide (EDNO), synthesized by endothelial isoform of nitric oxide synthase (eNOS). Endothelial dysfunction represents impaired EDNO bioactivity and it is an important early event in the development of many vascular diseases as hypertension, diabetes and atherosclerosis. Considerable evidence supports a casual role for enhanced production of reactive oxygen species (ROS) by vascular cells. ROS directly inactivate EDNO act as cell – signaling molecules and promote protein dysfunction, events that contribute to the initiation and developments of endothelial dysfunction [1]. **Oxidative stress** is defined as an imbalance between oxidants and antioxidants that favors the former, which is a prominent feature of vascular disease states [30,91,213,232] in the role of oxidative stress in vascular disease. Sies, who introduced the term in the title of the book he edited in 1985, *Oxidative Stress*, defined it in 1991 as *a disturbance in the prooxidant-antioxidant balance in favor of the former, leading to potential damage.* Such damage is often called **oxidative damage**. Original interest in the role of oxidative stress in vascular disease stems from the "oxidative – modification hypothesis" of atherogenesis [212]. This hypothesis proposed that a critical initiating event of atherogenesis is the oxidation of low-density lipoprotein (LDL). In recent times, it has become increasingly apparent that oxidative reactions, in addition to oxidative modification of lipoproteins, are important for the initiation and progression of vascular disease. The generation of reactive oxygen species (ROS) by cells of the blood vessel wall is currently an area of intense research focus. Several enzymatic sources appear responsible for the elevated production of ROS noted in vascular disease: NADPH oxidase, xanthine oxidase, mithocondria and "uncoupled" endothelial

nitric oxide synthase (eNOS) [232].This increased flux of ROS modulates the function and phenotype of vascular endothelial and smooth muscle cells that together contribute to vascular dysfunction.

REDOX SIGNALING

The generation of ROS is inevitable for aerobic organisms. Originally, ROS were considered to be random and destructive agents produced as unnecessary byproducts of aerobic metabolism or components of the innate immune defense against microorganisms. The initiation of ROS formation requires electrons (e^-), frequently generated by the mitochondrial electron-transport chain or by NADPH oxidase, that mediate the univalent reduction of molecular oxygen (O_2) to form $O_2^{\bullet -}$, which is subject to spontaneous or enzyme-catalyzed dismutation into hydrogen peroxide (H_2O_2) (*Reaction 1*).

$$\mathbf{O_2 + 2\ e^- \longrightarrow O_2^{\bullet -} + O_2^{\bullet -} + 2\ H^+ \longrightarrow H_2O_2 + O_2} \quad \mathbf{[1]}$$

Although the term ROS refers to all reactive species derived from the one-electron reduction of O_2, it is important to note that different ROS exhibit distinct chemical properties that have important implications from their biologic actions.Instead, ROS appear to act as signaling molecules that stimulate protease release [70]. These important findings add to the rapidly growing body of evidence that reduction and oxidation (redox) reactions control cell-signaling pathways that govern an array of physiological processes, including cell growth, transformation, senescence and apoptosis. Endogenous ROS and RNS with signaling potential are frequently synthesized by NADPH oxidase, mitochondria, or NOS and act by reversibly altering the function of a variety of target proteins, including phosphatases, kinases, small GTPases, transcription factors, ion channels, structural proteins and metabolic enzyme (Table 1). Exposure of cells to select ligands (*e.g.*, growth factors, cytokines, Angiotensin II) or hemodynamic force (*i.e.*, shear stress or cycling strain) induces cellular H_2O_2 production that stimulates cell signaling.

Molecular target of redox signaling: essential to characterize the importance and aim of action of redox cell signaling is the identification of the cellular targets that

sense and transduce the redox signal; another important aspect underlying redox signaling is the reversible, covalent modification of specific cysteine thiol residues that reside within active and allosteric sites of proteins, which results in alteration of protein function. Under physiological conditions, selected proteins contain cysteine thiolate anions that are obtained through the formation of salt bridges with surrounding positively charged amino acid residues and exhibit pK_a values ~ 5.0.Thus, an important aspect underlying the signaling properties of H_2O_2 is its ability to target proteins containing oxidation-susceptible deprotonated cysteines, critical for protein function [209]. Several classes of signaling proteins capable of conveying a broad spectrum of cellular signals and that contain conserved redox-sensitive cysteines have been identified. These latter include phosphatases, protein kinases, transcription factors, ion channels, structural proteins and metabolic and antioxidant enzyme.

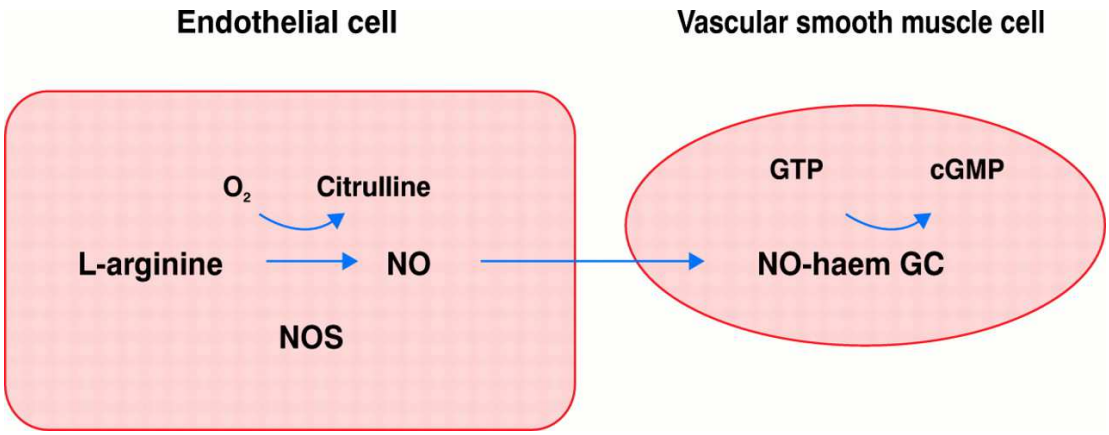
2.2 Vascular homeostasis and endothelium –derived nitric oxide

The endothelium is critical for the maintenance of cardiovascular homeostasis. Important for this function is EDNO, synthesized by eNOS, which controls vascular tone [129], arterial pressure [187], and inhibits platelet aggregation [10] and smooth muscular cells growth [79]. With respect to vascular tone, the extent to which EDNO mediates local vasodilatation differs among blood vessels of differing sizes [i. e., although under physiological conditions, EDNO is a primary vasorelaxant in mid- to large-sized conduit and resistance arteries, its contribution to vasodilatation in smaller vascular beds (e.g., coronary arterioles) is comparatively less [125]. The importance of EDNO in vascular homeostasis is highlighted by observations with eNOS gene knockout mice that exhibit spontaneous hypertension, defective vascular remodeling plus enhanced vascular thrombosis and leukocyte interactions [75,102,128]. Moreover, deficiency of eNOS results in accelerated arterial lesion formation in atherosclerosis-prone mice [118]. However, EDNO is not always beneficial. A recent study showed that eNOS gene deficiency protects mice against anaphylactic shock, highlighting that eNOS derived NO is a principal vasodilator in the acute hypotensive response [36]. Studies in endothelial cells indicate that cellular Snitrosylation is concentrated at the primary site of active eNOS [85,105].This support that, in addition to mediating changes in cGMP, EDNO bioactivity also relates to S-nitrosylation and activation of plasma membrane transientreceptorpotential ion channels (TRP) and potentiated Ca^{2+}

entry that may be important for sustained eNOS activity [256]. It is important to note that aberrant Snitrosylation has pathogenic potential. For example, mice with targeted deficiency of S-nitrosoglutathione reductase, an enzyme responsible for removal of S-nitrosoglutathione reductase and of S-nitrosothiols, is hypotensive and exhibits significant increases in cellular S-nitrosylation, tissue damage and mortality when subjected to a model of endotoxic shock.

2.3 Regulation of endothelium – derived nitric oxide production

In endothelium, EDNO is produced constitutively by eNOS, a 135 kDa protein that structurally consists of a C-terminal reductase domain (which binds NADPH, flavin adenine dinucleotide and flavin mononucleotide) linked by a regulatory calmodulin-binding site to an N-terminal oxygenase domain (*Fig.5*). The oxygenase domain contains the heme prosthetic group and binds (6R)-5,6,7,8-tetrahydrobiopterin (BH_4), molecular oxygen and the substrate L-arginine. The catalytic action of eNOS involves the flavin-mediated transport of electron from NADPH bound at the C-terminal reductase domain to the N-terminal heme, where molecular O_2 is reduced and incorporated into the guanidino group of L-arginine, resulting in the production of hydroxy-L-arginine. In a second catalytic step, the guanidino nitrogen of hydroxy-L-arginine is further oxidized to form L-citrulline, and NO is liberated.



(Figure 5)- Synthesis and bioactivity of EDNO –

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Active eNOS requires formation of a homodimer through a linkage between the N-terminal oxygenase domains. Dimer formation appears to be influenced by the binding of calmodulin, heme, L-arginine and BH_4. Stabilization of the eNOS homodimer and binding of BH_4 also appear to depend on the integrity of a zincthiolate cluster formed by a Zn^{2+} ion coordinated in a tetrahedral conformation with pairs of CysXXXXCys motifs at the N-terminal oxygenase domain and dimer interface [40,95,183].Vascular eNOS is subject to strict controls, that under physiological conditions ensure appropriate tonic regulation of NO output. In the endothelium, eNOS is subject to transcriptional control [134] and various forms of co- and post-transitional regulation that include substrate and cofactor availability, reversible enzyme acylation and subcellular localization, protein-protein interactions (*Table 1*) , phosphorylation and S-nitrosylation [58, 59,84,156]. Further criterria for the optimal activation of eNOS are the binding of heat shock protein 90 (Hsp90) , coordinated changes in the phosphorylation status of critical Ser and Thr residues (361) and Snitrosylation status of cysteines within the zinc-thiolate cluster, a modification that inhibits enzyme activity [58,69]. For instance, transient activation of eNOS by VEGF requires the rapid and reversible denitrosylation that is inversely related to Akt-dependent eNOS phosphorilation at Ser-1166 (human) or ser-1179 (bovine).

2.4 Oxidative stress, superoxide anion radical and endothelial dysfunction

Endothelial Dysfunction is an imprecise term that refers to a loss of a normal homeostatic function of endothelium (e.g. vasodilatation, inhibition of platelet aggregation and leukocyte adhesion) that is often apparent early in the course of vascular disease such as atherosclerosis, diabetes and hypertension. One important manifestation of endothelium dysfunction is a decrease in EDNO bioactivity. Considerable evidence indicates that endothelial dysfunction is caused by enhanced vascular ***Oxidative Stress*** [30,232]. As such, currently significant interest exists in identifying the responsible oxidative reactions. It exists different ways in which different oxidative reactions can affect EDNO bioactivity and hence endothelial function. Several initial findings suggested that impaired EDNO bioactivity is not the result of attenuated production of NO but is partly due, to the inactivation of EDNO before reaching its molecular target. Thus, diseased blood vessels from hypercholesterolemic rabbit produce substantial amount of nitrogen oxides (NO oxidation products) despite the impairment of NO-dependent vascular relaxation [149]. Also eNOS protein levels are paradoxically increased

rather than reduced in diseased blood vessels [100,126]. Subsequent studies have established that oxidative inactivation of EDNO frequently involves $O_2^{\cdot -}$.Hypertension, Hypercholesterolemia, diabetes and atherosclerosis are all associated with an increase in the steady-state flux of $O_2^{\cdot -}$ in the vascular wall (64), and $O_2^{\cdot -}$ reacts with NO at near diffusion controlled rates ($k=1.9 \times 10^{10}$ M/sec) to produce the potent oxidant peroxynitrite ($ONOO^-$) ***reaction 1*** [117]

$$O_2^{\bullet -} + {}^{\bullet}NO \longrightarrow ONOO^-$$

This constant rate for peroxynitrite formation exceeds both NO autoxidation ($k = 2\times10^6$ M/sec) and spontaneous $O_2^{\cdot -}$ dismutation ($k = 5\times10^5$ M/sec). Thus, peroxynitrite formation is kinetically favored over other NO reactions and likely occurs whenever both NO and $O_2^{\cdot -}$ are present. Because peroxynitrite inefficiently activates the soluble isoform of guanylate cyclase [228], its formation effectively decreases EDNO bioactivity in the vascular wall. Numerous studies indicate that direct inactivation of NO by $O_2^{\cdot -}$ represents a key event responsible for impaired EDNO bioactivity.

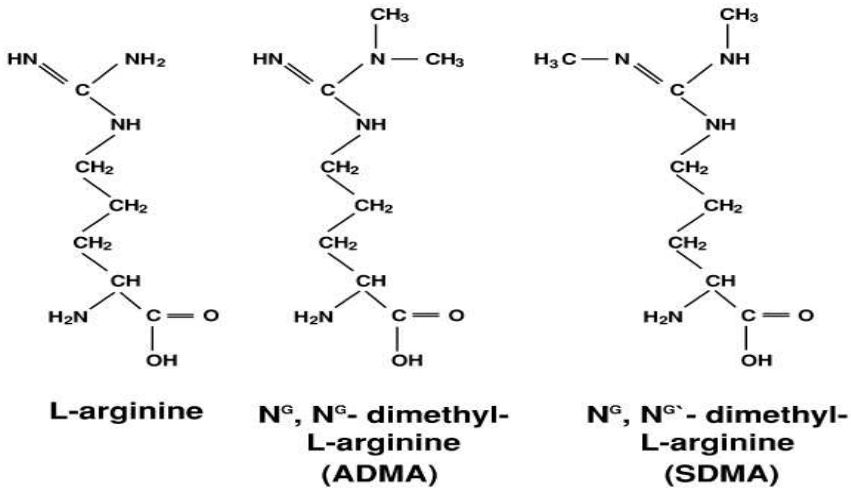
2.5 Interaction of oxidative pathways in endothelial cells

The preceding data indicate that increased endothelial $O_2^{\cdot -}$ can be derived from various enzymatic sources. As such, it is likely that the precise sources of pathologic superoxide anion production may depend on the nature of the vascular disease, the type of blood vessel in question and the stage of disease progression. Increasing evidence supports cross communication between different oxidative enzymes in endothelial cells, where an initial minor increase in ROS production from one enzyme can initiate a feed-forward, self-propagating pathway of amplified ROS production for endothelial dysfunction. NADPH oxidase derived H_2O_2 signals for the enhanced expression and activity of xanthine oxidase in endothelial cells exposed to oscillatory shear stress [145,146] or Angiotensin II [124]. NADPH oxidase-initiated reactions can also underscore enhanced mitochondrial ROS production [56] or the reduction of cellular BH_4 levels in endothelial cells, resulting in an amplification of ROS production by uncoupled eNOS [78,123]. Also H_2O_2 or mitochondria-derived ROS activate NADPH oxidase in endothelial cells, which may lead to a self-perpetuating cycle of enzyme activation [135,199]. Recent studies, however, support that overstimulation of local endothelial ROS production from NADPH oxidase acts to initiate and expand the uncontrolled production of pathogenic ROS from dysfunctional cellular sources, including uncoupled eNOS in hypertensive mice [123] or xanthine oxidase in CAD patients [124], leading to endothelial dysfunction.

Asymmetric Dimethyl Arginine and Oxidative Stress in Endothelial Dysfunction

3.1.Asymmetric Dimethyl arginine (ADMA): structure and functionality

Asymmetric dimethyl arginine (ADMA) represents a natural amino-acid that circulating in plasma, releases in urine and is present in tissue and cells [240,261] . ADMA revealed many interests because inhibits nitrix oxide synthase (NOS) [20] so it induces many biological effects, in particular matter on cardiovascular system (*Fig.6*). Recently, different studies suggest that plasmatic concentration of ADMA are an indicator of endothelial dysfunctional risk and cardiovascular disease [23, 240,261].

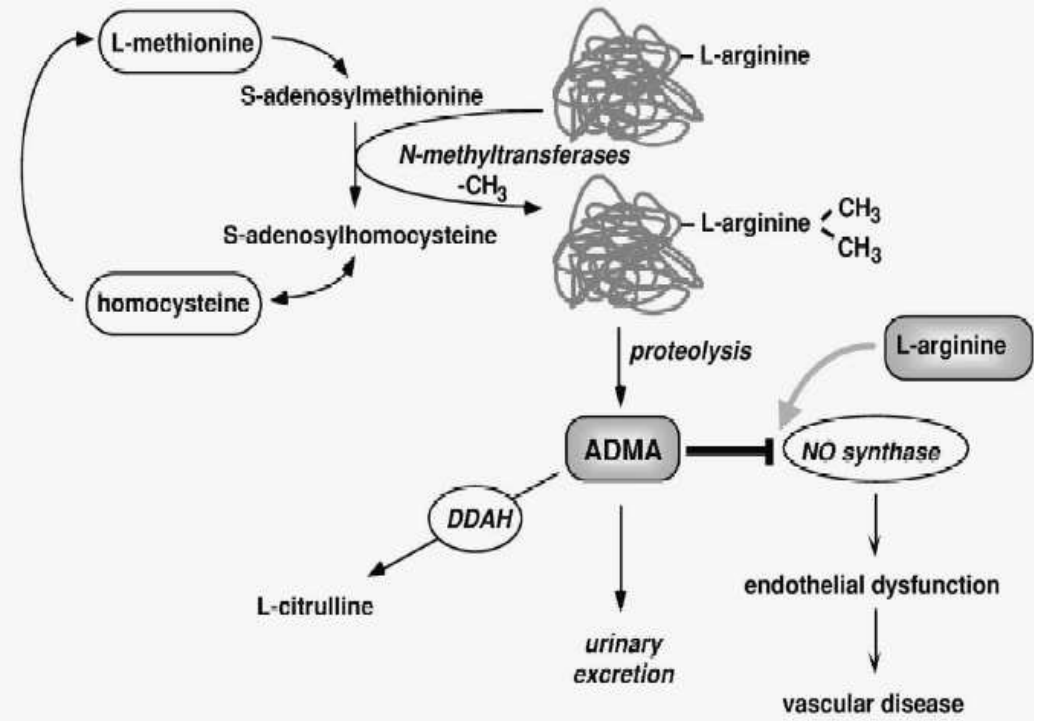


(Figure 6) -. Chemical Structure of L-arginine, of asymmetric dimethyl arginine (ADMA) and symmetric dimethyl arginine (SDMA) - L-arginine *is a natural substrate of NO synthase,* ***ADMA*** *is a competitor inhibitor of NO synthas, on the contrary* ***SDMA*** *is biologically inactive .*

3.2 Biosyntesis ofADMA

ADMA is synthetized when arginin residues of proteins are methylated by action of methyltransferasy arginin protein (PRMTs) [44,143]. The methylation of arginin is a post translational modification that addition 1 or 2 methyl groups to guanydinic nytrogens of arginine. It exists two types of PRMTs:

- type 1 catalysed the ADMA formation ;
- type 2 methyl both guanidinic atoms and generated formation of symmetric dimethyl arginine
- symmetric (SDMA; *Fig.7*).



(Figure 7) - Scheme of biochemical process refers to ADMA -

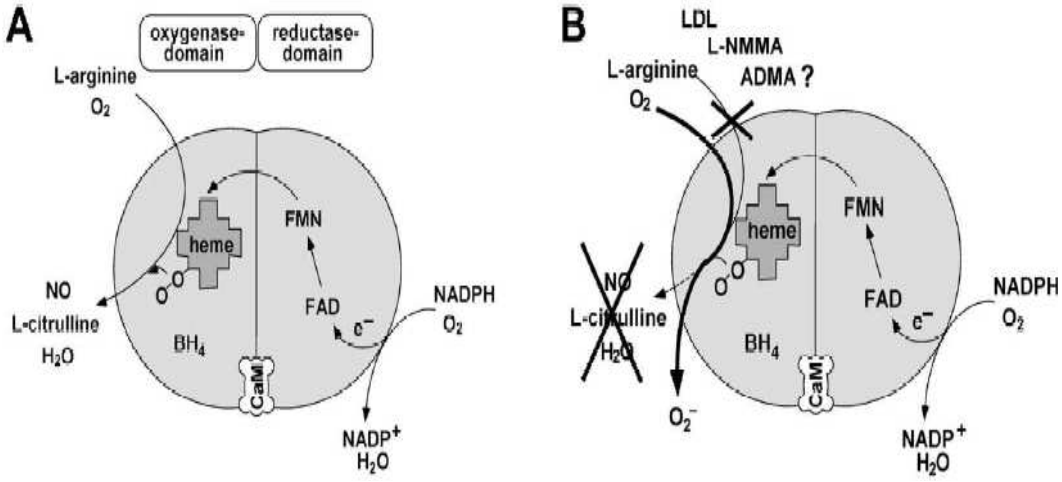
After proteolitic degradation of proteins, ADMA free is present in cytoplasmic compartment It can be present circulating in plasma portino in human blood. ADMA acts as inhibitors of NO synthetase to compete with L-arginine, it causes the endothelial dysfunction mechanisms and, successful atherosclerosis. ADMA is eliminated by organism between urinary excretion or alternatively, between enzyme metabolism of dimethylaminohidrolasy dimethylarginin (DDAH) in citrullin and dimethylammina. Both types of PRMT, of which different isoforms

exist, can be also mono-methylated, to produce the formation of N^G-Monomethyl-L-Arginin (L-NMMA) [3,143]. When the proteins are hydrolysed, free methylarginine appear in the cytosol. Asymmetric methylated arginin (ADMA and L-NMMA) are inhibitors of the NOS, on the contrary SMA is not.The role of methylation of arginine residues of the protein is not currently clear, but this process is implicated in the regulation of RNA in transcriptional regulation, DNA repair, protein localization, protein-protein interactions, signal transduction, and recycling or desensitization of cellular receptors. However, methylarginine free only appears after the degradation of the proteins in the cytoplasm; for now, no direct synthesis of ADMA from free arginine has been identified. Therefore, the amount of ADMA generated within a cell depends on the degree of methylation of arginine in proteins and the speed of protein turnover. Due to the complex process that guides the generation of free ADMA, it is unclear whether the generation of ADMA appears fairly constant, if the activity of PRMT is altered or if the rate of protein turnover is the most important. Recently, studies with relatively non-specific PRMT inhibitors and low power have suggested that the activities of PRMT 24 to 48 hours helps to generate free ADMA and that there is a relationship between the expression levels of PRMT and ADMA production of free radicals [26]. Further studies are needed to identify more clearly the likely changes in the rate of production of ADMA. In the cardiovascular system, PRMTs type 1 are expressed in heart, smooth muscle cells and endothelial cells. The pattern of expression has not been documented in detail, but the PRMT -1, 3, 4, and 6 (all PRMTs type 1) are all expressed in vascular cells. Interestingly, the expression of PRMT-1 in endothelial cells increased in response to shear stress and this effect can be blocked by the suppression of IKB kinase. This altered expression of PRMT-1 was associated with corresponding changes in the release of ADMA, suggesting that the generation of ADMA in the vessel wall should be partly regulated by the alteration of the expression of PRMT. The expression of PRMT1 is also the increased expression of low density lipoprotein (LDL), also in this case the effect seems to be correlated with impaired production of ADMA [26].

3.3 Molecular Target of ADMA

The ADMA inhibits all three isoforms of NOS and is approximately equipotent with L-NMMA . In addition to blocking the formation of NO, L-NMMA NOS and its releases can lead to the generation of superoxide, and it is likely that ADMA does the same. The 'decoupling' of 'catalytic activity of NOS was observed in experimental conditions in which

NOS is able to catalyze two-electron oxidation of L-arginine to nitric oxide, both in the presence of a suboptimal concentration of L-arginine [116], and when the enzyme is deprived of essential cofactors such as tetrahydrobiopterin. Under these conditions, the optimal flow of electrons within the two catalytic domains of NOS is impaired and molecular oxygen becomes the sole electron acceptor, making the inside of a generator NOS superoxide radical ('detachment NOS activity')(*Fig.8*)

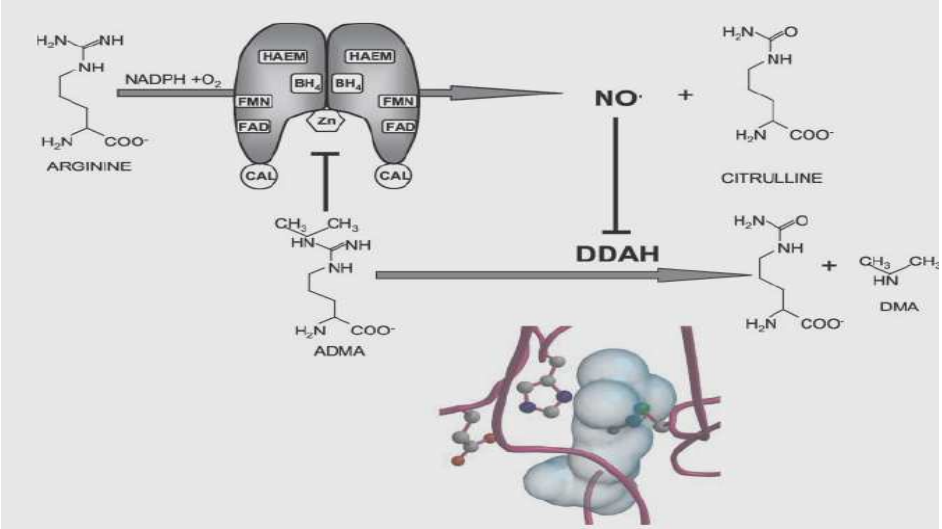


(Figure 8). *- NO synthase is a dimer formed by an oxygenase and a reductase domain. Under the ideal catalytic conditions (i.e. in the presence of optimal concentrations of the substrate L-arginine and co-factors (tetrahydrobiopterin (BH4), Calmodulin (CaM), NADPH, FMN and FAD), there is a transfer of electrons (e-) from molecular oxygen, along the cascade of co-factors to L-arginine (a). L-Citrulline and nO are the products of this reaction. in suboptimal conditions (e.g. relative deficiency of L-arginine in the presence of cholesterol LDL or L-NMMA), the catalytic mechanism is 'off '(B). The normal flow of electrons is disturbed, the molecular oxygen acts as an electron acceptor, with consequent formation of superoxide radical (O2-). The relative deficit L-arginine may also be the result of high levels of ADMA, the competitive NOS inhibitor. Further tests are required to test whether a high concentration of ADMA also leads to 'decoupling' of NO synthase, as suggested in this figure-*

Although NOS is the most obvious target of ADMA, we do not know yet whether it is the only one. At very high concentrations, ADMA and SDMA may compete with arginine for transport through the transport system y^+ and millimolar concentrations, guanidine compounds can inhibit the Na^+/ K^+ ATPase [142]. The effects of the system Y^+ methyl arginine probably only have at too high concentrations to be physiologically relevant. One potential target of 'ADMA may be the arginine-glycine amidino transferase, an enzyme with a structure similar to that of dimethylarginine dimethylaminohydrolase (DDAHs) that metabolizes ADMA [157]. Many microbes produce ADMA and express the enzymes needed to metabolize not expressing NOS, suggesting a possible mechanism of action of additional unknown 'ADMA.

3.4 Degradation of ADMA: DDAHs

The methylarginine is partly eliminated by renal excretion. However, although the SDMA (the methylarginine that does not inhibit NOS) is almost completely eliminated by the kidney, ADMA and L-NMMA are extensively metabolized [2,163]. In fact, in some species, more than 90% of the product, ADMA is metabolized rather than excreted [2,163,164,165]. The main metabolic pathway is mediated by L-citrulline and dimethylamine, a reaction catalyzed by DDAH [116]. The reaction probably involves a nucleophilic attack on the guanidine portion of the ADMA molecule by a cysteine in an active state seal in the tertiary structure of the enzyme [13]. This cysteine is undoubtedly involved because its replacement with a serine residue inactivates the enzyme [13,23]. In addition, it is susceptible to oxidation and the regulation of nitric oxide . It is not yet clear whether oxidative stress produces irreversible inhibition of the DDAH activity, but certainly the nitrosation involves reversibility. Thus, the production of NO, high capacity (for example, the expression of iNOS) inhibits the nitrous and the DDAH activity. This provides a potentially important homeostatic mechanism that increases or changes in the redox of NO where the 'NO is generated can stop a further generation of NO (*Fig.9*). The DDAHs seem to be predominantly cytosolic enzyme with no obvious subcellular localization. No clear call for co-factor has been identified, even if the activity is inhibited by some divalent cations [21] .



(Figure 9) - The metabolism of 'methylated arginine may be directly regulated by nitric oxide - *NOS catalyzes the conversion of L-arginine and molecular oxygen into citrulline and NO. The NOS enzymes are catalytically active as homodimers and require the binding of cofactors (flavin adenine dinucleotide [FAD], flavin mononucleotide [FMN], heme, and tetrahydrobiopterin [BH4]) and calmodulin for optimal activity. Each of the NOS dimer coordinates a single zinc atom. NO inhibits DDAH directly via S-nitrosation of the active site cysteine residue. Inhibition of DDAH results from the accumulation of ADMA and inhibition of NOS. Insert: structural model of the active site containing DDAH ADMA. E shows the triad catalytic residues of glutamine, histidine, and cysteine. The S-nitrosation of sulfur atom (green) active site cysteine residue and may disable this even occasionally hindering the binding of ADMA .*

The DDAHs are highly conserved during their evolution [235] and have been identified in primitive organisms including bacteria. In higher organisms, including humans, 2 isoforms of DDAH, encoded by genes located on chromosome 1 (DDAH-1) and 6 (DDAH-2) have been identified [235,130]. The two isoforms have distinct tissue distributions, but apparently similar activities [130]. Studies of literature described an overlap between the expression of DDAH-1 with neuronal NOS and DDAH-2 with endothelial NOS (eNOS), but it is clear that both are widely expressed and DDAHs are not limited to cells or tissues that express NOS [26]. Both isoforms have been identified within the cardiovascular system, although more DDAH-2 is expressed, at least at the level of mRNA. Analysis of the promoter region of DDAH-2 suggests that this gene has many of the characteristics of a gene known as "house-

keeping". However, even if that gene is highly expressed in many cells under basal conditions, it is also transcriptionally regulated and its expression levels can vary considerably. For example, treatment of endothelial cells with retinoic acid increases the expression of DDAH-2 2-fold. The fragmentation of the promoter of DDAH-2 has highlighted a number of control elements; a region that seems to promote basal gene expression was also identified, at least in some types of endothelial cells. Overall, available data suggest that the genes are widely expressed , DDAHs still regulated at the transcriptional level.

3.5 ADMA and Disease

Several clinical and scientific studies have also shown that elevated plasma levels of ADMA are found in diseases such as chronic renal failure, hypertension, diabetes,hyperhomocysteneimia and they are looking to identify this molecule as a possible marker for these conditions. Increased levels of ADMA has been identified in animal models of type 1 and 2 diabetes and in patients with type 2 diabetes . Although the mechanisms leading to this increase in relation to diabetes are not yet known. In patients with heart failure ADMA levels are high and this may reduce the contraction of heart rhythm [43], but it remains unclear whether there is a causal relationship with cardiac and endothelial function. The cardiac output arrest as well as that under strain can be attenuated following infusion of ADMA suggesting a role similar to the pathophysiology of heart failure [2]. ADMA levels are low during normal gestation, but increase in women with pre-eclampsia [43]. In these patients, it has been demonstrated a clear correlation between the levels of ADMA in early pregnancy and endothelial dysfunction [130], suggesting that ADMA may represent a new marker for early diagnosis of this anomaly of the gestational cycle. The precise mechanism by which it produces an increased concentrations of ADMA has not been set yet but it is thought that changes in renal function may explain the differences observed between normal pregnancy and those of patients with pre-eclampsia.

Antioxidant networks and redox signaling

1.1 New definition of Antioxidant

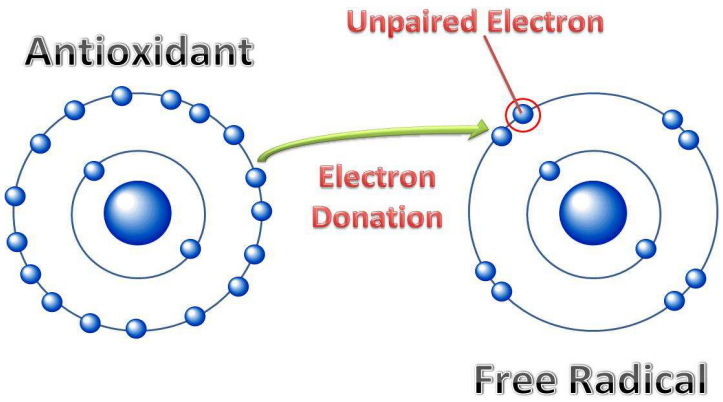
Antioxidant, like oxidative damage and oxidative stress, is a widely used term but surprisingly difficult to define clearly. Food technologists use antioxidants to inhibit lipid peroxidation and consequent rancidity in foods, so they often define an antioxidant as a good inhibitor of lipid peroxidation. Polymer scientists use 'antioxidants' to control process of polymerization in the manufacture of rubber, plastics and paint and for the protection of clear plastics against ultraviolet light. Combustion is a free-radical process: the oil industry makes extensive use of antioxidant and has a knowledge of free-radical mechanism in the design of better fuels and lubricating oils[162]. All these scientists have their own views on what a good antioxidant should be. When radical species (reactive oxygen and nitrogen species, RS) are generate *in vivo* and *in vitro*, many antioxidants come into play. Their relative importance depends upon:

- which RS is generated;
- how it is generated;
- where it is generated and
- what target of damage is measured.

So an ***antioxidants*** can be defined as *˝any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate˝*. This definition emphasizes the importance of the damage target studied and the source of RS used when antioxidant action is examined.

1.2 Cellular defense systems

The body fights against the presence of free radicals (ROS and RNS) by the presence of an anti-free radical system defined by its own natural antioxidant (*Fig.10*);



(Figure 10)- Molecular Actions of an Antioxidant-

Antioxidants are chemicals (molecules, ions, radicals) or physical agents that slow or prevent the oxidation of other substances. Oxidation is a chemical reaction of electron transfer from a molecular substance that gives electrons to a substance which acquires electrons. Oxidation reactions can produce free radicals, responsible for the initiation of a chain reaction that damages cells. The radical intermediates are generally buffered by antioxidant systems. Therefore, it follows that antioxidants are chemically defined as reducing agents - thiols or polyphenols - as catalyzing oxidation-reduction reactions [237]. A paradox in cellular metabolism is represented by the fact that the majority of complex organisms requires a certain amount of oxygen for its existence. However, oxygen is only compound under certain conditions, rather than a reactive molecule that can produce, if not well balanced, some of the cellular system damages leading to an increased production of reactive oxygen species [162]. Consequently, organisms contain a complex network of metabolites and enzymes that work synergistically to prevent oxidative damage to cellular components such as DNA, proteins and

lipids. In general, antioxidant systems either prevent the formation of oxidizing species or are removed before they can damage vital components of cells (*Fig.11*).

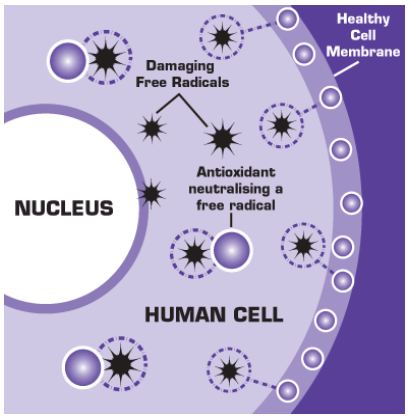


Figure 11 – Antioxidant effects at intracellular levels in Human cells-

The antioxidant system includes mechanisms to antioxidant enzyme activity and not enzymatic activity. There is the involvement of the enzyme superoxide dismutase (SOD), catalase and glutathione peroxidase. Substances do not include the enzyme α-tocopherol (vitamin E), ascorbic acid (Vitamin C), carotenoids, polyphenols, anthocyanins, etc.. [238]. The action of an antioxidant may depend on the correct functionality of the other members of the antioxidant system. The amount of protection provided by an antioxidant depends on:

I. its concentration;

II. its reactivity towards reactive oxygen species in question;

III. state of the antioxidants with which it interacts .

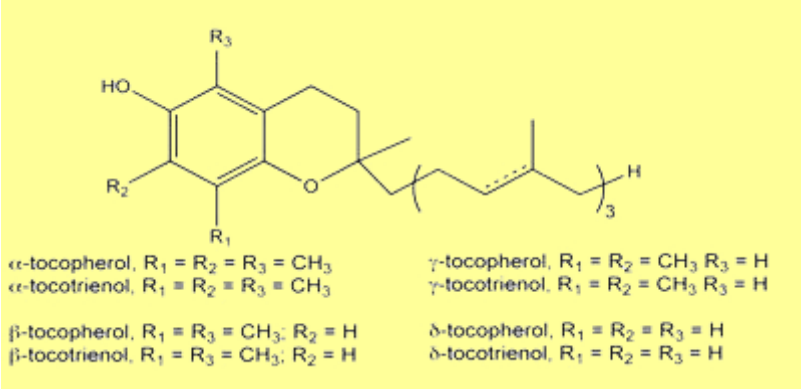
Non – enzymatic antioxidant activity

The non-enzymatic antioxidant function includes:

a) some compounds that contribute to antioxidant defense mechanisms, able to chelate transition metals thus preventing the catalytic effect they provide in the production of free

radicals in the cell. Particularly important is the ability to sequester iron, functional proteins used as its transport in the human transferrin and ferritin ;

b) non-protein fat-soluble antioxidants such as vitamin E and coenzyme Q10. Vitamin E has a pool of eight related tocopherols and tocotrienols among them, which are fat-soluble antioxidant vitamins (*Fig.12*). The α-tocopherol is the most studied isoform for its high bioavailability in the body. This protective effect modulates damages on cell membranes, removal of free radicals by intermediates and disruption in the chain propagation mechanism of the reaction during the process of lipid peroxidation [97].



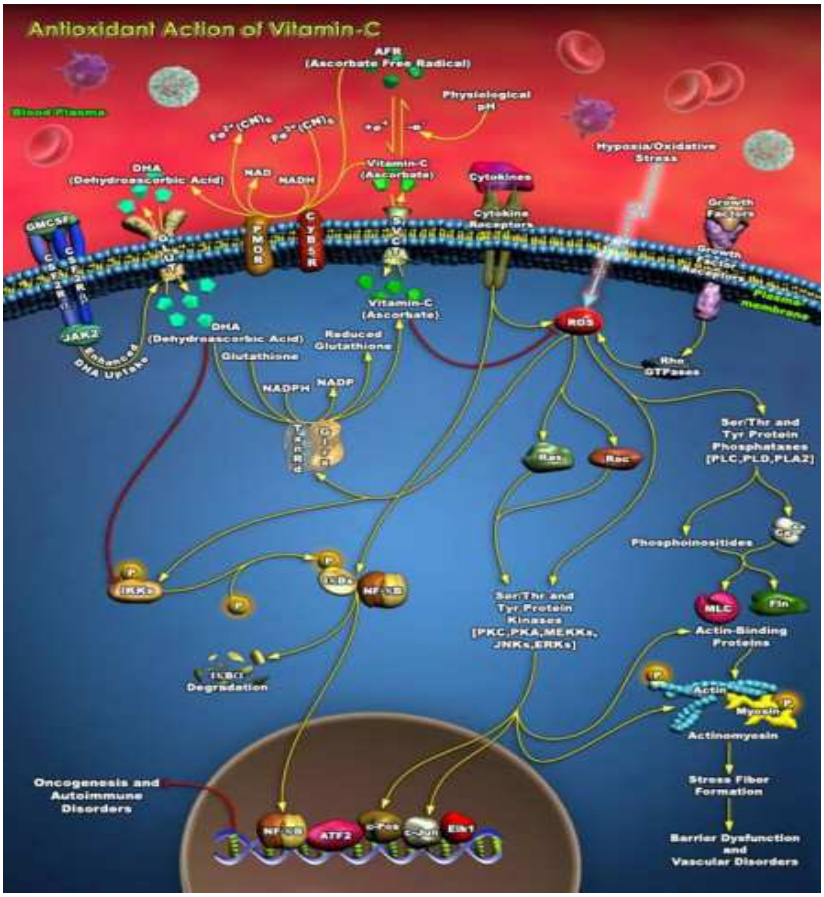
(Figure 12) – Different molecular conformation of Tocopherol-

Coenzyme Q_{10} (Ubiquinol) form a family of compounds synthesized by the body able to accept and donate electrons. Coenzyme Q10, is also present in three oxidants states.

c) water-soluble antioxidant compounds, including endogenous, such as uric acid and bilirubin; food-borne factors, such as **vitamin C** and a variety of flavonoids and polyphenols. Uric acid is the main product of purine catabolism. In vitro anti-radical role has only recently been discovered . It is assumed that uric acid has beneficial effects as it acts preserving the oxidation of vitamin C in plasma, complexing metal ions such as iron and copper. The flavonoids and polyphenols, widely present in the plant kingdom (fruit, wine and other), including compounds for which in vitro have reported beneficial effects on vascular function. But these are poorly absorbed from the intestine but metabolized in the body and have a modest antioxidant activity. [137]. Ascorbic acid (vitamin C) is a monosaccharide antioxidant found in both animals and plants (*Fig.13*).

In humans, it cannot be synthesized and it must be introduced for the diet .

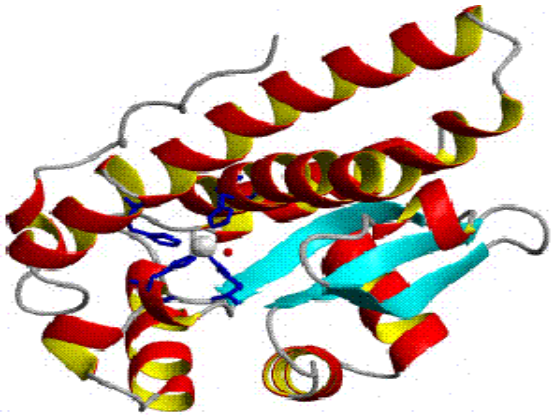
It neutralizes reactive oxygen species such as hydrogen peroxide [169] and it is a substrate for the enzyme ascorbate peroxidase [204].



(Figure 13) – Example of Antioxidant effects of Vitamin C-

Antioxidant defense enzyme activities

As with the chemical antioxidants, cells are protected against oxidative stress, thanks to an interactive network of antioxidant enzyme [237]. Superoxide anion derived from the process of oxidative phosphorylation is at first converted to hydrogen peroxide and subsequently reduced to water. This detoxification pathway is the result of the synergic action of more enzymes, with superoxide dismutase (SOD) that catalyze the first stage, and then catalase and peroxidase which remove any hydrogen peroxide in the second stage. As with antioxidant metabolites, the contributions of these enzymes are difficult to separate from each other [101]. The superoxide dismutase (SOD) represents a class of metalloproteins containing metal ions as cofactors (copper, zinc, manganese or iron).



(Figure 14)- Molecular structure of SOD enzyme-

The ***SOD enzymes*** (*Fig.14*)are present in almost all aerobic cells and in extracellular fluids. In the blood vessels, there are also three isoforms of SOD dismutation that is in the O2-H2O2;

- SOD copper / zinc (SOD1) is localized mainly in the cytosol and nucleus and Dismas superoxide derived from eNOS, increasing the half-life of nitric oxide. It represents 50-80% of the overall activity of SOD;

- the manganese-SOD (SOD2) is present in the mitochondria and Dismas superoxide derived from the respiratory chain. It represents 10% of the total activity of SOD [147];

- The third form of SOD in extracellular fluids (SOD3), which contains copper and zinc in its active sites [162], extracellular superoxide Dismas thus protecting nitric oxide from degradation. It accounts for 20-50% of the overall activity of superoxide dismutase. Vessels in the SOD3 is mainly synthesized by smooth muscle cells. Endothelial cells do not produce this type of SOD, but bind to heparan sulfate proteoglycans on the surface and can possibly be internalized only like this [75].

The ***Catalase enzyme*** is unusual because, although hydrogen peroxide is its only substrate, followed by a ping-pong mechanism: its cofactor is oxidized by one molecule of hydrogen peroxide and then regenerated by transferring the oxygen bound to a second molecule of substrate [99].

The ***Glutathione peroxidase***, are hemoproteins containing one heme oxidized (Fe^{3+}) and selenium applicants for their activities. There are at least four types and they act together with glutathione reductase . The glutathione peroxidase 1 is the most abundant and is an efficient scavenger of hydrogen peroxide, while glutathione peroxidase 4 is very active with lipid hydroperoxides. Surprisingly, glutathione peroxidase 1 is not essential, because mice lacking this enzyme have a normal course of life [101], but have a hypersensitivity to oxidative stress induced.

Antioxidants can eliminate the damaging effects that free radicals have on cells [237] and people who eat fruits and vegetables, rich in polyphenols and anthocyanins have a lower risk of cancer, cardiovascular diseases and neurological diseases [210].

Flavonoids:
Epigallocatechin-gallate
and its effects "*in vitro*" and "*in vivo*"

A growing interest exists in dietary flavonoid agents as protective agents against cardiovascular disease . Polyphenols are enriched in various dietary sources, including oils, fruits, nuts, vegetables, wine and tea. Mechanistically, flavonoid may improve endothelial function via their action as water – soluble antioxidants. However, the beneficial actions of polyphenol consumption on endothelial function in patients do not correspond to a reduction in plasma concentrations of F2-isoprostanes, marker of systemic lipid peroxidation, or 8-hydroxydeoxyguanosine, a marker of DNA oxidation, suggesting that actions other than antioxidant activity are involved . Instead, the beneficial activities of black tea flavonoid or polyphenols may relate to their ability to immediately activate eNOS. Activation of eNOS involved changes in the eNOS phosphorylation status at Ser-1177 and Thr-495 [185]. Signaling studies identified p38 MAPK as an upstream component of PI3-kinase/Akt-mediated eNOS activation in response to polyphenols .Interestingly, polyphenol-mediated induction of MAPK in endothelial cells occurs via activation of the estrogen receptor. Recent studies indicate that the green tea polyphenol, epigallocatechin gallate, or red wine polyphenols induce endothelium-dependent vasodilatation of isolated arteries *via* redox-sensitive PI3-kinase/Akt-dependent phosphorylation of eNOS. Also, the ability of resveratrol to alleviate cardiac dysfunction in diabetic rats related to the polyphenols' ability to stimulate Akt-dependent activation of eNOS. In addition to immediate effects, long term exposure of endothelial cells to red wine polyphenols enhances eNOS expression .Phenols on red wine were found to inhibit LDL oxidation *in vitro* and it was suggested that they could exert cardioprotective effects by limiting LDL oxidation *in vivo*. This was proposed as an explanation of the lower incidence of heart disease in certain areas of France (**French paradox**) despite the high prevalence there of risk factors, such as smoking and high fat intake.

However, alcohol alone has cardioprotective effects (at

moderate intakes) and the debate continues as to whether wine has any additional benefit due to its flavonoid content; an epidemiological study in the Netherlands (**Zutphen study**) suggested an inverse correlation between the incidence of coronary heart disease and stroke and the dietary intake of flavonoids which originated mainly from tea, fruits e.g. apples) and vegetables (e.g. onions) in the population examined. Since then, many studies have used biomarkers of oxidative damage, for example F2-Ips and 8OhdG, to see if flanoids exert antioxidant effects *in vivo* in humans. Some positive effects using these biomarkers have been found , for example with several studies involving green tea, soya, chocolate, dealcoholized wine, garlic extracts and grape juice and with some involving individual phenols, for example hydroxytyrosol. Since plasma levels of unconjugated flavonoids rarely exceed 1 μM and the metabolites tend to have lower antioxidant activity because of the blocking of –OH groups by methylation, sulphation or glucuronidation, it seems difficult to image a powerful antioxidant effect *in vivo*. However, high levels of phenols exist in the stomach, small intestine and colon and could conceivably exert antioxidant, and other protective effects there.

2.1 The Epigallocatechin-gallate: polyphenol antioxidants.

Polyphenols are a diverse group of natural substances, particularly known for their positive action on human health. In nature, the polyphenols are produced by secondary metabolism of plants, where in relation to the chemical diversity that characterizes them cover different roles: defense against herbivores (impart unpleasant taste) and pathogens (phytoalexins), mechanical support (lignins) and barrier against microbial invasion, attracting pollinators and dispersal of the fruit (anthocyanins), inhibitors of plant growth in competition [7]. From the chemical point of view, the polyphenols are molecules composed of multiple cycles condensed phenolic (organic compounds that possess one or more hydroxyl groups - OH - bound to an aromatic ring). Depending on their structure, they can be schematically divided into three different classes .

The **SIMPLE PHENOL**: in this class phenolic acids, coumarins and benzoic acids. They can result in condensation polymers such as lignin and are widely distributed in foods and beverages (e.g. caffeic acid in coffee).

TANNINS: belong to that class of two categories: hydrolysable tannins and condensed tannins. The former are also known as proanthocyanidins because of strong acids by hydrolysis originate anthocyanidins. The latter are heterogeneous polymers containing phenolic acids (eg. Gallic acid) and simple sugars.

FLAVONOIDS: the largest class of natural phenols have all the reference structure as the 2-phenyl benzopirone (or flavonone). Structural changes in the aromatic rings allow you to subdivide the flavonoids into several families: flavonols, flavones, isoflavones, anthocyanins and other .

In nature exist many kinds of polyphenols, which are characterized by a marked structural and functional variability. In principle, however, the activity of polyphenols can be summarized as follows:

- ***Antioxidant*** polyphenols protect cells from damage caused by free radicals, which are developed with the normal cellular metabolism and because of stressful events such as radiation, smoke, pollutants, UV rays, physical and emotional stress, chemical additives, bacterial and viral attacks, etc.

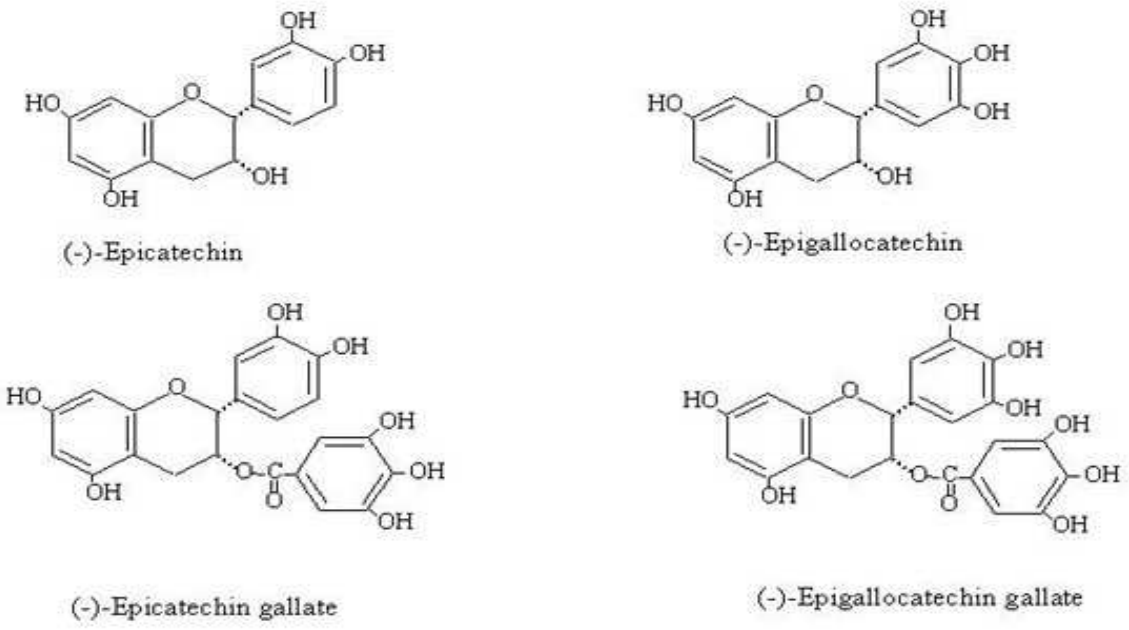
- ***Anticarcinogenic*** generally shows an impact on the initiation step of cancer development, protecting the cells against the direct attack by carcinogens or altering the mechanism of activation in vitro. Experimental evidence explaining the existence of a correlation between the consumption of fresh vegetables and reduced incidence of certain cancers (skin, lung, stomach, esophagus, duodenum, pancreas, liver, breast and colon).

- ***Antiatherogenic*** lipid oxidation of LDL in particular is the cause of development of atherosclerosis and its related diseases (stroke, thrombosis and cardiovascular disease in general). The main mechanism is the reduction in platelet clotting blood and LDL, other mechanisms are the inhibition of the oxidation of lipoproteins, the elimination of the radicals and the modulation of eicosanoid metabolism.

- ***Anti-inflammatory***: the process of inhibiting the arachidonic acid cascade. Antibacterial and antiviral [7].

Catechins are the major tea flavonoid with powerful antioxidant, which actually belong to the flavonoid family. Catechins are antioxidant substances extremely important to counteract the action of free radicals by acting specifically on both hydroxyl radicals and peroxyl. In addition, they neutralize environmental radicals and promote the regeneration of other antioxidants such as vitamin E .The catechins as metabolites of plant origin, such as Flavon 3-oils, are abundantly present in many plant species but also in tea or as a derivative of green tea Camellia sinensis in chocolate, fruits, vegetables, wine, etc.., they are present in trace amounts . These epimers (stereoisomers), where (-) Epicatechin and (+) catechins are the optical isomers common in nature . Catechin was extracted for the first time from a compound catechu, which takes its name. If you heat the catechin past its degradation, we get the formation of pyrocatechol .

(*Fig.15*).



(Figure 15) – Different Conformation of Catechins-

The epigallocatechin gallate (ECG) is a type of catechin, the most abundant in tea. Antioxidants can help in protecting the skin from damage induced by ultraviolet radiation, and by the formation of tumors . Tea contains a number of bioactive substances, represented by its catechins, of which the 'epigallocatechin gallate (ECG) is the most abundant. Tea catechins and polyphenols are effective scavengers of reactive oxygen species (ROS) *in vitro* and may function indirectly as antioxidants mediating their effects on transcription factors and enzyme activities. Catechins are rapidly metabolized extensively and this underlines their functional importance *in vivo*. Through studies in humans, it has been shown that catechin has a positive effect on metabolic syndromes such as obesity, type II diabetes mellitus and cardiovascular disease [231]. In animal models, there was a progressive reduction of atherosclerotic plaques. In the last few years, the beneficial effects of green tea have been increasingly recognized for health. Among the advantages offered in recent scientific studies, there is the maintenance of vascular endothelial function properly in the presence of flavonoids and especially of catechins. Epigallocatechin-gallatete have antioxidant activity against free radicals, metal ions activate, inhibit transcription factors and induce redox reactions of the antioxidant enzymes. They are capable of inhibiting the key enzymes involved in lipid biosynthesis, thereby reducing the intestinal absorption and improving the quantity present in the bloodstream. These substances also have the ability to regulate vascular tone by activating endothelial nitric oxide (eNOS), which prevents the process of vascular inflammation that plays a critical role in the progression of atherosclerotic lesions. The anti-inflammatory activity may be due to inactivation of the accession of leukocytes to the endothelium and subsequent migration through the inhibition of nuclear transcription factor NF-kb [98].

Therapeutic Opportunities

for treatment of Endothelial Dysfunction

3.1 Antioxidants

Enhanced vascular oxidative stress represents a primary mechanism underlying endothelial dysfunction. Therefore, it is not surprising that many researchers have attempt to normalize EDNO synthesis and bioactivity in the setting of vascular disease through the administration of various antioxidant strategies, including small-molecular-weight antioxidants or inhibitors of ROS-producing enzymes. Other strategies include treatment with eNOS substrate and cofactors to enhance EDNO production, novel drugs that target heme free sGC or improving endothelial progenitor cell (EPC) number/function to aid in the repair of dysfunctional endothelium. It is also becoming increasingly apparent that pharmacological agents known to improve clinical outcome in cardiovascular disease patients may act, in part, by ameliorating endothelial dysfunction. Next, we discuss in more details the mode through which specific strategies act to improve endothelial function that may provide therapeutic potentials.

3.2 Polyphenols

Mechanistically, polyphenols may improve endothelial function *via* their action as water-soluble antioxidants. However, the beneficial actions of polyphenol consumption on endothelial function in patients do not correspond to a reduction in plasma concentrations of F2-isoprostanes, markers of systemic lipid peroxidation, or 8-hydroxydeoxyguanosine, a marker of DNA oxidation, suggesting that actions other than antioxidant activity are involved.[185,198,39,210] Instead, the beneficial activities of black tea polyphenols may relate to their ability to immediately activate eNOS. Activation of eNOS involved changes in the eNOS phosphorylation status at Ser-1177 and Thr-495. Signalling studies identified p38 MAPK as an upstream component of PI3-kinase / Akt-mediated eNOS activation in response to polyphenols .Interestingly, polyphenol-mediated induction of MAPK in endothelial cells occurs via activation of the estrogen receptor.Recent studies indicate that the green tea polyphenols induce endothelium –dependent vasodilation of isolated arteries via redox-sensitive PI3-kinase /Akt-dependent phosphorilation of eNOS. Also, the ability of epigallocatechin gallate or of resveratrol to alleviate cardiac dysfunction in diabetic rats related to the polyphenols ability to stimulate Aktdependent activation of eNOS. In addition to immediate effects, long-term exposure of endothelial cells to red wine polyphenols enhances eNOS expression . The extent to which these mechanisms are important for the beneficial activities of dietary polyphenols also in patients with vascular complications is currently unknown.

***Part II*: "In vitro" study on Angiotensin IV receptor and Angiotensin IV involved in stress pathway**

Experimental protocols

Angiotensin IV and

Renin Angiotensin-System

“ *in vitro*„ models

1.1 Angiotensin IV (3-8 esapeptide)

Angiotensin IV can be generated by aminopeptidase M in Renin Angiotensin System by degradation of Angiotensin III. This biologically active peptide, has attracted a growing interest in the discovery and cloning of the insulin receptor-regulated aminopeptidase (IRAP), a binding site of Ang IV and likely receptor (AT_4) [246]. Many studies indicate that Ang IV have different functions in the central nervous system, suggesting its role as a neuropeptide or neuromodulator. The initial interest about Ang IV stems from its ability to improve the recall of memory and learning [215] in rodents (behavioral models). Even reverses memory deficits caused by scopolamine, mecamylamine, alcohol abuse or destruction of ischemia on hippocampal piercing. In vitro and in vivo improve the long-term potentiation (LTP) [241]. In addition to its title in favor of memory, Ang IV (depending on dose) [215], reduces seizures induced by pentilentetrazolo (PTZ) and pilocarpine [241] and plays an antiepileptic effect. It is also skilled in influencing dopaminergic neurotransmission in the striatum and in protecting brain ischemia caused by neurological damage. It also promotes cell survival in the hippocampus [215]. Ang IV increases renal blood flow, which is blocked by the antagonist of AT_4, divalinal-Ang IV, and is not affected by AT_1 receptor antagonists. This effect is also accompanied by an increase in urinary excretion of sodium. In other discordant results, Ang IV decreases renal blood flow and increases blood pressure. These effects are dependent on AT_1 and in fact they are repressed by the antagonists of this receptor [241]. This peptide also regulates cell growth of cardiac fibroblasts, endothelial cells and vascular smooth muscle cells. It seems that Ang IV is involved in vascular and inflammatory response could play a

role in cardiovascular pathophysiology [246]. Ang IV participates in various stages of atherogenesis including the initial formation of plaque and subsequent stages such as plaque rupture and thrombus formation [192]. In atherosclerotic lesions, the bond increases in the average of Ang IV, in the neointima and in the layer of cells re-endothelialization, suggesting a role for this in hexapeptide vascular remodeling in response to damage [215]. In particular, angiotensin 3-8 hexapeptide interacting with AT2 decreases superoxide, therefore plays an important role in reducing oxidative stress. It increases the expression of nitric oxide synthase by endothelial post-transcriptional modulation, so it increases the release of NO which in turn stimulates soluble guanilciclasi by the accumulation of cGMP (effector molecule). Then, it increments the endothelium-dependent vasodilation. The phenomena just mentioned, derived from the interaction with the AT_4 receptor of Ang IV and AT_2 receptor.Consequently, the Ang IV evokes a clear vasoprotective effect [246]. Ang IV up-regulates several pro-inflammatory factors. In vascular smooth muscle cells, Ang IV increases the production of the protein chemotactic for monocytes-1 (MCP-1), the main chemokine used in the recruitment of monocytes and the expression of ICAM-1 (intracellular adhesion molecule) responsible attack and transmigration of circulating cells into the damaged tissue. Ang IV also increases cytokines such as interleukin-6 and tumor necrosis factor-α and stimulates the production of the prothrombotic factor plasminogen activator inhibitor-1 (PAI-1) and could thus participate in the perpetuation of the inflammatory response and thrombus formation. Finally, Ang IV activates NF-kB, a transcription factor centrally involved in inflammatory and immune responses. The mechanism by which Ang IV produces these effects in vascular smooth muscle cells is unclear but the involvement of the AT1 receptor was excluded since the same effects occur in mice Knock-out for the AT1 receptor [215].

1.2 The AT_4 receptor subtype.

Before 1988, the shorter Angiotensins IV were considered biologically inactive and therefore of little physiological importance. This hypothesis was based on two events [253]:

1. Ang IV revealed very low affinity for the AT_1 and AT_2 sites [158] because [^{125} I] Ang IV does not displace agonists or antagonists of the AT_1 receptor and AT_2, as Sar1, Ile8-Ang II, Losartan and PD123319 [241] ;

2. Ang IV and shorter fragments, are considerably less powerful of Ang II and Ang III in triggering the classic angiotensin-dependent functions [253].

Two discoveries have changed this hypothesis. First, ***Braszko et al. (1988)*** have reported that Ang IV facilitates the acquisition of a conditioned avoidance response in rats. Secondly, a distinct binding site for Ang IV has been identified and subsequently classified as subtype AT_4 because it binds ligands known for the AT_1 and AT_2 sites. This subtype was originally isolated bovine adrenal membranes used [253] by ***Swanson et al***. ***(1992)*** . It was established that the [^{125}I] Ang IV binds reversibly all AT_4 receptor, saturating it with high affinity. The AT_4 site was found [253] in various tissues of different species of mammals (uterus, lung, aorta , heart, adrenal gland, bladder, colon, kidney, prostate, brain, spinal cord) [253]. Incubation of [^{125}I] Ang IV in brain slices of guinea pig, reveals that a predominant distribution of binding sites is localized in the hippocampus, thalamic nuclei, cerebral cortex, cerebellum and brain stem. A similar distribution was observed by radioreceptor autoradiography, in both sections of the brain of *Macaca fascicularis* and man [241]. As a small peptide is able to activate the AT_4 site, and as the vast majority of the small size of peptides receptors are coupled to G proteins, it was logical to expect that the AT_4 receptor could be a receptor protein associated with serpentine G. However, it is not the case, because the link to that site was found to be insensitive to guanine nucleotides. In addition, the AT_4 receptor subunit has a molecular weight of 100kDa in the range determined by polyacrylamide gel electrophoresis in the presence of SDS. A molecular weight equivalent for this receptor has been reported in other bovine tissues including heart, thymus, kidney, bladder, aorta, and hippocampus. In addition, ***Bernier et al. (1995;1998)*** discovered a m

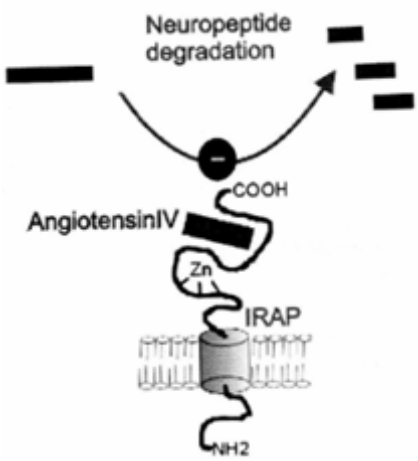
olecular weight similar to the AT_4 receptor binding subunit in bovine aortic endothelial cells. The absence of coupling to G proteins is also supported by the observation that GTPγS (non-hydrolysable analogue) fails to alter the link [^{125}I] Ang IV in the heart of rabbit, pig brain and rat vascular smooth muscle. ***Dúlin et al.*** have shown that GTPγS inhibits the binding of the receptor in renal cell AT_4 possum. Thus, to date there is little evidence linking the AT_4 receptor to G proteins; however, as already occurred for the AT2 receptor, for a definitive conclusion, we must await the sequencing of this receptor protein [253].

2.1 The AT_4 receptor is the receptor c-Met or insulin-regulated aminopeptidase (IRAP)?

A potentially important turning point in the understanding of the AT_4 receptor system was the identification of the receptor as insulin-regulated aminopeptidase, an enzyme associated with the membrane that co-distributes with the transporter GLUT4. This assumption based initial sequence homology between a tryptic fragment derived from human brain AT_4 receptor and human IRAP, and masses almost identical protein subunits of the AT4 receptor involved in binding and IRAP. The subsequent expression of IRAP in HEK293T cells resulted in a binding site similar to the native AT_4 receptor with affinity for Ang IV . IRAP has different names such as gp60, vp165, P-LAP, or cystine aminopeptidase, which is an integral membrane protein type II homologous to A aminopeptidase (AP-A), aminopeptidasi N (AP-N), and Zn^{2+} -aminopeptidase to other employees who belong to the large family of aminopeptidase gluzincine. It is a protein of 916 amino acid residues, which includes an intracellular region (109A) followed by a hydrophobic transmembrane segment (22aa α-helix) and an extracellular domain of 785aa containing the HEXXH-XE motif highly conserved zinc-binding and this is the reason why GXMEN exopeptidase are central to the business. IRAP is skilled in the amino acid N-terminals, which cleave several bioactive peptides *in vitro* including oxytocin, vasopressin, lys-bradykinin, met-enkephalin, dynorphin 1-8, neurochinina A, neuromedina B, somatostatin and cholecystokinin-8. Lately, ***Wallis et al. (2007)*** have shown that vasopressin injected intravenously is cleaved from IRAP *in vivo* [241].

1.4 Evaluation of IRAP as the AT_4 receptor

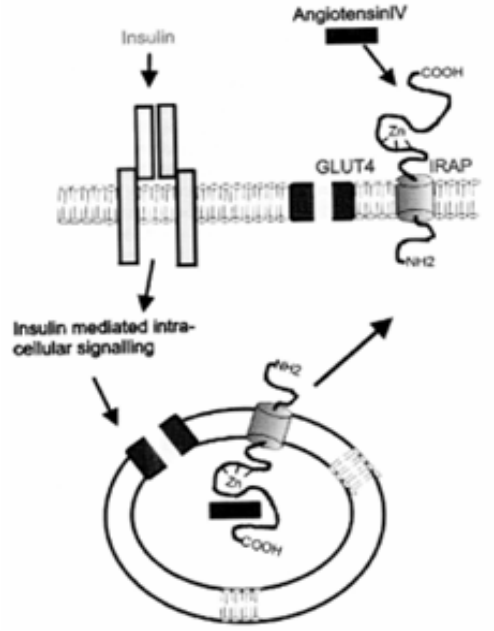
After the identification of IRAP as a binding site of AT_4, we tried to understand the molecular mechanism of this enzyme bound to the membrane that could mediate the intracellular signaling as well as a variety of biological effects in vivo, triggered by the ligands of AT_4, three mechanisms mediated IRAP are briefly hypothesized to explain these effects and illustrated in (*Fig. 16;17; 18;19*) [241]. The first mechanism that supports the binding of ligands at AT_4, causes an accumulation of different neuropeptides [241]. So it was proposed that the multiple physiological actions of AT4 receptor ligands are due to their ability to inhibit competitively the activity of peptidase IRAP, that prevent their degradation [241]. This model predicts that the effectiveness of all the ligands of the receptor in question, Ang IV, LVV-H7 and Nle1-Ang IV , must be equivalent in quality as their action is due to binding with 'IRAP and IRAP to prevent the ability to catabolize endogenous peptides *in vitro* [241]. At the moment, there is no evidence that supports this hypothesis *in vivo*. However, in a recent study, it was reported that the anticonvulsant effect of Ang IV and the increase in the concentration of extracellular dopamine in the striatum of rats can be inhibited by a somatostatin receptor antagonist, suggesting that accumulation *"in vivo "* somatostatin average these effects. In line with this hypothesis, it has also been shown that IRAP helps in the metabolism of vasopressin "*in vivo*" [241].



(Figure 16) - Illustration of possible mechanism by which Ang IV and AT_4 ligands mediate their effects-

Inhibition of the enzymatic activity of IRAP by Ang IV causes the accumulation of extracellular substrates neuropeptide.There are several problems with this first mechanism [253]: (1) This concept is difficult to reconcile with the results obtained from compounds which are agonists and antagonists opposing physiological actions; (2) the beginning of the physiological effects must be slow since this action requires an accumulation of the endogenous ligand of AT_4. This prediction agrees with the observation that the ligands of AT_4 have rapid effects on signaling molecules. For example, the AT_4 receptor activation can lead to increased 20-fold activation of extracellular signal regulated kinases (ERK) in C6 glioma cells within 30s. Similarly, *in vivo* studies indicate rapid changes of AT_4 mediated blood flow, renal oxygen consumption, and long-term potentiation (LTP), which typically occur in less than 1 min. It is unlikely that the peptide accumulates sufficiently to trigger physiological responses in such a short period of time. In fact, the typical time in which they act in vivo peptidases are inhibitors of hours or days, not seconds. (3) The concentrations of the ligands of AT_4 are necessary to make the changes in physiological function and are of the order of subnanomolar subpicomolare: concentrations much lower than those reported for any known enzyme inhibitor. This concern is relevant for IRAP as the reported Ki (inhibition constant) of Nle1-Ang IV (Ang IV-Norleucine1: metabolically stable derivative of Ang IV and the agonist peptide) for IRAP (> 0.3μM) is several orders of magnitude higher than the biologically effective dose of AT_4 ligands. (4) It is also questioned whether the ligands function as substrates of AT_4, competitive in a study by ***Caron et al. (2003)*** indicating that the allosteric ligands Ang IV interact with IRAP at a site distinct from the active site. The specific characteristics of the structure of Ang IV, of its analogues and other Angiotensins such as Ang III, do not make them substrates of IRAP, but still able to bind, they are currently unclear. (5) This proposal is in contrast to earlier work by ***Tsujimoto et al. (1992)*** in which it was shown that Ang III is an excellent substrate for human placental leucine aminopeptidase (IRAP homologous rat).The discrepancy between the primary mechanism for IRAP and laboratory observations suggests two likely

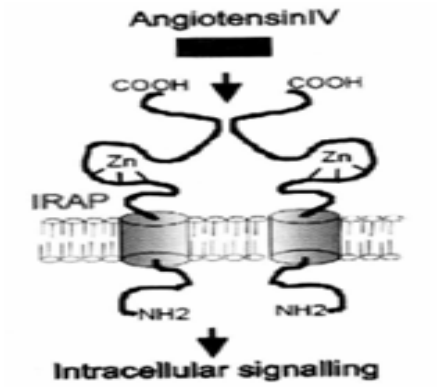
possibilities. First, IRAP is not responsible for the AT_4 receptor signal transduction, but is involved in the regulation of extracellular levels of endogenous receptor ligands AT_4. Second, IRAP may be a signal transduction receptor, but is based on activities that go beyond its ability to aminopeptidase. If the second possibility is correct then IRAP should possess in its short hydrophilic segment with 109 amino acid residues, the N-terminal, the information required for signal transduction [253]. It gives credence to this possibility, a previous study indicating that the N-terminus of IRAP contains two dileucine motifs and several regions of the acid, which play important roles in vesicle trafficking [253]. A peptide consisting of 55-82 residues to the N-terminus, containing one of the reasons AA dileucine and acidic groups, has proved sufficient to cause translocation of GLUT4. In addition, ***Ryu et al. (2002)*** showed *in vitro* phosphorylation of serine in position 80 (Ser80) of IRAP, which is involved in the regulation of insulin to stimulate translocation of GLUT4 [24]. The binding of ligands to AT_4, may prolong the superficial location of IRAP as GLUT4 and therefore the resulting increase in glucose uptake could be responsible for the biological effects of AT_4 [22].



(Figure 17) - Illustration of possible mechanism by which Ang IV and AT_4 ligands mediate their effects.IRAP is co-localized with insulin-regulated glucose transporter-

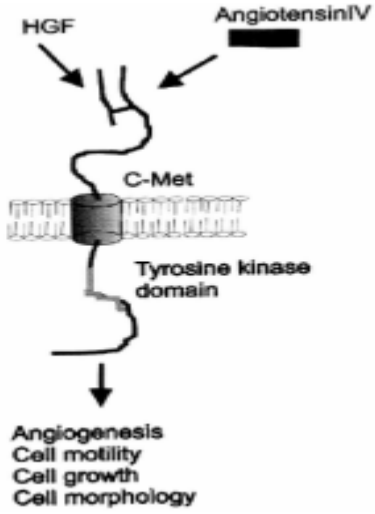
The binding of Ang IV to IRAP may increase the translocation of IRAP and GLUT4 on the cell surface, causing an increase in intake of glucose by the cells.

The poly (ADP-ribose) polymerase tankirasi was identified in a yeast two-hybrid system and aa96-100 interacts with IRAP. It is interesting to note that the acyl-coenzyme A dehydrogenase (ACD), identified by the fusion of glutathione-S-transferase (GST) with IRAP (GST-IRAP55-82), is probably involved in maintaining vesicles containing GLUT4 in intracellular compartments designated [253]. IRAP can increase the concentration of intracellular calcium, some modular MAPK kinase, activate the signaling of NF-kappaB, increase production of cGMP and downstream to create hanges in DNA synthesis [253].



(Figure 18)- Illustration of possible mechanism by which Ang IV and AT_4 ligands mediate their effects-IRAP could be a membrane-bound receptor. The binding of Ang IV can activate signaling pathways.

No matter the exact role of IRAP in AT_4 signaling ligands, the affinity of IRAP for the latter suggests that its function is somewhat important. Furthermore, it was assumed the hypothesis that Ang IV and its analogues, should have structure homology with one or more natural ligands that work through similar receptors to mediate physiological actions triggered by those who remember similar of Ang IV. Based on such thinking a search has been done for homology, which produced a partial match with the anti-angiogenic protein, angiostatin, and hepatocyte growth factor (HGF) [253]. HGF is a pleiotropic factor with mitogenic activity, motogena, morphogenic acting through the type I receptor tyrosine kinase, c-Met [217]. c-Met is the membrane-bound and ligand binding triggers the trans-phosphorylation of specific tyrosine residues, resulting in activation of multiple signaling pathways downstream. [253].



(Figure 19)- Illustration of possible mechanism by which Ang IV and AT4 ligands mediate their effects. Ang IV can bind and activate the HGF receptor called c-Met-

Classically it has been noted for its ability to direct the proliferation and differentiation of stem cells, to induce tubular morphogenesis in many organs including the kidney and to support angiogenesis by activating vascular endothelial cells [132]. Recently, c-Met has attracted considerable attention because of its role in multiple cancers [106], weakening its ability to neurodegenerative changes and its potential involvement in learning and memory consolidation [205,206]. The known ability of analogues of Ang IV to alter cognitive function, increased the development of neurites and activated vascular endothelial cells that direct the angiogenic process, encouraged it to study how similar Ang IV exert their biological activity through the system HGF / c-Met.

The results of the experiments reported that the AT_4 receptor antagonist, Norleucine (Nle-Tyr-Leu-(CH2-NH2) 3-4-His-Pro-Phe), is able to inhibit the proliferation, the 'HGF-dependent invasion and spread in different cell lines at concentrations pmol. In addition, Norleucine crashes [^{125}I]-HGF bound to c-Met with a Ki of 3pm, while the contrary can effectively block the HGF [^{125}I]-norleucine in the membranes of HEK cells (human embryonic kidney). As anticipated from these results, the norleucine shows potent anti-angiogenic and anti-tumor activity. In particular, the norleucine inhibits the growth of cells in vivo murine myeloma B-16, cancer murine c-Met-dependent, and induces apoptosis of human glioblastoma cells U-87 and survival is highly dependent on the active system c-Met signaling

Characteristics	AT_1	AT_2	AT_4–c-Met
Affinity	AngII > AngIII > AngI	AngIII > AngII > AngI	Nle^1, $Leual^3$-AngIV > HGF = AngIV > LVV-H7[a]
Selective antagonists	CGP46027, DuP753, DuP 532, EXP3174, L158809, GR117289, SK/F108566, SC51316, UP269-6, LR-B/081	CGP42112A, PD123177, PD121981, PD123319, PD124125	Nle^1, $Leual^3$-AngIV, Divalinal-AngIV
Coupling to	G-protein	G-protein	Tyrosine kinase
Signal transduction	$\uparrow Ca^{2+}$, ↑IP3, DAG, ↓Adenylyl cyclase, Src, JAK/STAT, ↑Prostaglandins, PL-A, -C, and -D	↓cGMP/↑cGMP, ↑Prostaglandins PL-A_2, NO	Gab1, Grb2, Grb10, PI3K, PLC-8, SHP2, Shc
Structure	359 amino acids; 7 transmembrane domains	363 amino acids; 7 transmembrane domains	dimer linked by disulfide bonds
Molecular size	41–42 kDa	40–41 kDa	α 50 kDa; β 140 kDa

Adapted from Birchmeier et al. (2003), de Gasparo et al. (2000), Ma et al. (2003), Mehta and Griendling (2007), Speth et al. (2003) and Wright and Harding (1997, 2004).

3.1Relationship between structure and activity of competitive ligands (Pharmacological profile)

The above association has helped to define the structural requirements of the angiotensin-derived peptides to compete with the binding of [^{125}I] Ang IV. It was revealed that deletion of the N-terminal valine of Ang IV, the N-terminal extension and replacement of L-valine with D-valine completely prevent the binding. The same study found that the C-terminal extension with the corresponding amino acids of Angiotensinogen or deletion of amino acids at the C-terminus only gradually decrease the binding affinity. So it was noticed that the three residues to the N-terminus of Ang IV are essential for the binding of the ligand, while the C-terminal domain has a crucial role [241]. In this regard, the recent isolation and identification of proAngiotensin-12 could serve as a possible precursor to generate peptides elongated C-terminal end . In addition, Ang-Nle1 IV has been identified as an excellent and powerful AT_4 receptor ligand. The Val1.3-Ang IV obtained with the introduction of two methylene bonds, appears to block the cognitive improvement, the long-term potentiation and release of acetylcholine evoked by K^+, mediated by Ang IV. Similarly, this compound inhibits the increase in blood flow in rat renal cortex as well as the increase in urinary excretion of sodium caused by Ang IV. This peptide called divalinal-Ang IV does not bind the AT_1 receptor or AT_2, and is therefore known as the first AT_4 receptor antagonist [241]. However, the intrinsic activity of the Ang IV-

divalinal (agonist or antagonist) appears to depend on the physiological processes and / or cell types. In bovine kidney epithelial cells and human, the Ang IV-divalinal as Ang IV causes a rapid increase in intracellular calcium. In the same way the divalinal-Ang IV exerts a partial agonistic effect in the insulinoma cell line of rat (INS-1) which is caused by the uptake of extracellular calcium. In addition, the local perfusion divalinal-Ang IV or Ang IV in the striatum of rats causes a similar increase in the concentration of extracellular dopamine in the brain structures. Several studies showing differences is unclear, but the existence of an AT_4 receptor, yet to be defined, cannot be excluded. On the other hand, there is recent evidence obtained from the receptors with seven transmembrane domains that a particular ligand may be able to stimulate receptor activity without affecting other activities triggered by the same receptor. These so-called "partial ligands" may well be able to expound an agonist activity in a tissue or cell type, while being an antagonist in another [241].Another peptide derived from Ang IV, a fragment structurally distinct β chain of hemoglobin, emorfina LVV-7 (LVV-H7) was isolated from the brains of sheep and competes with the high affinity binding of [Iodine-125] Ang IV [54]. LVV-H7 is clearly an AT_4 receptor agonist which originates the same effects as central of Ang IV, as the facilitation of cholinergic neurotransmission in hippocampal slices, the cognitive improvement and increased extracellular dopamine in the striatum of rats [216].

3.2 The ligand activates the intracellular signalling in cell culture

An important property for a receptor is to be able to transduce the binding of endogenous ligand (ie, the agony) into a signal that includes the cell and causes it to produce a physiological response. In this regard, many efforts have been made to characterize the intracellular signalling in various cell types of Ang IV. The signalling induced by Ang IV is heterogeneous but is highly dependent on cell type studied. These effects can be grouped into categories. The first is an effect on intracellular calcium concentration or by the rapid release from intracellular stores or influx from the extracellular medium. The secondly Ang IV has an impact on cell proliferation shown especially by the extent of incorporation of [3H]-thymidine. The third Ang IV appears to influence the activation of some MAPK (mitogen-activated protein kinase). MAPK is a family of serine / threonine kinase that responds normally to a variety of intracellular signals such as cellular stress, inflammatory signals and

different cellular responses to mitogens as well as gene expression, mitosis, differentiation and survival / apoptosis. In addition, a number of different responses are observed and include the activation of NF-kB, activation of tyrosine kinases, formation of cAMP and cGMP accumulation (related to the release of NO). If a particular effect induced cell by Ang IV is mediated through the AT_4 in a right way, it is important that:

1. it should not be inhibited by the AT_1 or AT_2 receptor antagonists. This is certainly important because Ang IV at micromolar concentrations is a full agonist for the AT_1 receptor;

2. a similar effect is mediated by different ligands, including analogues of AT_4 Nle1-IV as well as Ang IV LVV-H7. In addition, the corresponding EC_{50} values (concentrations that produce 50% maximal effect) must be correlated with the corresponding values of Ki binding experiments;

3. it is obvious that these functional experiments are skilled in determining the intrinsic activity of some ligands. As mentioned above, it was found that the Ang IV-divalinal inhibits the *in vivo* effects of Ang IV. In agreement with this, at least some of the cellular effects generated by Ang IV are inhibited by the fact divalinal-Ang IV. On the other hand, in other cells such as those of Mardin-Darby bovine kidney (MDBK) and the human proximal tubule epithelial (HK-2), the Ang IV-divalinal behaves like an AT_4 receptor agonist [253].

3.3 Future Prespectives

It is evident that Ang IV is not only a metabolite of the hormone Ang II, but cardiovascular exercises have interesting biological effects. While some of these effects are mediated by the AT_1 receptor, an important number of effects could not be blocked by the AT_1 and AT_2 receptor antagonists [241]. These effects include the improvement of learning and memory , anticonvulsant effects (in animal models of epilepsy) [214, 229], protection against cerebral ischemia [184,186,188] and improvement of endothelial function in animal models with atherosclerosis [83,241]. These observations in combination with the description of the binding sites of Ang IV have high affinity with a clear distinct pharmacological profile and is consistent with the concept of the AT_4 receptor. Despite these observations, the biochemical and cellular mechanism by which these effects are produced, is not yet completely established. In this regard, three possible mechanisms have been advanced and are based on the binding of ligands of AT_4 to aminopeptidasi insulin-regulated (IRAP). It is obvious that further work is required to identify which cell types and under what mechanism is responsible

for different biological effects mediated by Ang IV [241]. In this connection it may be important to use the newly synthesized ligands of AT_4 that instead of Ang IV are more stable and exert an improved selectivity for IRAP and AT_1 receptors [9, 42,136].

MATERIALS AND METHODS

4. *Cell Culture*

Human umbilical vein endothelial cells (HUVECs) were harvested from fresh human umbilical cord veins and cultured until the third passage, as previously described. Purity of the endothelial cell monolayer was confirmed by the cobblestone morphological pattern and by cell staining with a specific monoclonal antibody by von Willebrand factor. When the HUVECs, after microscopy observation, are in a confluent state on flasks surfaces they are trypsinized under a laminar flow hood, so the old culture medium is eliminated and the cells were washed with a specific posphate saline buffer (PBS) that present a value of ph ~ 7.4 at 37° C and after resuspended in a new culture medium. After HUVECs put to a sterile falcon (10 ml) and cell suspension was centrifuged (1100 rpm over a period of 10 minutes at + 20°C), the supernatant was removed and HUVECs were resuspended in culture medium (3 mL EGM2® Bullet Kit) (Lonza,Basel, CH). The cell cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air. Confluent cells were trypsinized and reseeded in culture medium in a specif sterile flasks pre-treated with factors of adhesion on surfaces. Cells used were at passages 2–8. Cells at different cycles of trypsinization were seeded in multi-well plates at a density of about 2 x 10^4 cells/well and used for the described experiments.[17]

4.1 Chemicals

Study I

Reagents:

Angiotensin II; Losartan (AT_1R antagonist); PD123319(AT_2R antagonist); Asymmetric Dymethil Arginine (ADMA);Apocynin (NADPH oxidase inhibitor) (Calbiochem ® Merck KgaA, Darmstadt, Germany) ; Epigallocatechin-gallate (all from Sigma St. Louis, MO, USA).

Label and Reagents:

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazoliumbromide], Acridin Orange (AO); 2'-7'-dichlorofluorescein-diacetate H_2DCF-DA (all from Sigma St. Louis, MO, USA) and DiidroEthidium (DHE: 2,7-Diamino-10-ethyl-9-phenyl-9,10-dihydrophenanthridine) (Calbiochem ® Merck KgaA, Darmstadt, Germany) ; DAF-2DA (Alexis Biochemicals, Lausanne, Switzerland); Malondialdehyde and Propidium Iodide (Sigma St. Louis, MO, USA).

Study II

Reagents :

Angiotensin IV)(Enzo BIOCHEMICAL, Vinci-Biochem s.r.l., Italy); Losartan (AT_1R antagonist); PD123319(AT_2R antagonist) (Sigma St. Louis, MO, USA);

Label and Reagents:

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazoliumbromide], Acridin Orange (AO); H_2DCF-DA(Sigma St. Louis, MO, USA) and AK-171 Kit for Detection of Conjugates Dienes(Enzo BIOCHEMICAL, Vinci-Biochem .r.l., Italy);

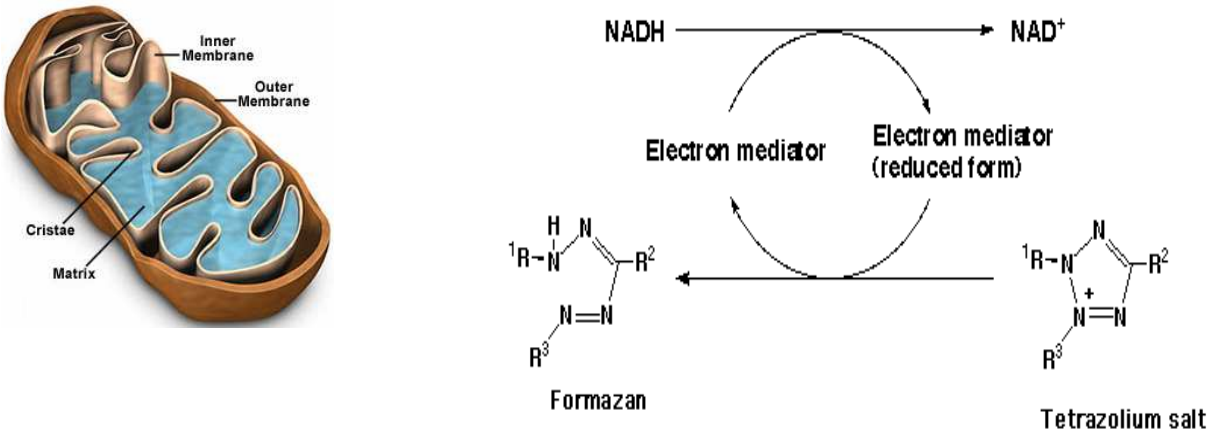
4.2 Experiment Protocol

4.2.1 Study 1: Evaluation of Angiotensin II effects on Endothelial Dysfunction in HUVEC's protocol

Study 1.1

MTT

Cell viability is assessed by JASCO v.630 Spectrophotometer using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazoliumbromide] at a final concentrations of 5 mg /ml that is dissolved in a EGM Bullet Kit without red phenol (incomplete medium) according to Bernas T. Et al 2002*(Fig.21).*HUVECs are plated in 24 wells and after eliminating specific culture medium are incubated with the MTT (1mL) at 2×10^4 cells/well. After four hour of incubation at the HUVECs is additioned a sterile dimethylsulphoxide (DMSO) (300 μL) and incubated for another hour. The cells are studied by JASCO Spectrophotometer and the program used is defined SpectraManager measurements. The data evaluation is after 1, 3 and 7 days of culture in the presence of Ang II either with or without Losartan or PD 123319 at 10^{-7} M. An aliquot of cells after damaging in a sterile watherbath under a laminar flow hood for a specific period of time (30 min) in Hyperthermic (40°-41°C) or Hypothermic (33°-34°C) conditions and after subjected to MTT cell viability assay.



(Figure 21) - Mitochondrial Structure and reaction in the development of MTT test-

Reactive Oxygen Species Assay

Reactive oxygen species (ROS) are a by product of a number of natural cellular processes such as oxygen metabolism and inflammation. ROS are generally 'kept in check' by antioxidant proteins and systems within cells, but overproduction of ROS can lead to cell death. ROS are highly reactive and can damage DNA, protein and lipids. DCF-DA has been used as a detector of ROS for a number of years in many applications such as fluorescence microscopy and flow cytometry. But actually is used a derivativ dye similar to DCF-DA defined H_2DF-DA. The generation processes of reactive oxygen species (ROS) are monitored using fluorescence methods. The intracellular ROS generation of cells can be investigated using the 2',7'-dichlorofluorescein-diacetate (H_2DCF-DA). The conversion of the non fluorescent 2',7'- dichlorodihydrofluorescein – diacetate (H_2DCF-DA) to the highly fluorescent compound dichlorfluorescein (DCF) happens in several steps. First, H_2DCF-DA is transported across the cell membrane and secondly deacetylated by esterases to form dichlorfluorescein (DCF) with the loss of two hydrogen groups. Third, this compound is trapped inside the cells and when binding reactive oxygen species becomes fluorescent, so this label is defined fluorogenic. The label was excited at 502 nm whereas emitted fluorescence was recorded at 523 nm.

The protocol for ROS detection by this dye is straightforward:

1) Human umbilical endothelial vein cells were plated in 6 well plates at 2×10^4 in full serum media;

2) Then after 1, 3 or 7 days, the cells were treated in Hyperthermia or Hypothermia for 30 minutes (as a positive control);

3) Later HUVECs were incubated also with Ang II in presence or absence of AT_1 or AT_2 receptor antagonists at 10^{-7} M .

4) Untreated and treated cells were harvested in full serum media, spun down for 5 minutes at 1000 rpm and resuspended in culture medium toghether to PBS containing 10 μM H_2DCFDA;

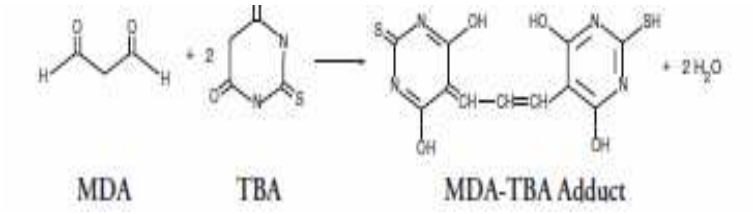
5) Cells were maintained at 37°C in the dark in incubator for 2 hours to 'load' the dye (N.B. Untreated cells not loaded with dye were used as a negative control to examine cellular autofluorescence.)

6) Analysis by microfluorymetry with reverted microscope Olympus IX 50 (objective 20X, oculare 10X).

Biomarkes of lipid peroxidation: The TBA assay

Malondialdehyde (MDA) is a naturally occuring product of lipid peroxidation. Lipid peroxidation is a well-estabilished mechanism of cellular injury in both plants and animals and is used as an indicator of Oxidative Stress in cells and tissue [1,2]. Lipid peroxides, derived from polyunsatured fatty acids, are unstable and decompose to form a complex series of compounds, which include reactive carbonyl compounds, such as MDA. The measurements of thiobarbituric acid reactive substances (TBARs) is a well-estabilished

method for screening and monitoring lipid peroxidation . Modification of the TBARs assay by many researchers has been used to evaluate several types of samples including human and animals tissue and fluids, drugs and food. Even though there remains a controversy cited in literature regarding the specificity of TBARs toward compounds other than MDA, it still remains the most widely employed assay used to determine lipid peroxidation. Lipid with greater unsaturation will yield higher TBARs value. Malondialdehyde reacts in the TBA test to generate a coloured product, a (MDA- TBA adduct) (*Fig.22*).



(Figure 22)- Molecular reaction in MDA Assay-

The MDA-TBA adduct formed by reaction of MDA and TBA at high temperature (90°-100°C) and acid conditions is measured colorimetrically at 586 nm according to (Gérard-Monnier D et al. 1998).

MDA protocol

▪ HUVECs, at mean number of 10^7 cells for a single spectrophotometric determination, are washed to removed protein and other costituents from the media in PBS, then lysed by 2 or 3 freeze/thawing cycles. Cell debris are removed by centrifugation at 3000 × g for 10 minutes at 4°C.

Preparation of TMOP (MDA) Standard

A MDA standard is provided as tetramethoxypropane (TMOP) because MDA is not stable. The TMOP is hydrolyzed during the acid incubation step at 45°C, wich will generate MDA. The TMOP Standard is provided as a 10mM stock solution. Just prior to use is diluted 1/500 (v/v) in water to give 20µM stock solution and is placed at 0°- 4°C until use.For the standard

curve, pipet the indicatedvolumes of Standard and water (or buffer) to the reaction tube to give a total of 200 µL. Final Concentration value is the MDA concentration in the reaction mixture (1 mL total volume).

Assay

1. Add 10 L of probucol to each assay tube;
2. Add 200 L of sample to the respective assay tubes;
3. Add 640 L of diluted N-methyl-2-phenylindole in acetonitrile (R1 reagent) to each tube.
4. Mix by briefly vortexing each tube;
5. Add 150 L of Hydrochloric acid (R2 reagent);
6. Stopper each tube and mix well by vortexing;
7. Incubate at 45°C for 60 minutes;
8. Centrifuge turbid samples (e.g. 10,000 × g for 10 minutes) to obtain a clear supernatant;
9. Transfer the clear supernatant to a cuvette;
10. Measure absorbance at 586 nm, (Color is stable for a least two hours at room temperature).[80]

Study 1.2

SuperOxide Evaluation

Superoxide production was assessed in HUVECs (2×10^4 for each tested samples) by the dihydroethidium fluorescent method. Dihydroethidium is a cell – permeable compound that can undergo a 2-electron oxidation to form the DNA-binding fluorophore EB [30]. Cytosolic dihydroethidium displays blue fluorescence, however, following oxidation to ethidium and subsequent intercalation to DNA, a bright red fluorescence is achieved. It is used to determine the level of Superoxide anion in cells by microluorimetry or flow cytometry.

SuperOxide Protocol

▪ Human umbilical endothelial vein cells were plated in 12 well plates at 2×10^4 in full serum media;after 1, 3 or 7 days, the cells were treated in Hyperthermia or Hypothermia for 30 minutes ; Huvecs were incubated also with Ang II in presence or absence of AT_1 or AT_2 receptorial antagonists at 10^{-7} M in a first series of experiments. In a second part HUVECs were incubated with two different concentration of Asymmetric DymethilArginine [10^{-4} and $0.5 * 10^{-4}$ mol/L] containing PSS alone or in presence of Ang II, Losartan or PD123319[10^{-7} M] or Apocynin [10^{-5} M] for 30 minutes .PSS is a physiological salt solution composed of (in nmol/L) 110.0 NaCl, 5.0 KCl, 2.5 $CaCl_2$, 1.0 MgSO4, 1.0 KH_2PO4, 5.5 glucose and 24.0 NaHCO3 equilibrated with a gas mixture balanced with nitrogen a pH 7.4. This solution in wich is diluited ADMA is preparated on the day of each experiment. Then, dihydroethidium (5 × 10-6 mol/L) was added to the vials and incubated for a further 10 minutes. Intensity of EB fluorescence of HUVECs culture was measured and quantified by NIH Image J software. Relative EB fluorescence intensity was counted by extracting the intensity of background from a standar HUVECs control culture. Measurements was repeated 6 times, and relative intensity of EB[...] fluorescence was presented as the percentage of control.

Study 1.3

Cells continuously produce free radicals and reactive oxygen species (ROS) as part of metabolic processes. These free radicals are neutralized by an elaborated antioxidant defense system consisting of enzyme such as catalase, superoxide dismutase , glutathione peroxidase and numerous non-enzymatic antioxidant, including vitamins A, E and C, ubiquinone and flavonoids [20]. In this study is considered an specific flavonoid as **Epigallocatechin gallate** (ECG), also known as epigallocatechin 3-gallate, that is the ester of epigallocatechin and gallic acid and it is a type of catechin that is the most abundant in tea and is a potent antioxidant that may have therapeutic applications in the treatment of many disorders (e.g. cancer). The aim is to evaluate effects of epigallocatechin gallate on HUVECs treated in Hyperthermia or Hypothermia in free radicals generation and nuclear cellular damage. In this study, beside considering aformentioned protocols on ROS assay after addition an important compound with function of a natural antioxidant as epigallocatechin-gallate, were evaluated also the effect of this flavonoid on Nitrix Oxide generation and also in nuclear damage development in HUVECs .

ROS protocol

▪ Human umbilical endothelial vein cells were plated in 6 well plates at 2×10^4 in full serum media; after 1, 3 or 7 days, the cells were treated in Hyperthermia or Hypothermia for 30 minutes (as a positive control); later HUVECs were incubated also with Ang II in presence or absence of AT_1 or AT_2 receptorial antagonists at 10^{-7} M and in presence of 50 μM of epigallocatechin-gallate for each samples [21] . Untreated and treated cells were harvested in full serum media, spun down for 5 minutes at 1000 rpm and resuspended in culture medium toghether with PBS containing 10 μM H_2DCFDA; Cells were maintained at 37°C in the dark in incubator for 2 hours to 'load' the dye. Analysis by microfluorymetry with reverted microscope Olympus IX 50.

Nitrix Oxide Evaluation

For Nitrix Oxide evaluation experiments were performed with a specific label :DAF-2DA. Diaminofluorescein –2 Diacetate (DAF-2DA) is a cell permeable reagent that can measure free Nitric Oxide (NO) in living cells under physiological conditions. Once inside the cell, the diacetate groups on the DAF-2DA reagent are hydrolyzed by cytosolic esterases thus releasing DAF -2 and sequestering the reagent inside the cell. Production of nitric oxide converts the non-fluorescent dye, DAF-2, to its fluorescent triazole derivative, DAF-2T.

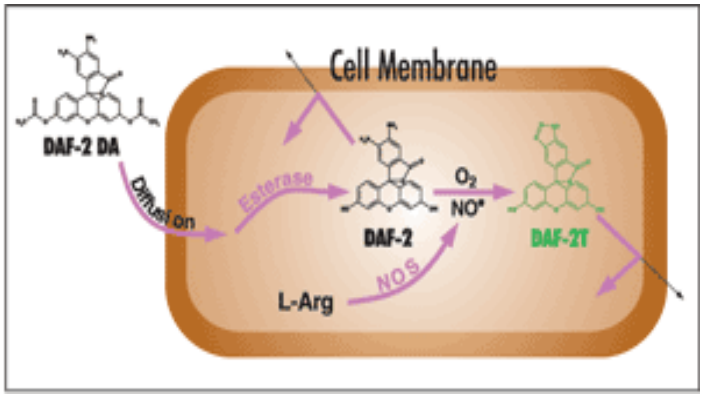


Fig.23- Functionality mechanisms of DAF-2DA-

Thus, in the present study, the DAF-2T fluorescence intensity will reflect the NO concentration in EC.

NO protocol

▪ The label was dissolved in DMSO (1 mg/ 0.45 ml) and diluted to 10 µM in a phosphate buffer (0.1M, pH 7.4). After 1, 3 or 7 days, the cells were treated in Hyperthermia or Hypothermia for 30 minutes. Then the cells were incubated in EGM2 culture buffer containing DAF-2DA at 10^{-7}M. To this medium was added Ang II [10^{-7} M] with either AT_1R or AT_2R antagonists at 10^{-7} M and 50 µM of epigallocatechin -gallate . After 2h incubation in one of these reaction media the fluorescence from the reaction of DAF-2DA with NO released under theeffect of Ang II and Epigallocatechin-gallate was studied on Olympus IX 50 fluorescence microscope calibrated for excitation at 495 nm and fluorescence emission at 515 nm.

Nuclear Damage Generation

Propidium Iodide Protocol

▪ **Propidium iodide** (or PI) is an intercalating agent and a fluorescent molecule that can be used to stain cells. When excited with 488 nm wavelength light, it fluoresces red. Propidium iodide is used as a DNA stain for both flow cytometry to evaluate cell viability or DNA content in cell cycle analysis [1] and microscopy to visualise the nucleus and other DNA containing organelles. It can be used to differentiate necrotic, apoptotic and normal cells [2]. After 1, 3 or 7 days, HUVECs were treated in Hyperthermia or Hypothermia for 30 minutes. Then the cells were incubated in EGM2 culture buffer and later incubated in a solution containing 16 μM Propidium Iodide. To this medium was added Ang II [10^{-7} M] with either AT_1R or AT_2R antagonists at 10^{-7} M and 50 μM of Epigallocatechin -gallate . After 1 hour of incubation the samples are analyzed by microfluorimetry.

4.2.2. Study 2: Evaluation of Angiotensin IV and AT_4 receptor effects on Endothelial Dysfunction in HUVEC's protocol

Study 2.1

MTT

Cell viability is assessed by JASCO v.630 Spectrophotometer using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazoliumbromide] at a final concentrations of 5 mg /ml that are dissolved in a EGM Bullet Kit without red phenol (incomplete medium) according to Bernaas T.HUVECs are plated in 24 wells multiwell and after discarding specific culture medium are incubated with the MTT (1mL) at 2×10^4 cells/well. After four hours of incubation to the HUVECs are additioned with a sterile dimethylsulphoxide (DMSO) (300 μl) and incubated for another hour. The cells are studied by JASCO Spectrophotometer and the program used is SpectraManager. The data evaluation is after 1, 3 and 7 days of culture in the presence of Ang IV at three different concentrations [10^{-6}; 10^{-7} and 10^{-8} M] either with or without Losartan and PD 123319 at [10^{-7} M].

Reactive Oxygen Species Assay

The protocol for ROS detection is described below:

- Human umbilical endothelial vein cells were plated in 6 well plates at 2×10^4 in full serum media;

- Then after 1, 3 or 7 days, the cells were treated in Hyperthermia or Hypothermia for 30 minutes (as a positive control);

- Later HUVECs were incubated also with Ang IV at three different concentrations [10^{-6}; 10^{-7} and 10^{-8} M] either with or without Losartan and PD 123319 at [10^{-7} M] ;

- Untreated and treated cells were harvested in full serum media, spun down for 5 minutes at 1000 rpm and resuspended in culture medium toghether to PBS containing 10 Mm H_2DCFDA;

- Cells were maintained at 37°C in the dark in incubator for 2 hours to 'load' the dye (N.B. Untreated cells not loaded with dye were used as a negative control to examine cellular autofluorescence.)

- Analysis by microfluorymetry with reverted microscope Olympus IX 50.

Nitrix Oxide Evaluation

NO protocol

▪ The label was dissolved in DMSO (1mg / 0.45ml) and diluted to 10 μm in a phosphate buffer (0.1M, pH 7.4). After 1, 3 or 7 days, the cells were treated in Hyperthermia or Hypothermia for 30 minutes. Then the cells were incubated in EGM2 culture buffer containing DAF-2DA at 10^{-7}M. To this medium was added Ang IV at three different concentrations [10^{-6}; 10^{-7} or 10^{-8} M] either with or without Losartan and PD 123319 at [10^{-7} M]. After 2h incubation in one of these reaction media the fluorescence from the reaction of DAF-2DA with NO released under the effect of Ang IV and AT_1 and AT_2 receptorial antagonists was studied on Olympus IX 50 fluoromicroscope calibrated for excitation at 495 nm and fluorescence emission at 515 nm.

Calcium Intracellular Production

$[Ca^{2+}]i$ levels in HUVECs were determined using the label Fluo-3AM. Fluo-3 acetoxymethyl (AM) ester was used as the fluorescent indicator. It is a fluorescent chelator excited by visible light (488 nm) emits a yellowish green fluorescence (525 nm) when bound to one calcium ion[4]. Fluo-3 does not fluoresce unless bound to calcium ions and because it is a water-soluble regent it cannot pass through the cell membrane. Fluo-3 AM ester is a derivative of fluo-3, it is a fat-soluble reagent and is also non-fluorescent. In contrast to fluo-3, this ester

can be passively loaded into the cell across the cell membrane. Once introduced into the cell, intra cellular esterases break down the Fluo-3 AM ester into acetoxymethyl and fluo-3 which can then readily combine with free intracellular calcium ions. This acetoxymethyl ester loading technique significantly reduces the time needed for carrying out the calcium fluorescence observations.[5].The cells are treated with the label for a period of 2 hours before observation to determine Ca^{2+}. The cells were observed after 1, 3 or 7 days only in presence of Ang IV at three different concentrations [10^{-6}; 10^{-7} or 10^{-8} M] either with or without Losartan and PD 123319 at [10^{-7} M] at physiological conditions.

Cell Viability (Acridine Orange)

Cell viability is assessed by fluorescence microscopy using the label Acridin Orange at 0,2% that is dissolved in PBS stain according to Darzynkiewicz Z. Acridine Orange (AO) is a nucleic acid selective metachromatic stain useful for cell cycle determination. AO interacts with DNA and RNA by intercalation or electrostatic attraction, respectively. DNA not intercalated AO fluoresces green (525nm); damaged DNA intercalated and RNA electro statically bound AO fluoresces red (>630nm). AO recognizes the quiescent phase and the activated one, during the cells proliferation, and it may also allow differential detection of multiple G1 compartments. AO staining may also be useful as a method for evidencing apoptosis, and for detecting pH gradients.[3].

AO protocol

▪ HUVECs are plated in 24 wells multiwell and are incubated with the label and EGM2® Bullet Kit medium at 2×10^{4} cells/well. After 10 minutes the cells are studied by microfluorometry (200x). The data evaluation is after 1, 3 and 7 days of culture in the presence of Ang IV at three different concentrations [10^{-6}; 10^{-7} and 10^{-8} M either with or without Losartan and PD123319 at [10^{-7} M]. An aliquot of the cells was treated in Hyperthermia or Hypothermia for 30 minutes (as a positive control) and after addition of Ang IV at three different concentrations [10^{-6}; 10^{-7} or 10^{-8} M] either with or without Losartan and PD123319 at [10^{-7} M].

Conjugates Dienes Assay

Lipid peroxidation is a well-estabilished mechanism of cellular injury in both plants and animals, and is used as an indicator of Oxidative Stress in cells and tissue. Lipid peroxides, derived from polyunsaturated fatty acid are unstable and decompose to form a complex series of compounds. These include reactive aldehydes of wich the most abundant is malondialdehyde (MDA). The AK-171 method is designed to assay free MDA or, after a hydrolysis step, total MDA (i.e. free and protein-bound Schiff base conjugates) . The assay conditions are useful to minimize interferences from other lipid peroxidation products, such as 4-hydroxyalkenals [80].

Fluorescence microscopy and Spectrophotometric Analysis .

Cell fluorescence is observed by means of fluorescence microscope Olympus IX50 (Japan) with UV source (20X objective, 10X oculars). Emission is acquired by UC30 digital camera and Cell-A program (Olympus) and Images analyses and data elaboration are performed by **NIH *ImageJ program v.1.61*.** For evaluation of Cellular state of HUVECs is used an optical microscope MOTIC AE21(China).For spectrophotometric measurements is used JASCO Spectrophotometer v.630 and for elaboration of data acquisitions is used the program ***Spectramanager*** (Jasco, Japan***)***, all instruments are present in Laboratory of Cell Physiology and in Department of Cell Biology, UNICAL.

Statistics

For statistical Analysis are used two different tests:

- **One-way ANOVA**
- **Two-ways ANOVA**

both statistical analyses are performed by Bonferroni test for multiple comparison between all samples. All data analized during experimentation are reported as means ± SEM.

The values are considered significant for a level of $p < 0.05$.Data are shown as means ± SEM. The n values indicate the number of independent experiments. Statistical Analysis was performed by a statistical programs :***Graph Pad Prism v.5*** .

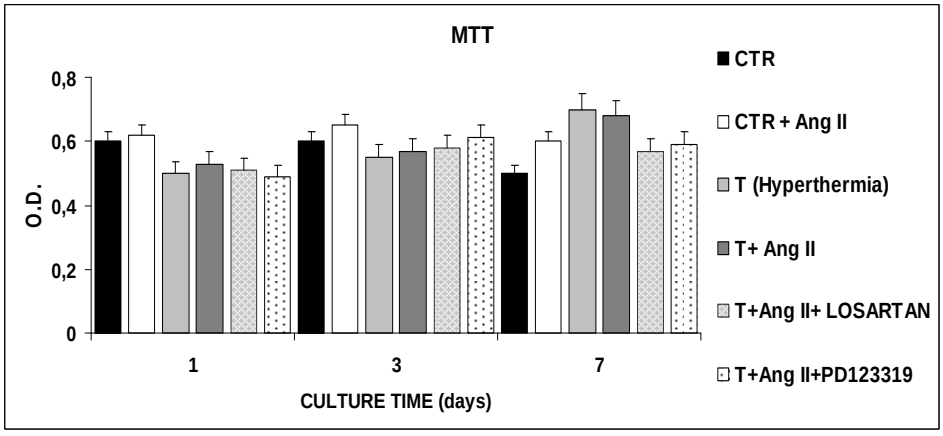
RESULTS AND DISCUSSIONS

Study 1

5.1 Effects of Angiotensin II in Hyperthermia and Hypothermia on Cellular Viability

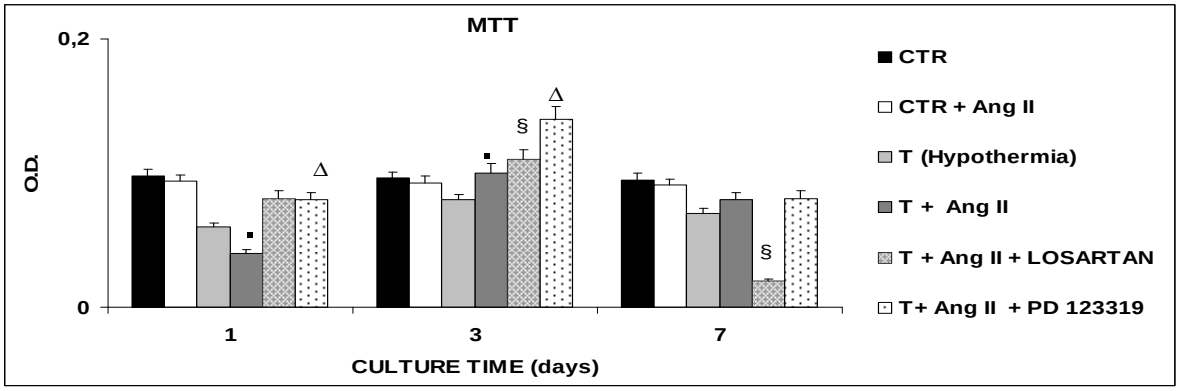
In a firts series of experiment conducted, viability test with MTT is performed. Analysis is based on evaluation of effects of stimulation by Angiotensin II and receptorial antagonists (Losartan and PD123319) in HUVECs placed in culture for a time of 1, 3 and 7 days. Cells are to be considered in normal condition and also after physical thermic damages. In this experimentation thermic treatments concerns Hypothermia (40°- 41°C) or Hypothermia (33°-34°C). They are achieved through a thermostatic sterile waterbath situated under sterile laminar flow hood. When the cells are in a confluent state, after microscope observation, are tripsinized and setting up (2×10^4.) for each sample, a number of ten replicas is performed. Heat treatment used on our cells lasts a time of 30 minutes, at the end of this period the cells are incubated after stimulation with Angiotensin II [10^{-7} M]and receptorial antagonists (Losartan and PD123319) [10^{-7} M] with MTT .

The ***(Fig.24a)*** and ***(Fig. 24b)*** shows the results obtained:



(Fig.24a) - MTT cell proliferation assay HUVECs healthy and damaged cells by hyperthermia after 1,3 or 7 days - *(One way ANOVA) no significant difference*

As can be seen from the figure Hypothermia is a condition that not influenced HUVECs viability and in presence of Angiotensin alone and in presence of Losartan or PD123319.



(Fig.24b) Cell viability evaluation in healthy and damaged samples after 1, 3 or 7 days of culture.

· p< 0,05 (comparison 1-3 is significant in Ang II treated samples);

∆ p< 0,05 (comparison 1-3 is significant in Ang II and PD 123319 treated samples);

§ p< 0,05 (comparison 3-7 is significant in Ang II and Losartan treated samples).

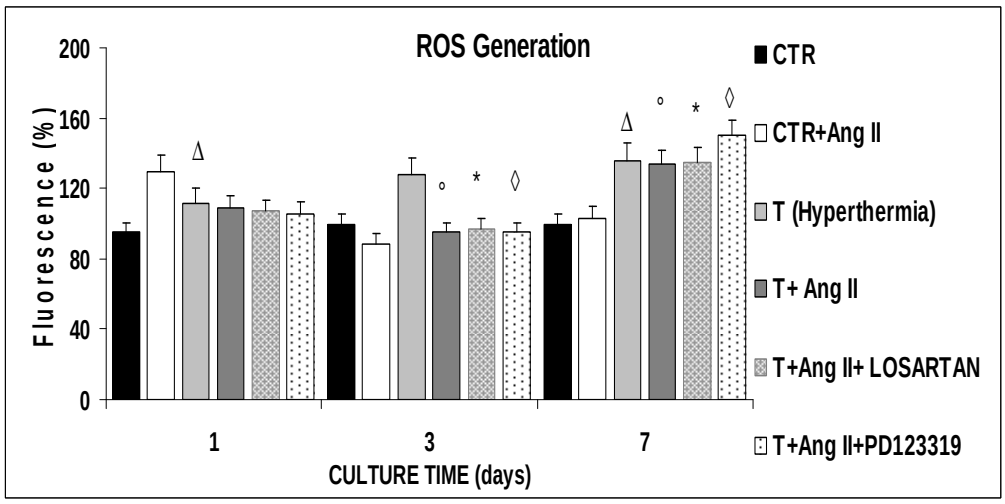
(One way- ANOVA)

Viability of HUVECs in Hypotermia, after stimulation with Angiotensin II or receptorial antagonists, increases cell proliferation between 1 and 3 days of culture in samples damaged and stimulated with Angiotensin II alone. Especially it appears to be modulated by the Angiotensin type 1 receptor pathway.

5.1.1 Effects of Angiotensin II on ROS generation in Hyperthermia and Hypothermia

For ROS evaluation, analysis is based on observed effects of stimulation by Angiotensin II and receptorial antagonists (Losartan and PD123319) in HUVECs in culture for a time of 1, 3 or 7 days. Cells are considered in normal condition and after physical thermic damages. In this experimentation thermic treatments concern Hypothermia or Hypothermia, they are achieved by a thermostatic waterbath under sterile laminar flow hood. When the cells are in a confluent state, after microscope observation, are tripsinized and setting up (2×10^4) for each sample, a number of ten replicas are performed. Heat treatment used on our cells lasts a time of 30 minutes, at the end of this period the cells are incubated after stimulation with Angiotensin II [10^{-7} M]and receptorial antagonists (Losartan and PD123319) [10^{-7} M] with a specific label H_2DCF-DA to detect of reactive oxygen species.

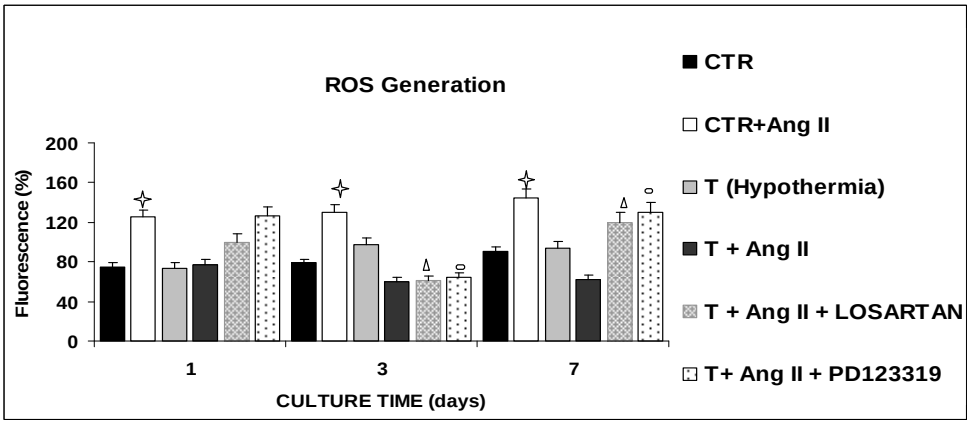
The ***(Fig.25 a)*** show results obtained:



(Fig.25 a) - Fluorescence microscopy Analysis of HUVECs in Hyperthermia.
∆°*◊p<0.05 (significant difference in comparison with treated cells and treated cells in presence of Ang II, Losartan, PD 123319)- *(One way ANOVA)*

In this figure is present an significant increase of ROS generation in samples damaged by Hypothermia between 1 and 7 days of culture, and especially in samples treated in Hypothermia after stimulation with Angiotensin II alone and in combination with Losartan or PD123319 between 3 and 7 days of HUVECs culture.

In ***(Fig.25b)*** are reported the obtained results:



(Fig.25b) Fluorescence microscopy Analysis of HUVECs in Hypothermia

✧ p<0.05 (control and cells with Ang II after 1-3-7 days comparison is significant)

∆° p<0.05(treated cells with or without Ang II, Losartan, PD 123319 comparison after 3-7 days is significant).

(One way ANOVA)

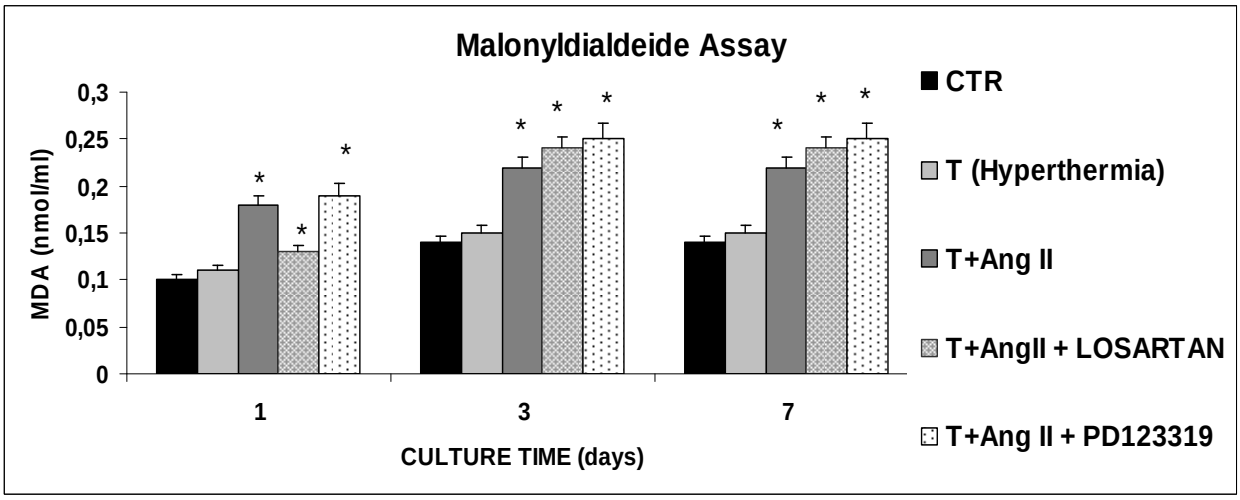
Reactive Oxigen Species production decreases in Hypothermic condition but after 7 days of culture in samples treated and stimulated with Angiotensin II and Losartan or Angiotensin II and PD123319 final levels of ROS increase.

5.1.2Effects of Angiotensin II on Lipid peroxidation products in Hyperthermia and Hypothermia

To test formation of Lipid Peroxidation products and evaluate effect of Angiotensin II stimulation in presence or absence of receptorial antagonist, after Hyperthermic or Hypothermic treatments and in normal conditions is used a specific Kit to monitoring

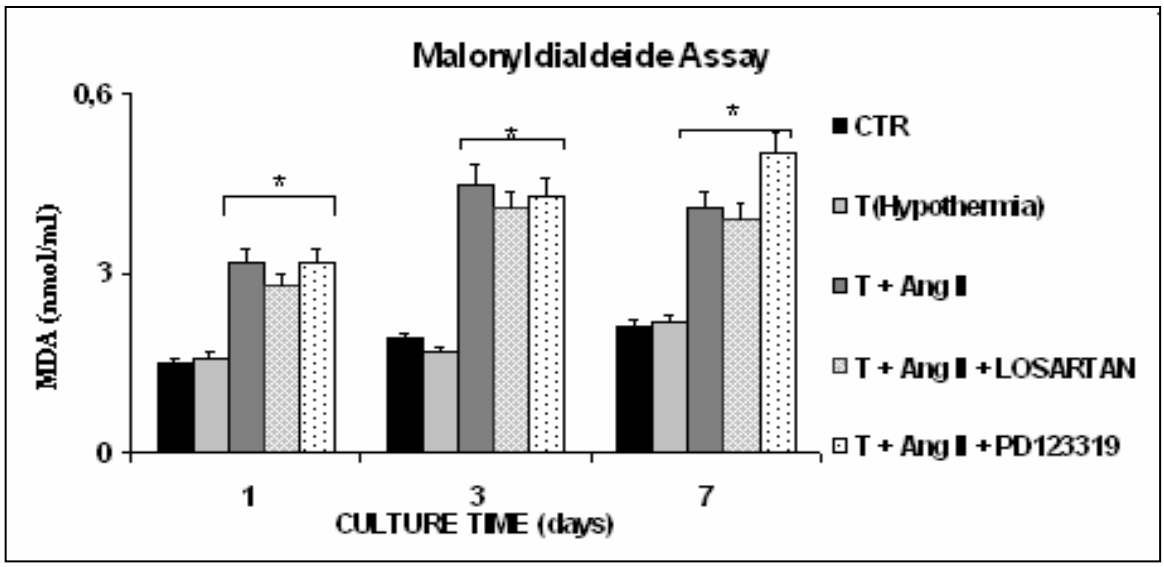
Malondialdehyde generation in culture cells. So, after 1, 3 and 7 days of culture HUVECs are analyzed with different assays performed with JASCO Spectrophotometer v.630.

Results are reported in ***(Fig.26a)*** and in ***(Fig.26b)***:



(Fig.26a) Assessment of ROS effects by MDA method for 1-3-7 days:

****p <0.05 (comparison between damaged cells and damaged cells in the presence of Ang II, Losartan, PD 123319).*** *(One way ANOVA)*



(Fig.26b) Evaluation of ROS effects by MDA method for 1-3-7 days in hypothermic conditions:

****p <0.05 (comparison between damaged cells and damaged cells in the presence of Ang II, Losartan, PD 123319).*** *(One way ANOVA)*

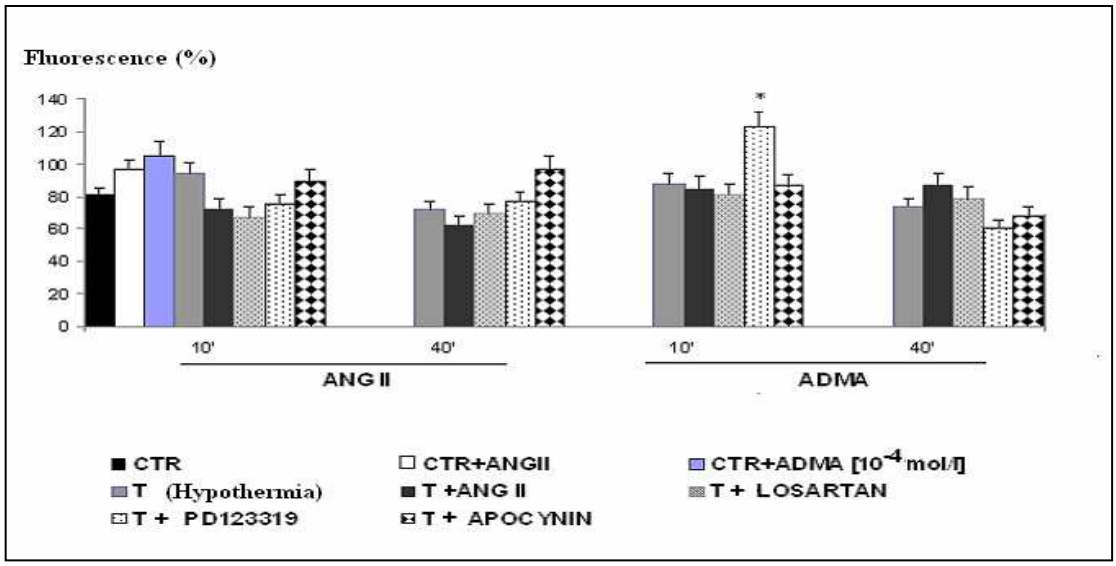
In Hyperthermic conditions malondialdehyde is produced by HUVECs and in all samples treated after stimulation of Angiotensin II alone or in presence of both receptorial antagonist it increase during the time confirming results of Fluorescence microscopy.

Malondialdehyde, as adduct of lipid peroxidation mechanisms in HUVECs, in Hypothermic conditions increases after 1, 3 and 7 days of culture in samples stimulated with Angiotensin II alone, Angiotensin II and Losartan, Angiotensin II and PD123319.

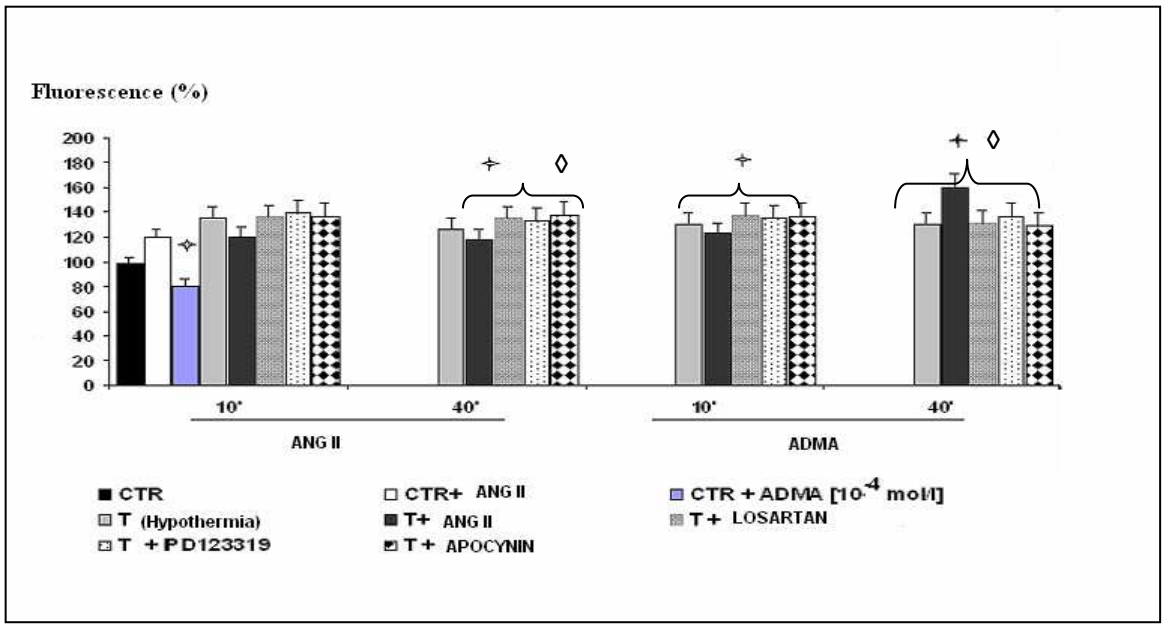
5.2 Effects of ADMA on Angiotensin II pathways in Hyperthermic and Hypothermic conditions in HUVECs

Asymmetric Dymethil Arginine (ADMA) is a structural analogue of L-Arginine aminoacid and is an inhibitor of eNOS, is considered to evaluate the interaction with Angiotensin II pathways in presence of Hyperthermia (40°-41° C)or Hypothermia (33°-34°C). ADMA is tested alone at two different final concentrations: [10^{-4} M], [0.5×10^{-4} M] and after stimulation with Angiotensin II [10^{-7} M], Losartan [10^{-7}], PD123319 [10^{-7}] or Apocynin (NADPH oxidase inhibitors) [10^{-5} M] .

The ***(Fig.27 a)*** and ***(Fig.27b)*** reported the results for evaluation of ADMA at [10^{-4} M] in Hyperthermia and Hypothermia:



(Fig.27a)Evaluation of SUPEROXIDE production :*p <0.05 significant difference after 10 minutes in the hyperthermic preparations in the presence of Ang II and AT_2R in the presence of ADMA [10^{-4} mol] in comparison to the same preparations without ADMA.
(Two ways ANOVA)



(Fig.27b) Analysis of Superoxide production in hypothermic treatments with Ang II [10^{-7} M], Losartan [10^{-7} M], PD 123319 [10^{-7} M], Apocynin [10^{-5} M], ADMA [10^{-4}M] after 10 and 40 minutes of incubation.

✧ ***p< 0,05 (significant comparison in thermic treated samples with Ang II or Losartan or PD123319 or Apocynin after 10 and 40 min respect to control samples in presence of ADMA [10^{-4} M]).***

◊ ***p< 0,05 (significant comparison in thermic treated samples with Ang II after 40 min in comparison to thermic treated samples in presence of ADMA [10^{-4} M] after 40 min).***

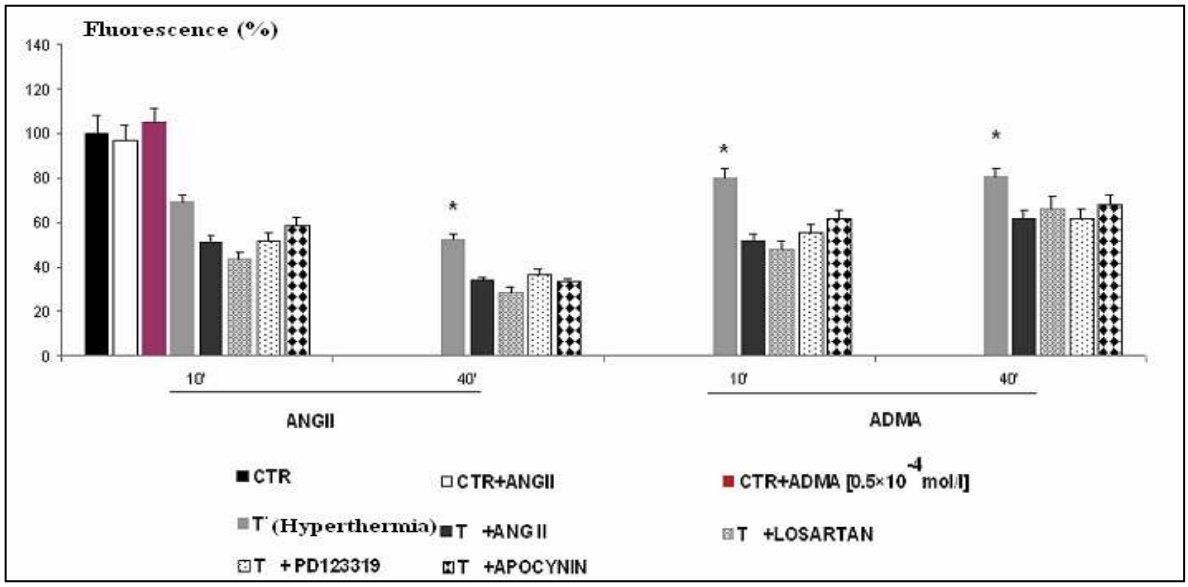
(Two ways ANOVA).

Considering results, reported in ***(Fig.27a)*** and ***(Fig.27b),*** we observed that:

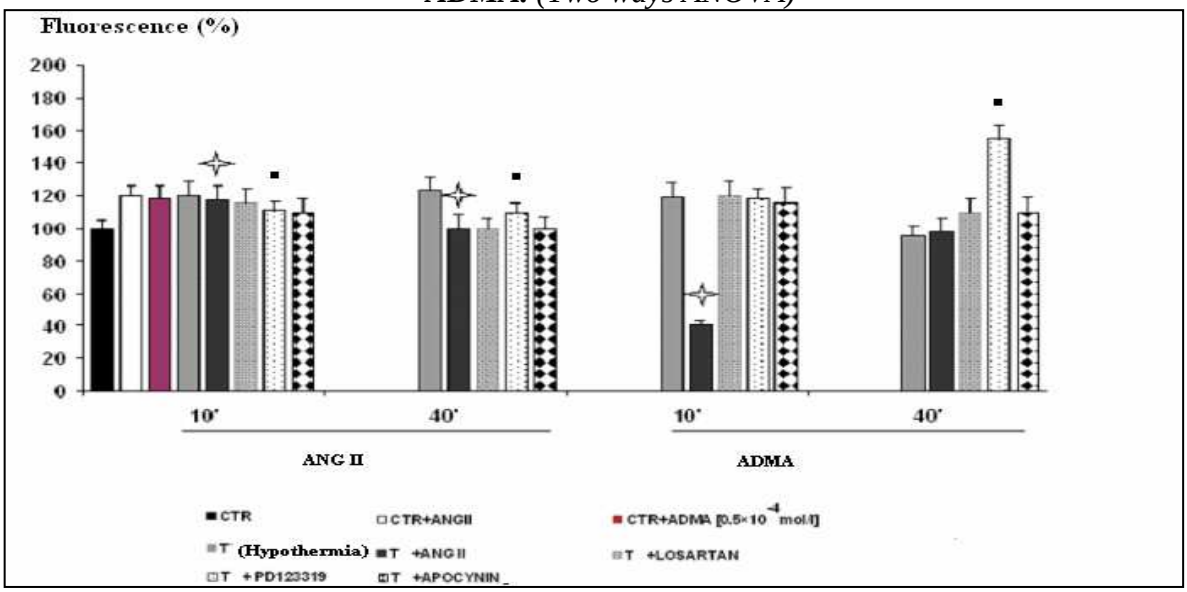
In Hyperthermic conditions is present an interaction, at 10 minutes of observation with Dihydroethidium label, between ADMA [10^{-4} M] and Angiotensin II [10^{-7} M] confirmed by Superoxide generation in HUVECs ;

Also in Hypothermic condition this effect is evidenced but interactions regards samples stimulated with Angiotensin II alone and both receptorial antagonist and samples stimulated with addition of ADMA [10^{-4} M] after 10 and 40 minutes of label exposition. In this case is present an increase of Superoxide productionin comparison to control samples with idem final concentration of ADMA .

After idem analysis is performed with ADMA [0.5×10^{-4} M] and in ***(Fig.28a)***, ***(Fig.28b)*** are reported results obtained:



(Fig.28a) Evaluation of SUPEROXIDE production:
****p <0.05 significant difference after 10 minutes in the hyperthermic preparations in the presence of ADMA [0.5 × 10^{-4} mol]in comparison to the same preparations without ADMA.*** *(Two ways ANOVA)*



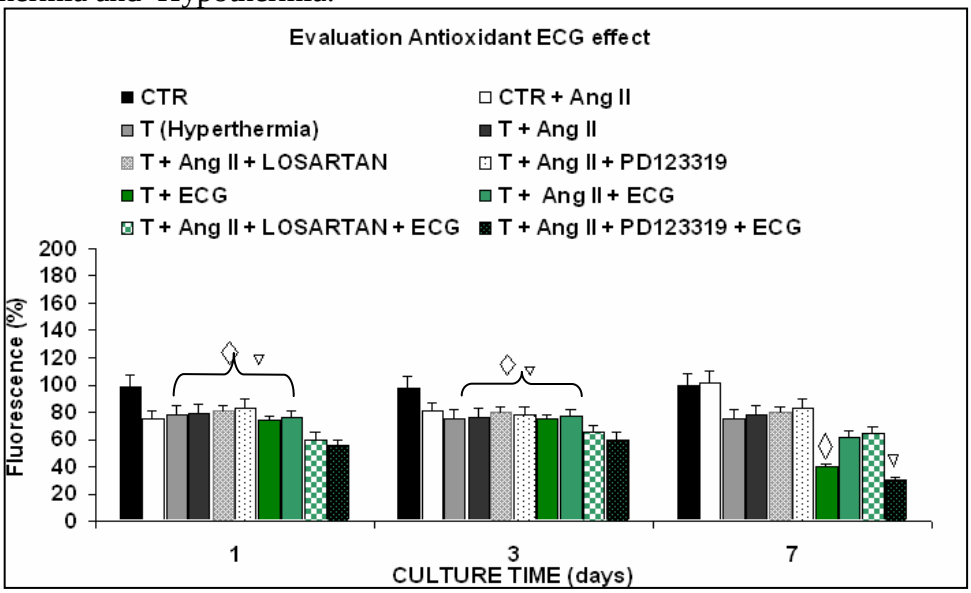
(Fig.28 b)Analysis of Superoxide production in hypothermic conditions with Ang II [10^{-7} M], Losartan and PD123319 [10^{-7} M], Apocynin [10^{-5} M], ADMA [5×10^{-4} M] after 10 and 40 min of incubation:

✧ ***p<0,05 (significant difference between treated samples with Ang II after 10 and 40 min and treated samples in presence of Ang II + ADMA);***
▪ ***p<0,05 (significant difference between samples with Ang II and PD123319 after 10 and 40 min and samples with Ang II + PD123319 + ADMA after 40 min;*** *(Two ways ANOVA)*

Superoxide levels in samples subjected at Hyperthermic shock in presence of ADMA [0.5 $\times 10^{-4}$ M] alone show an increase at 10 and 40 minutes of treatments; samples subjects at Hypothermic shock stimulated with Angiotensin II and ADMA [0.5× 10^{-4} M] decrease superoxide levels after 10 minutes of observation.

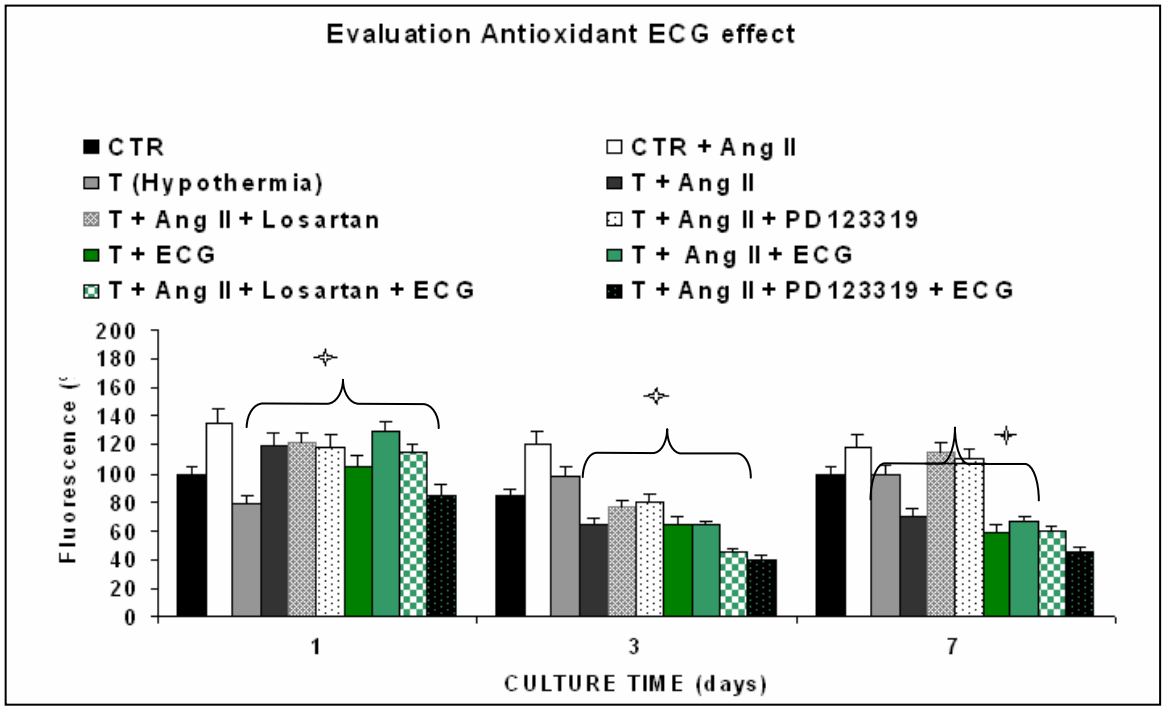
5.3 Effects of Epigallocatechin-gallate on ROS generation in Hyperthermia and Hypothermia in HUVECs

In a second experimetal phase HUVECs are tested in presence of a powerful natural reductan: Epigallocatechins-gallate to evaluate the free radical generations (ROS and RNS) and to analyze nuclear damage if observable. Effects of Epigallocatechin-gallate are analyzed on Angiotensin II pathways in presence of Hyperthermia (40°-41° C)or Hypothermia (33°-34°C). Epigallocatechin-gallate is used at a final concentration of 50 μM and after stimulation with Angiotensin II [10^{-7} M], Losartan [10^{-7}] or PD123319 [10^{-7}.The ***(Fig.29a)*** and ***(Fig.29b)*** reported the results for evaluation of Epigallocatechin-gallate in Hyperthermia and Hypothermia:



(Fig.29a)Evaluation ROS production in hyperthermia with Ang II [10^{-7} M], Losartan and PD123319 [10^{-7} M] and natural flavonoid Epigallocatechin-gallate

.◊Δ p<0.05 (comparison between treated samples with Epigallocatechin or ECG and PD123319-Angiotensin II in comaprison to all treated samples after 1-3-7 days of culture.
(Two ways ANOVA)



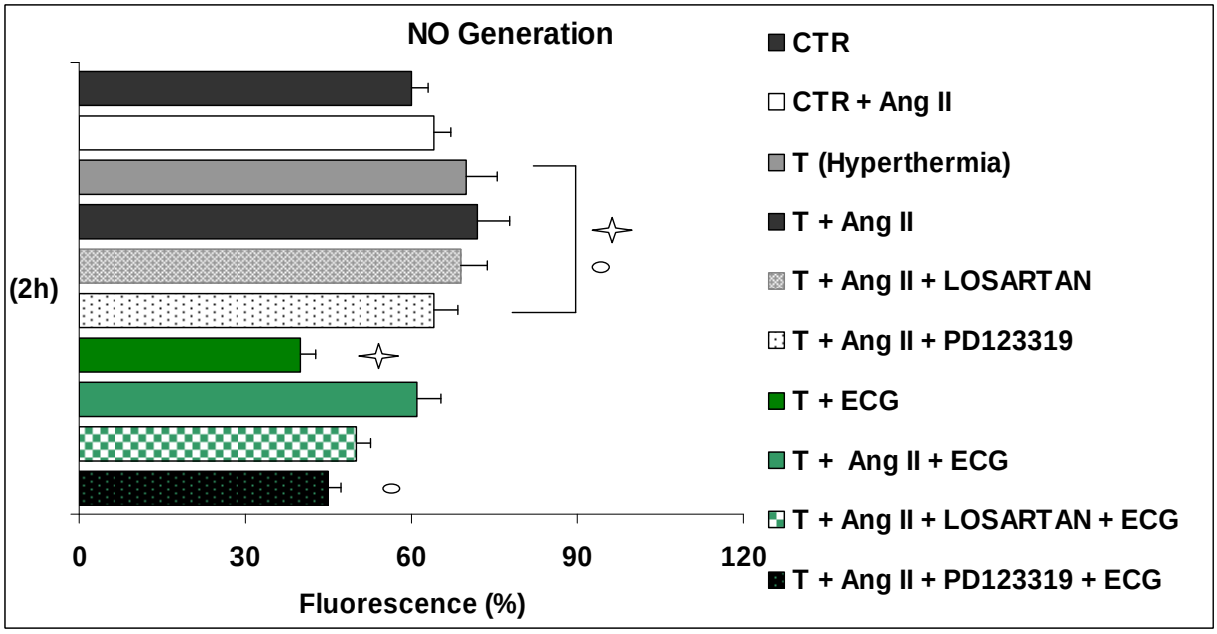
(Fig.29b)Evaluation ROS effects in Hypothermia with flavonoid:

✧ ***p<0.05 (comparison between treated samples with Epigallocatechin or Angiotensin II or Losartan or PD123319 and ECG in comparison to all treated samples with or without ECG after 1,3 or 7 days of culture.***

(Two ways ANOVA)

Previously was also assessed cell viability of HUVECs in presence of this natural reductants cultured for 1, 3 or 7 days, and the data, that were not reported graphically showed that: Hyperthermia increases HUVECs viability especially in samples treated in presence of Epigallocatechin-gallate after stimulation with Angiotensin II alone, also Hypothermia increases HUVECs viability for the samples treated with Angiotensin II and PD123319 . Then , our data show that Hypertermia modulates ROS generations infacts this parameter decreases in damaged samples in presence of Angiotensin II stimulation or Losartan or PD123319 so in this mechanisms are involved both Angiotensin II pathways. In Hypothermia ROS levels are also modulate and decrease in all tested samples .

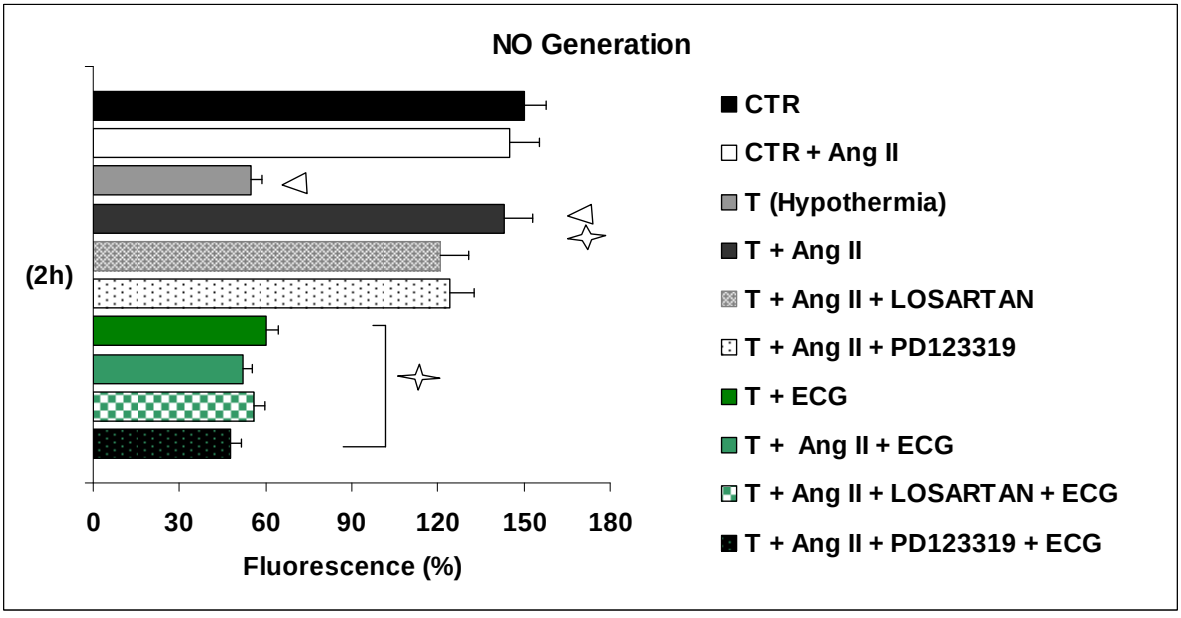
For Nitric Oxide evaluation is used a specific label DAF-2DA and the data obtained show that:



(Fig.30a)Evaluation of NO production levels in HUVECs

° p<0.05 (comparison between Hyperthermic sample with or without Ang II and AT_1R a, AT_2Ra respect to samples treated with Epigallocatechins and ECG + Ang II +PD123319.

(One way ANOVA)



***(Fig.30b)* Evaluation of NO production levels in HUVECs**

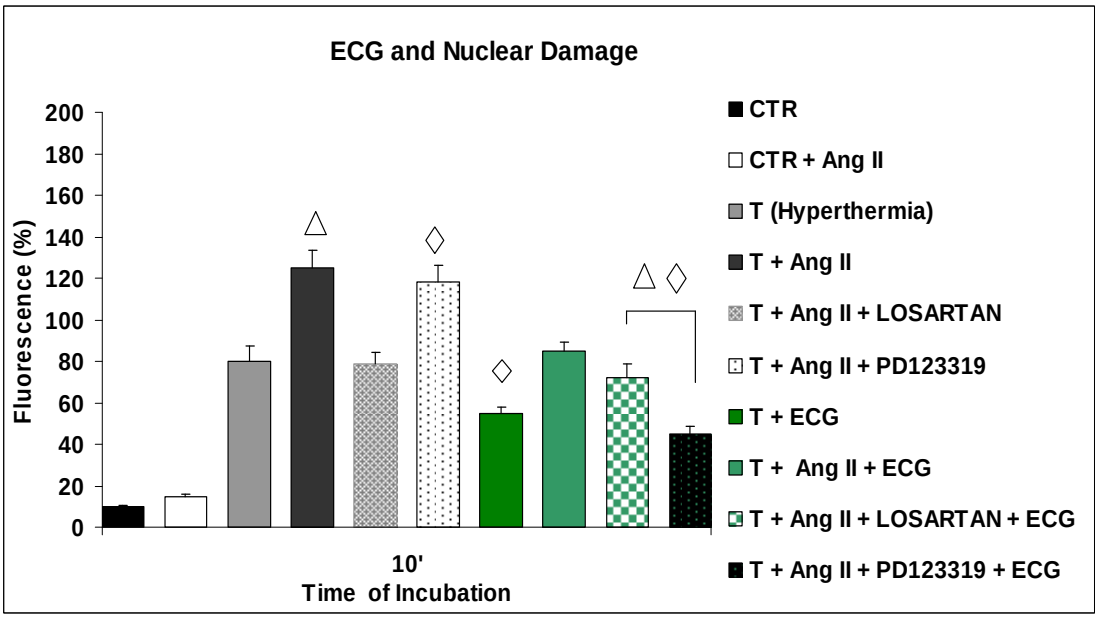
∆ *p<0.05 (comparison between Hypothermic samples and samples treated with Ang II)*

✧*p<0.05 (comparison between Hypothermic sample with Ang II respect to samples treated with ECG.*

(One way ANOVA)

In ***(Fig.30a)*** and ***(inFig.30 b)*** are reported Nitric Oxide levels generated :

in Hypothermia Nitrix Oxide production levels are modulates by presence of ECG by Angiotensin type 1 receptor pathway; in Hypothermia NO levels increase in presence of Angiotensin II and decrease in presence of ECG in all samples tested with this specific antioxidant.Consecutively, effects of Epigallocatechin-gallate are monitored with the aim of research a presence of a nuclear damage in HUVECs after Hyperthermic or Hypothermic damage in presence of hormone and Losartan or PD123319 and effects of riparation or rigeneration at nuclear level.

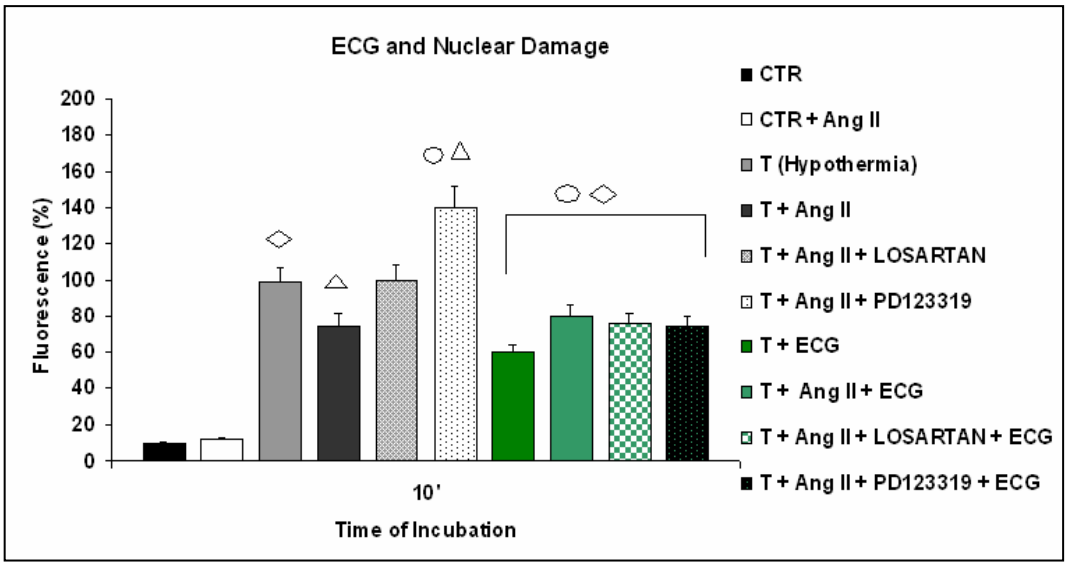


(Fig.31a) Analysis of Propidium Iodide for nuclear damage:

∆ p<0.05 (comparison between hypertermic sample with Ang II and hyperthermic sample with Ang II, Losartan or PD123319 and ECG);

◊p<0.05 (comparison between treated sample with Ang II and PD123319 and hyperthermic sample with ECG or Ang II+ Losartan or PD123319 and ECG).

(Two ways ANOVA)



(Fig.31b) Analysis of Iodide Propidium for nuclear damage:

◊ p<0.05 (comparison between samples with Ang II and PD123319- hypothermic samples with ECG and Ang II +Losartan or PD123319 and ECG);

°p<0.05 (comparison between treated sample with Ang II and PD123319 and hypothermic sample with ECG or Ang II+ Losartan or PD123319 and ECG);

∆p<0.05 (comparison beetween hypothermic samples with Ang II and idem samples and PD123319).

(Two ways ANOVA)

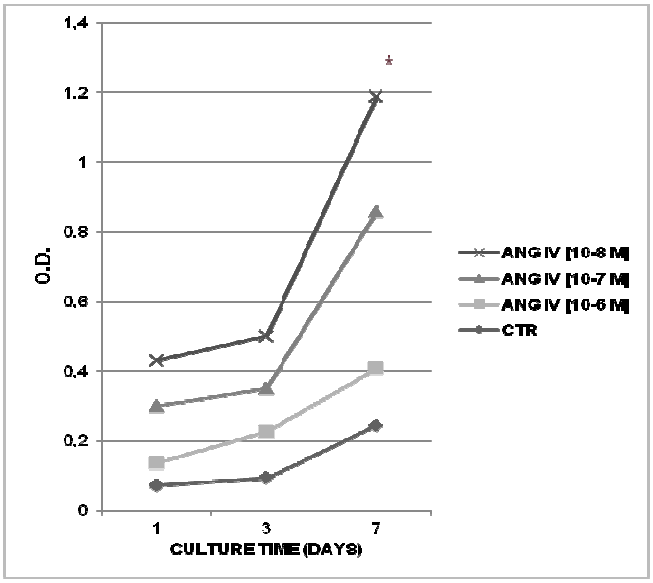
Our analysis confirme that exisist a nucear damage in Hyperthermic and Hypothermic conditions on HUVECs nuclei but it is less present in samples treated with Epigallocatechin-gallate after stimulation of Angiotensin II and both receptorial antagonists. In the ***(Fig.31a)*** is reported this effect specially for both Angiotensin II pathways; in the ***(Fig.31b)*** nuclear damage in HUVECs after 10 minutes of incubation with Iodide Propidium is attenuated with the addition of Epigallocatechin-gallate.

Study 2

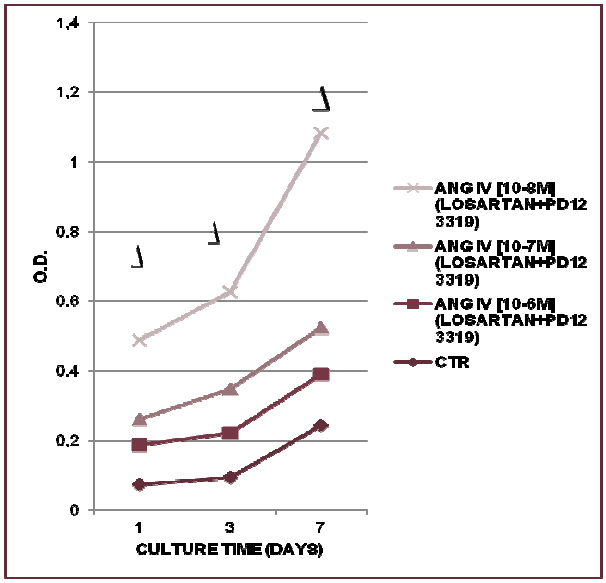
5.4 Effects of Angiotensin IV on Cellular Viability

In a third series of experiments conducted, Viability test with MTT is performed. Analysis is based on evaluation of effects of stimulation by Angiotensin IV and receptorial antagonists (Losartan and PD123319)of Angiotensin II in HUVECs placed in culture for a time of 1, 3 and 7 days. Cells are considered at physiological condition .When the cells are in a confluent state, after microscope observation, are tripsinized and setting up (2×10^4.) for each sample, a number of ten replicas are performed. HUVECs are incubated after stimulation with Angiotensin IV considering three different final concentration [10^{-6}; 10^{-7}; 10^{-8} M]and receptorial antagonists (Losartan and PD123319) [10^{-7} M] with MTT .

The ***(Fig. 32 a)*** and ***(Fig. 32b)*** shows the results obtained:



(Fig,32a) Evaluation of HUVECs Viability in physiological conditions in presence of Angiotensin IV:
****p<0.05(comparison between Ang IV[10^{-8}M] after 7 days and all samples).***
(Two way ANOVA)



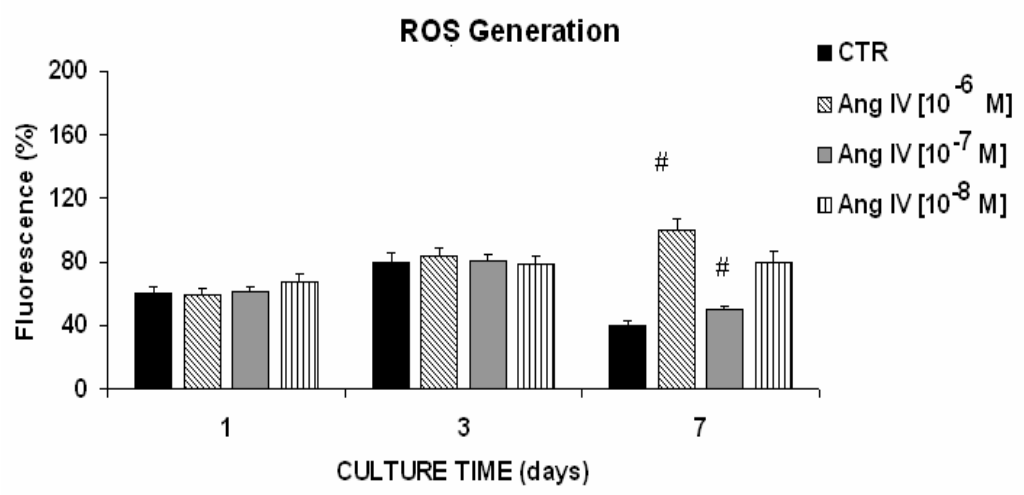
(Fig.32b)Evaluation of HUVECs Viability in physiological conditions in presence of Losartan and PD123319- Angiotensin IV :
Δp<0.05(comparison between Ang IV [10^{-8} M] after 1-3 and 7 days with samples is significant. (Two ways ANOVA)

This data show that only Angiotensin at a final concentration of [10^{-8} M] increase HUVECs viability after 7 days of culture in physiolocical conditions and this effect is confirmed by Losartan and PD123319 in addition to Ang IV [10^{-8} M] stimulations in the same samples at 1, 3 and 7 days.

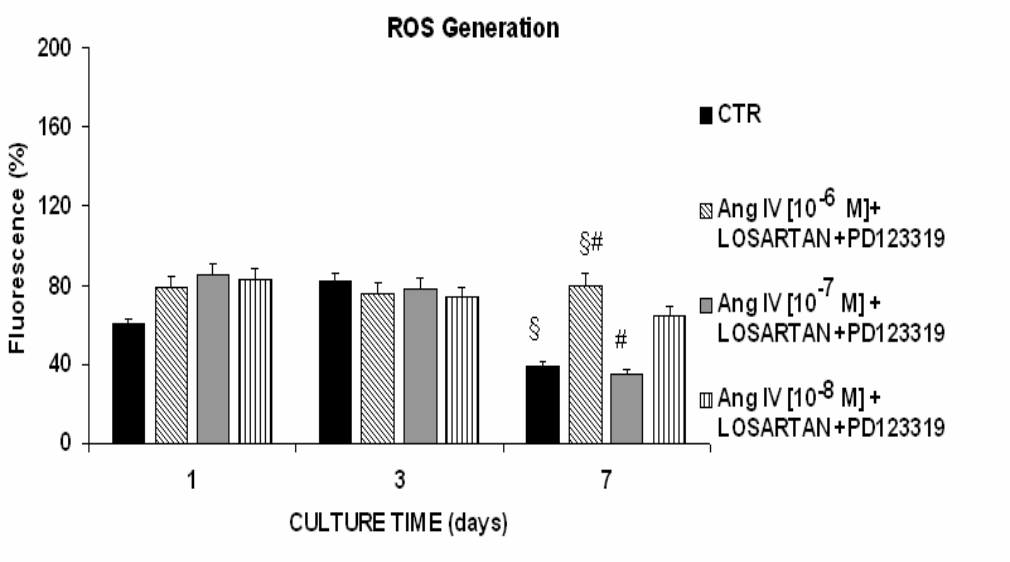
5.3.1 Effects of <u>Angiotensin IV</u> on free radicals generation (ROS and RNS)

Different final concentration of Angiotensin IV [10^{-6}; 10^{-7};10^{-8} M] are analyzed alone or in presence of both Angiotensin II receptor antagonists on evaluation of reactive oxygen species production and reactive nitrogen species, especially Nitric Oxide generation . HUVECs (2×10^{-4} M) are tested after 1, 3 and 7 days of culture:

- by H_2DCF-DA label for ROS detection, ***(Fig.33 a)*** and ***(Fig.33 b)*** encompass analysis performed in fluorescence by Olimpus IX50 microscope;
- by DAF-2DA label for Nitric Oxide detection, ***(Fig.34a)*** and ***(Fig35 b)*** show results obtained.



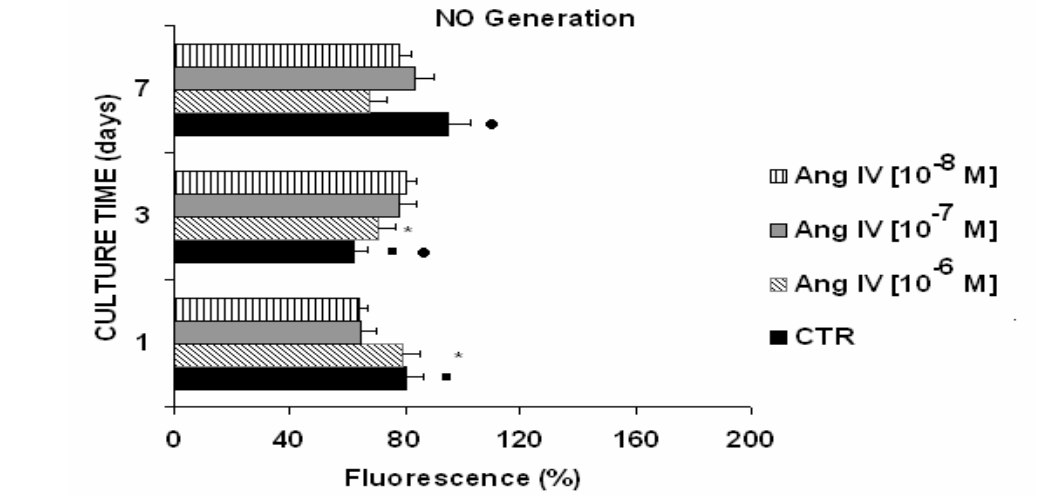
<u>(Fig.33 a)</u>Evaluation of ROS generation in presence of Ang IV in physiological conditions: #p<0.05 (comparison between Ang [10-6 M] and Ang [10-7 M] after 7 days is significant. (Two way ANOVA)



(Fig.33 b) Evaluation of ROS generation in presence of Ang IV , Losartan and PD123319 in physiological conditions:

§# p<0.05 (comparison between control and Ang IV [10^{-6} M] is significant;
Ang IV [10^{-6} M] and Ang IV[10^{-7} M] after 7 days is significant) (Two ways ANOVA)

Results obtained show that ROS generation increased in presence of higher concentrations of Angiotensin IV [10^{-6} and 10^{-7} M] especially at 7 days of culture and also in presence of Angiotensin II receptor antagonists these results is significant in physiological conditions in HUVECs.

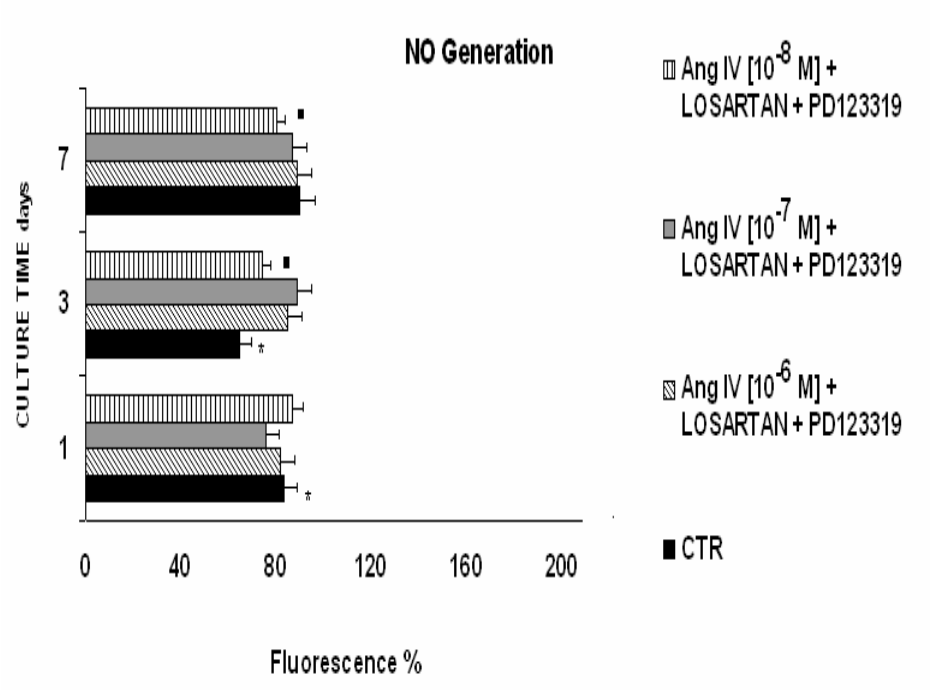


(Fig.34a)Evaluation of Nitric Oxide levels in HUVECs after 1-3 and 7 days of culture in presence of Ang IV at different concentrations:

▪p<0.05 (significant difference between control samples after 1 and 3 days)

****p<0.05(significant difference between samples with Ang IV [10^{-6} M] after 1 and 3 days)***

●p<0.05(significant difference between control samples after 3 and 7 days of culture)



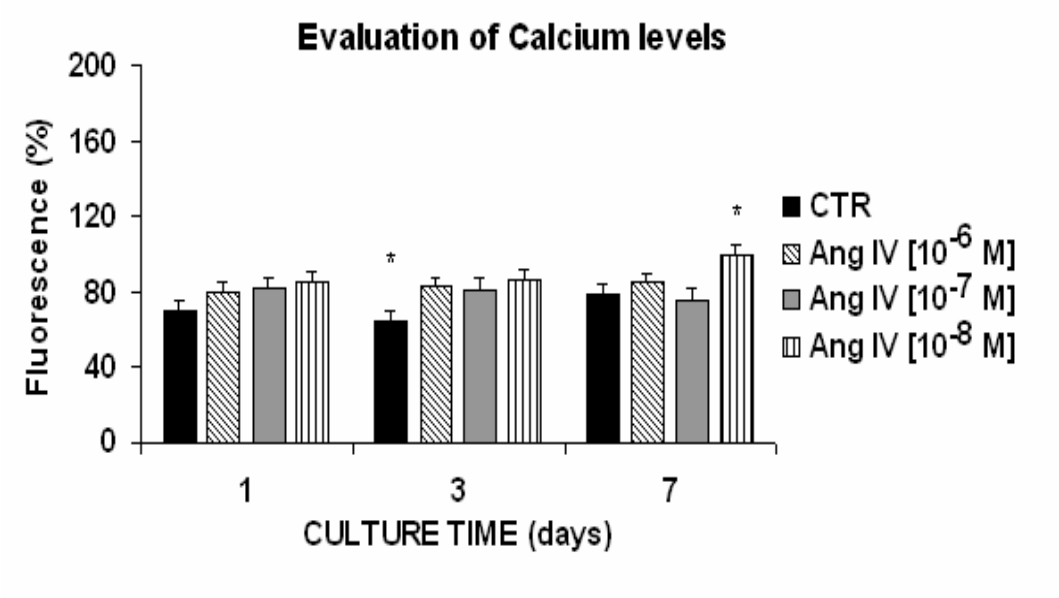
(Fig 34b)Evaluation of Nitric Oxide levels in HUVECs after 1-3 and 7 days of culture in presence of Ang IV at different concentrations and receptorial antagonists of Ang II (Losarrtan and PD123319):
****p<0.05(significant difference between control samples after 1 and 3 days)***
▪p<0.05 (significant difference between treated samples in presence of Ang IV [10-8 M]+ Losartan + PD123319 after 3 and 7 days).

Observing different Nitrix Oxide levels after stimulation with Angiotensin IV at three different concentrations in HUVECs the physiological response at intraceòlular levels increase alone in samples stimulated with Ang IV [10^{-8} M] alone and this effect is present also in presence of Losartan and PD123319 between 3 and 7 days.

5.3.2 Effects of Angiotensin IV on Calcium Levels

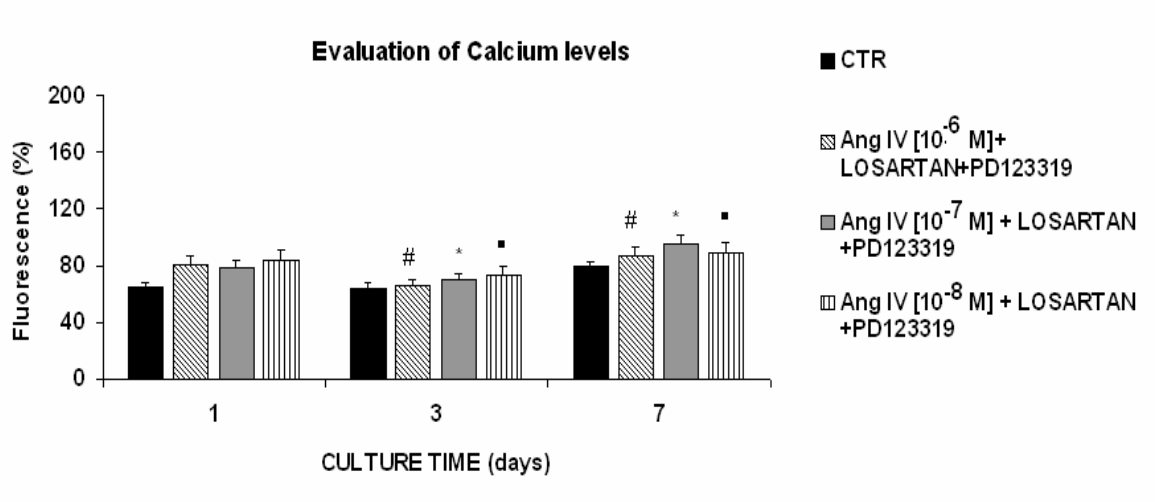
Analysis of Calcium levels is based on evaluation of effects of stimulation by Angiotensin IV and receptorial antagonists (Losartan and PD123319)of Angiotensin II in HUVECs placed in culture for a time of 1, 3 and 7 days. Cells are utilized at physiological condition .When the cells are in a confluent state, after microscope observation, are tripsinized and setting up (2×10^{4}.) for each sample, a number of ten replicas are performed. HUVECs are incubated after stimulation with Angiotensin IV considering three different final concentration [10^{-6}; 10^{-7}; 10^{-8} M]and receptorial antagonists (Losartan and PD123319) [10^{-7} M] with Fluo-3AM.

The ***(Fig. 35a)*** and ***(Fig.35b)*** shows the obtained results :



***(Fig.35a)** Analysis of Calcium generation in presence of Angiotensin IV in physiological conditions:*

p<0.05 (significant difference between control samples after 3 days of culture and samples treated with Ang IV [10^{-8} M]after 7 days of culture)



(Fig.35b)Analysis of Calcium levels in presence of Angiotensin IV, Losartan (AT_1Ra) and PD123319 (AT_2Ra) in physiological conditions:

#p<0.05 (significant difference between controls after 3 days with the same samples after 7 days of culture)

****p<0.05 (significant difference between samples with Ang IV [10^{-6} M] after 3 days and those with Ang IV [10^{-6} M] after 7 days of culture)***

▪p<0.05 (significant difference between samples with Ang IV [10^{-7} M] after 3 days and those after 7 days of culture)

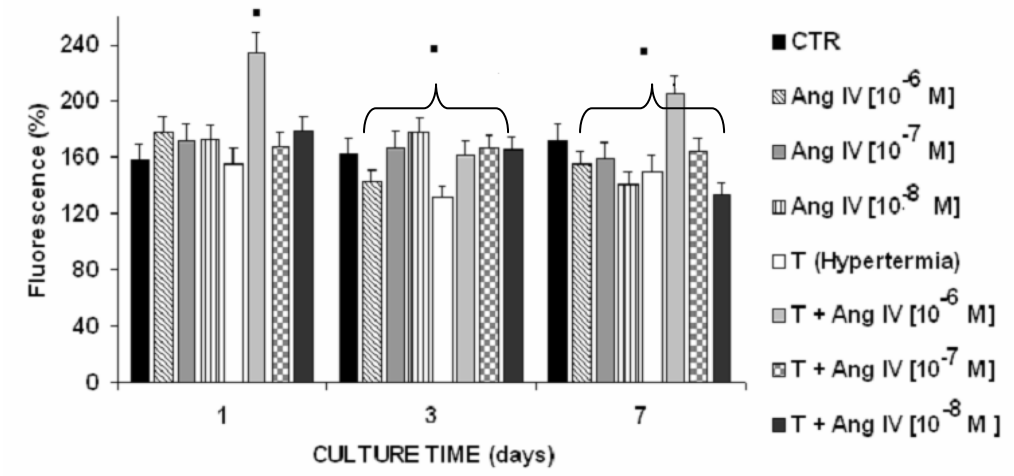
Data show that only Angiotensin IV [10^{-8} M] increses Calcium intracellular levels after 7 days of culture if considered alone, when analyzed also interaction with Losartan and PD123319 toghether with Angiotensin IV [10^{-8} M] between 3 and 7 days of culture Calcium levels in HUVECs increase.

5.3.3 Evaluation of Angiotensin IV stimulation in Hyperthermia and Hypothermia on Cellular Viability

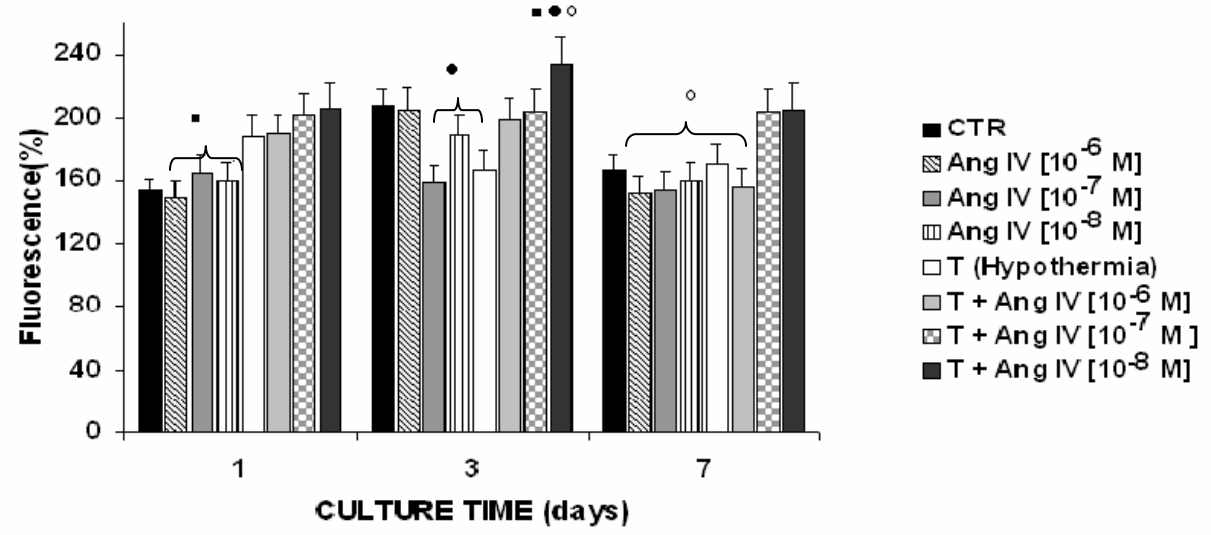
In a final series of experiment conducted, Viability test with Acridine Orange (AO) is performed. Analysis is based on evaluation of effects of stimulation by Angiotensin IV and receptorial antagonists (Losartan and PD123319)of Angiotensin IV on HUVECs placed in culture for a time of 1, 3 and 7 days. Cells are considered both at physiological condition and

in Hyperthermia or Hypothermia after treatments in a specific sterile waterbath under a laminar flow hood for 30 minutes .HUVEcs are tripsinized and setting up (2×10^4) for each sample, ten replicas are performed. HUVECs are incubated after stimulation with Angiotensin IV alone considering three different final concentration [10^{-6}; 10^{-7}; 10^{-8} M]alone with Acridine Orange .

The ***(Fig.36a)*** and ***(Fig.36b)*** shows the results obtained:



(Fig.36a)Evaluation of HUVECs viability in control samples and Hyperthermic samples in presence of several concentrations of Angiotensin IV after 1-3-7 days of culture
▪p<0.05 (significativ difference between Hyperthermic samples in presence of Angiotensin IV [10^{-6} M] after 1 day of culture and all samples considered after 3 and 7 days).



(Fig.36b)Evaluation of HUVECs viability in control samples and Hypothermic samples in presence of several concentrations of Angiotensin IV after 1-3-7 days of culture.

▪ p<0.05 (significantv difference between Hypothermic samples in presence of Ang IV [10-8 M] after 3 days of culture and all control samples considered after 1 days);

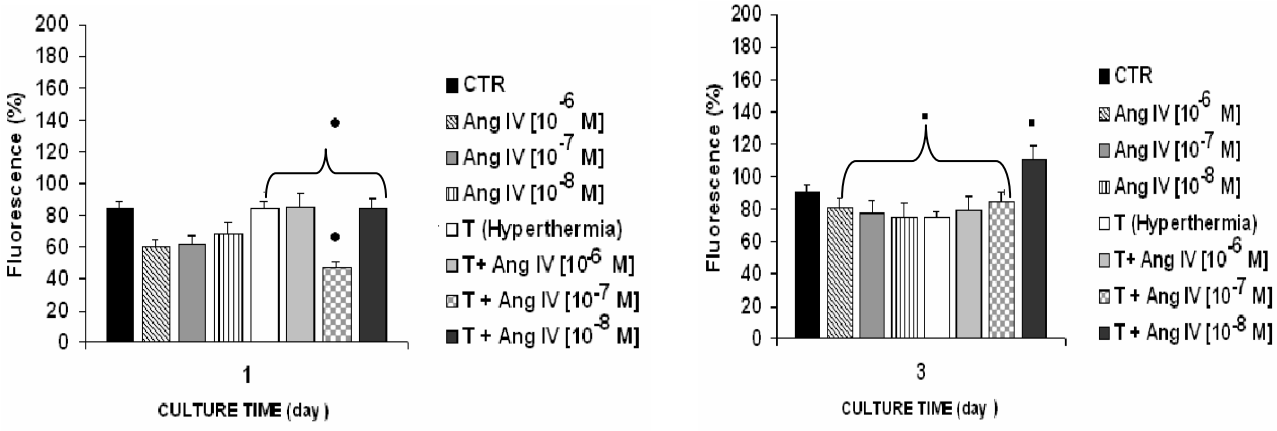
●p<0.05 (significant difference between Hypothermic samples in presence of Ang IV [10^{-8} M] after 3 days of culture and control samples in presence of Ang IV [10^{-6},10^{-7} M] considered after 3 days);

°p<0.05 (significant difference between Hypothermic samples in presence of Ang IV [10^{-8} M] after 3 days of culture and control samples in presence of several concentrations of Ang IV + Hypothermic samples + samples treated with Ang IV [10^{-6} M] after 7 days).

As evidenced by the results analyzed by NIH J programs v.1.61 , in Hyperthermia HUVECs viability in the time increases only in presence of Angiotensin IV [10^{-6} M] in tested samples and in treated samples. On the contrary Hypothermia modulates HUVECs Viability in samples stimulated with Ang IV [10^{-8} M] after hypothermic damage.

5.3.4 Evaluation of Angiotensin IV stimulation in Hyperthermia and Hypothermia on ROS generations

Evaluation of ROS generation by Angiotensin IV and Angiotensin II receptorial antagonists (Losartan and PD123319) stimulation in HUVECs placed in culture for a time of 1, 3 and 7 days is performed by selective H_2DCFDA labeling. Cells are observed at physiological condition and in Hyperthermia (40°-41°C) or Hypothermia (33°-34°C) after treatments in a steril waterbath in a laminar flow hood for 30 minutes .HUVEcs are tripsinized and setting up (2×10^4.) for each sample, a number of ten replicas are performed. HUVECs are incubated after stimulation with alone Angiotensin IV considering at three different final concentration [10^{-6}; 10^{-7}; 10^{-8} M] and observed by labelling and microfluorescence analysis .

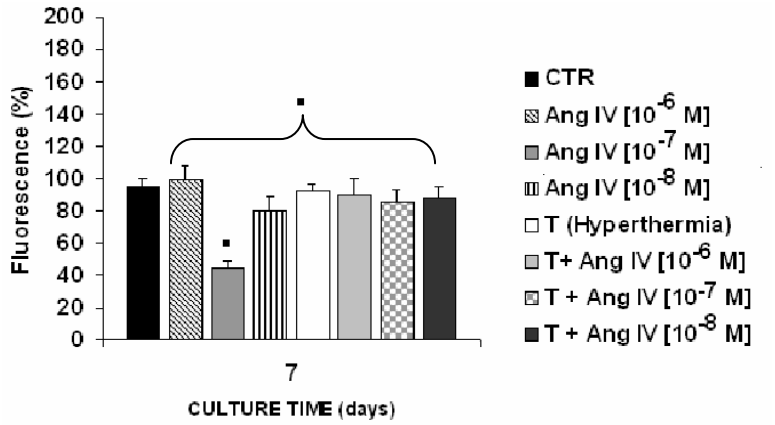


(Fig.37a) Evaluation of ROS generation in HUVECs in presence of several concentrations of Angiotensin IV after 1 days of culture.

● p<0.1 (significant difference between hyperthermic samples in presence of Ang IV [10^{-7}M] and all treated samples at different concentrations of Ang IV).

(Fig.37b) Evaluation of ROS generation in HUVECs in presence of several concentrations of Angiotensin IV after 3 days of culture.

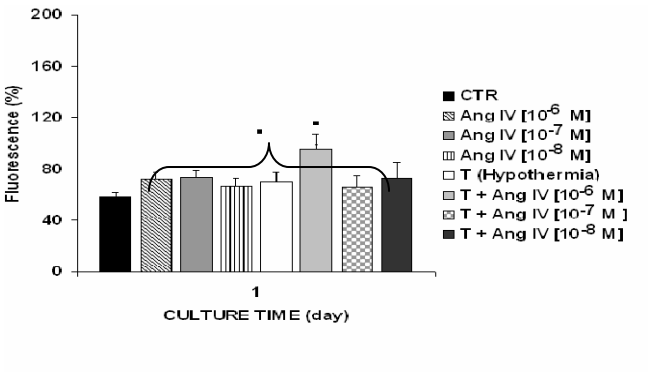
▪ p<0.05 (significant difference between hyperthermic samples in presence of Ang IV [10^{-8}M] and all samples: controls and treated with different concentrations of Ang IV.

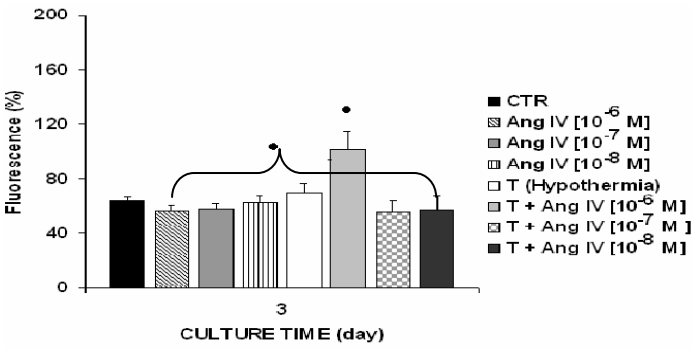


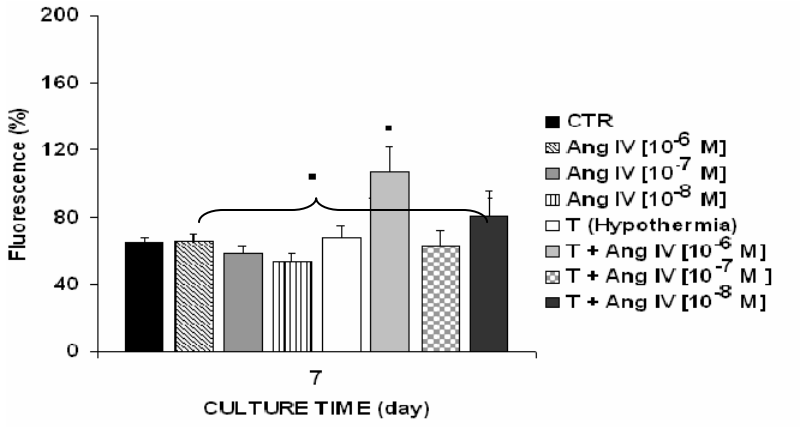
(Fig.37b) Evaluation of ROS generation in HUVECs in presence of different concentrations of Angiotensin IV after 7 days of culture.

▪p<0.05 (significant difference between control samples with Ang IV [10-7M] and all other samples).

ROS generation decreases in Hyperthermic samples and especially in presence of Ang IV [10^{-7} M] after 1 day of culture, but at 3 day of culture samples manifested an increase in percentage fluorescence only for treated samples after stimulation with Angiotensin IV [10^{-8} M], finally at 7 days of culture also the control samples after stimulation with Angiotensin [10^{-7} M] show a decrease in this free radical species.







(Graph 38)

(a)Evaluation of ROS generation in HUVECs in presence of different concentrations of Angiotensin IV after 1 day of culture.

▪p<0.05 (significant difference between Hypothermic samples with Ang IV [10-6M] and all other samples).

(b)Evaluation of ROS generation in HUVECs in presence of different concentrations of Angiotensin IV after 3 days of culture.

●p<0.05 (significant difference between Hypothermic samples with Ang IV [10-6M] and all other samples).

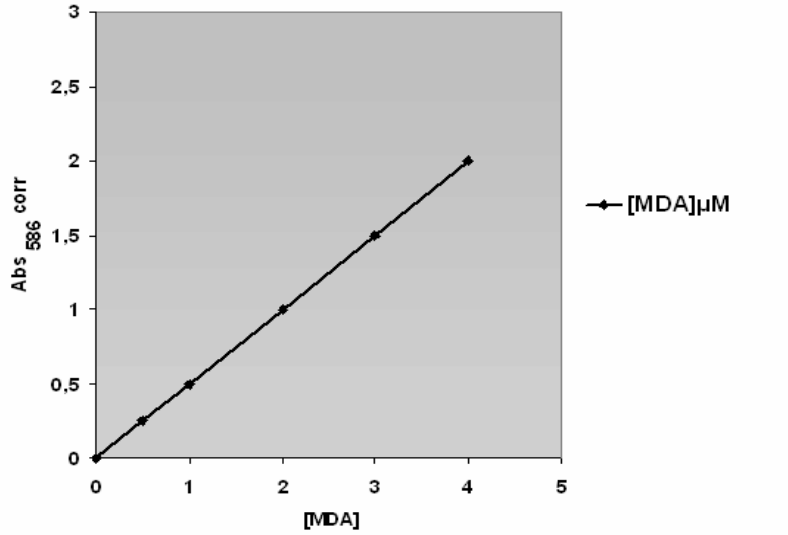
(c)Evaluation of ROS generation in HUVECs in presence of different concentrations of Angiotensin IV after 7 days of culture.

▪p<0.05 (significant difference between Hypothermic samples with Ang IV [10-6M] and all other samples).

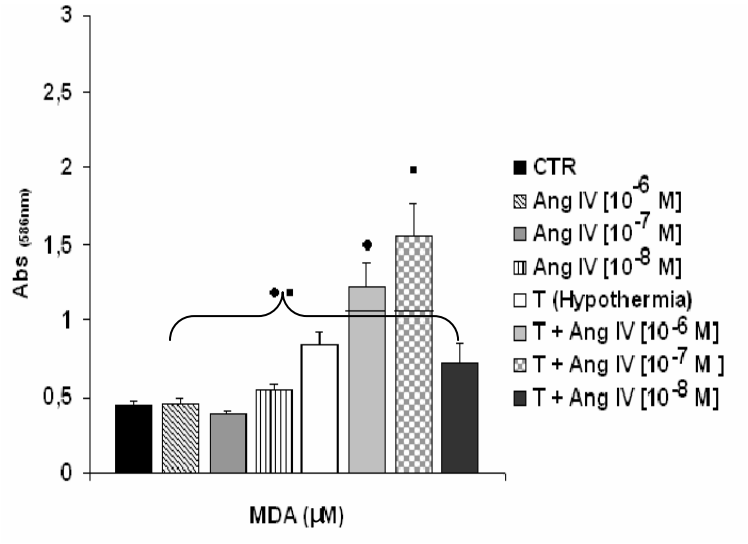
ROS generations in Hypotermia increases in culture of HUVECs treated after stimulation of Angiotensin IV [10^{-6} M] alone at 1, 3 and 7 days. On the basis of these results in Hypothermia we wanted to extend our testing to check any damage on cell membranes of HUVECs to verify that the stimulation with Ang IV at a final concentrations of [10^{-6} M]was really involved in the oxidative stress mechanisms in Hypothermia.

5.3.5 Evaluation of Angiotensin IV stimulation in Hypothermia on Lipid peroxidation products

After Hypothermic damage induced as decribe previously and after stimulation with Angiotensin IV [10^{-6};10^{-7};10^{-8} M] HUVECs are treated in base MDA and Conjugated Dienes Protocols with AK-171 kit. Data obtained shown that:



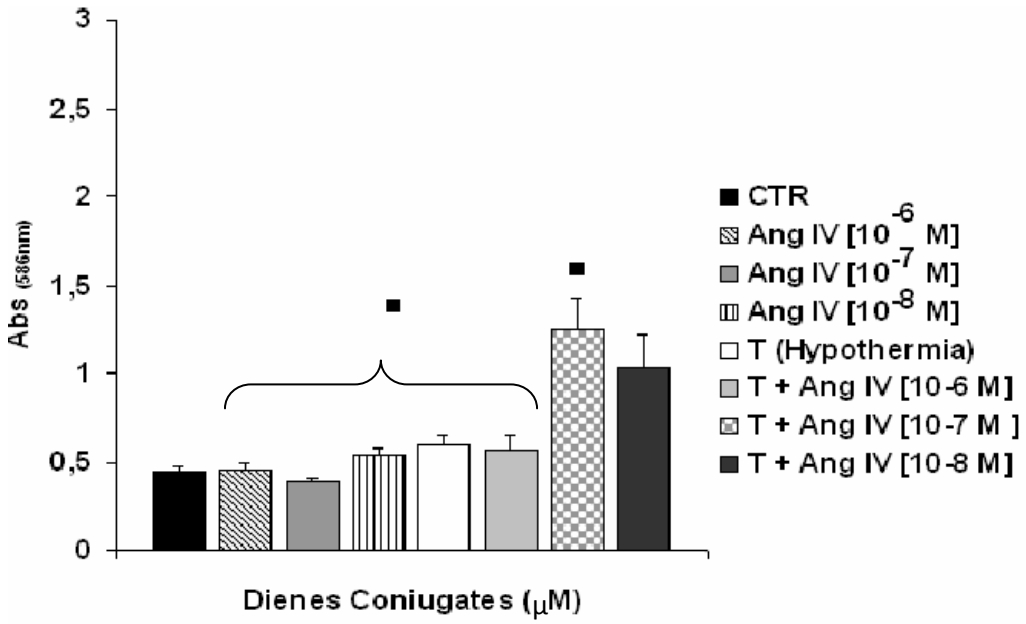
(Fig.39) Plot of A586 corr vs [MDA] for the standard in the reaction



(Fig.40)Evaluation of [MDA] formation in presence of different concentrations of Ang IV in Hypothermia.

▪p<0.05 (significant several between Hypothermic samples with Ang IV [10^{-7} M] and all samples);

●p<0.05 (significant several between Hypothermic samples with Ang IV [10^{-6} M] and all samples);



(Fig.41)Evaluation of Conjugated Dienes formation in presence of several concentrations of Ang IV in Hypothermia.

▪ ***p<0.05 (significant difference between Hypothermic samples with Ang IV [10^{-7} M] and all samples);***

Also in this experimentations results confirm that is present an increase of malondialdehyde adduct in samples treated with Ang IV [10^{-6} M] in Hypothermia after 1,3 and 7 days of culture toghether increase of Conjugates Dienes product in samples treated in Hypothermia at idem final concentrations. Further work shall use the decribed methods to verify also the contribution of Nitric Oxide and Calcium levels under the action of both Ang IV and physical or chemical oxidative stress

Discussion

About the effects of Angiotensin II on metabolic alterations in the production of peroxides in Human umbilical vein endothelial cells we observed that they induce a condition similar to endothelial dysfunction, the achieved results, in the context of this research thesis, show that the induction of dysfunction by hyperthermia in endothelial cells the viability does not appear to be significantly altered, whereas after hypothermic damage a relevant increase in cell proliferation is observed, as evaluated by MTT test. Specifically the pathway that appears to be involved is mediated by Angiotensin II type 1 receptor. In Hyperthermia the ROS levels increased under the action of Angiotensin II [10^{-7} M] and under the action of both AT_1 and AT_2 receptorial antagonists.

After Hypothermia the ROS levels decrease after 1 day of HUVEcs culture, but tend towards increase after 7 days under the action of Angiotensin II by AT_1 receptor pathway. About the mechanisms of lipid oxidation (studied by MDA test) we observe an increase in products of oxidation damage after hyperthermia, as analyzed using spectrophotometric methods, in the presence of such damage. The hypothermic effects stay unchanged as evaluated by the aforementioned analysis on HUVECs in culture after 1-3 or 7 days. The induction of a state similar to Endothelial dysfunction, in the presence of the selective inhibitor of eNOS. ADMA is used to determine the contribution of the radical Nitric Oxide response to oxidative stress. In hyperthermic conditions and in the presence of ADMA [10^{-4}, 0.5×10^{-4} M] and Angiotensin II [10^{-7} M] there is still an effect of increased ROS levels, especially Superoxide radicals, in a particular way, is analyzed by specific probe dihydroethidium in the cellular compartment. At the final concentration [0.5×10^{-4} M] of ADMA in presence of Angiotensin II we observed an increase in SuperOxide levels. In Hypothermia in presence of ADMA [10^{-4} M], Angiotensin II [10^{-7} M] and both receptorial antagonists (Losartan or PD123319) an increase in SuperOxide production in HUVECs also is present. This is evidenced in HUVECs even in presence of Apocynin [10^{-5} M] (inhibitor of NADPH oxidase) after 10 and 40 minutes of treatment. ADMA [0.5×10^{-4} M], in combination with Hypothermic damage and Angiotensin II, modulates a decrease of SuperOxide generation in HUVECs especially after 40 minutes preincubation.

In order to mitigate the damages to synthesis of Superoxide and their derivatives we chosen to use the natural reducing agent epigallocatechin gallate, that in the literature is regarded as the more effectiv flavonoid for its high reducing power. In fact, the heat treatment in the presence of Angiotensin II and both its receptor antagonists. This is also verified in the presence of the ECG antioxidant, that allows an increase in cell viability of HUVECs in culture after all studied incubation times after Hypothermia. Increased Angiotensin II concentrations further enhanced ROS in the samples under the action of PD123319. A similar heat treatment in the presence of Epigallocatechin gallate and Angiotensin II evidences a decrease in the production of ROS pathway by selective inhibition of receptor AT_2. The generation of Nitric Oxide is AT_1 pathway mediated, so that under Epigallocatechin gallate action is present a significant decrease in the generation of this metabolite. In fact the nuclear damage detected by Propidium Iodide in the presence of Epigallocatechin-gallate furthelyr decreases in the culture media of HUVECs.

In the second step of the study the action of Angiotensin IV, also known as Ang II (3-8), was analyzed. This peptide has a more specific membrane receptor called AT_4 receptor. Since in subcellular preparations the binding of Angiotensin IV is effective between [10^{-5} and 10^{-8} M] probably it improves the viability of HUVECs after 7 days of culture, we have explored the effectiveness of Agiotensin IV between [10^{-6} and 10^{-8}M] on HUVECs. We also tested the Angiotensin IV [10^{-8} M] after 7 days of culture and it still improves the viability of HUVECs. At the same concentration Angiotensin IV, ROS generation in HUVECs is not altered but at higher final concentrations increases ROS levels both by Angiotensin IV alone and Angiotensin II receptorial antagonists. At the concentration of [10^{-8}M] are present increased levels of intracellular Calcium after 7 days of culture in physiological conditions. However Nitric Oxide generation of the same final concentrations of Angiotensin IV increases after 3 and 7 days of culture. On the basis of these preliminary data Angiotensin IV acts on isolated viable cells without alteration on ROS levels. Nitrix Oxide levels increase only between 3 and 7 days of culture. Then we went to check these parameters in the presence of heat treatment: in Hyperthermia and we observe increased viability levels only in samples stimulated, after thermal treatment, with Angiotensin IV [10^{-6} M], ROS generation decreases after stimulation with Ang IV [10^{-7} M] and increases with Ang IV [10^{-8} M]. In Hypothermia viability of HUVECs increases at lower final concentration of Ang IV [10^{-8} M].

On the contrary ROS generation increases in samples stimulated with Ang IV [10^{-6} M], condition. These data are confirmed by increase at the same concentration of Angiotensin IV in idem samples studied for malondialdehyde production and Conjugates Dienes formation.

Conclusion

In conclusion we can say that with little effect on cell viability peptide, Angiotensin II induces a significant increase in ROS levels and lipid oxidation products in both thermal conditions, but more effectively in Hypothermia. Considering the eNOS inhibitor ADMA in our experimental data we obtained an intracellular response of increased superoxide radical levels. This happens also after thermal treatment by both Angiotensin II pathways. This effect is mainly due to the interaction of selective e NOS inhibitor ADMA and Angiotensin II, mainly in reference to the mechanisms for the generation of reactive oxygen species. The contribution of Nitric Oxide and combination products of ROS are negligible in the presence of ADMA [10^{-4} M]. Only at a lower concentrations of ADMA is possible to observe a decrease in the production of Superoxide. Overall, the action of the main changes due to thermal damage and the action of Angiotensin II is mediated by AT_1 receptor, the recovery of such damages has been accomplished through the action of the reducing natural agent ECG even at relevant concentrations. Once again we can confirm that the peroxidative damage is primarily mediated via AT_1R in all tested (thermal and chemical) treatment combinations. The way to reduce the effect is to add reducing agents of natural origin. If the levels used *in vitro* reproduced *in vivo* ones, however, high doses of this flavonoid should be required . Only the use of many reducing agents from different sources could achieve the equivalent effect without causing collateral damages due to the means of conveyance (e.g. alcohol). On the basis of the described data, Angiotensin IV [10^{-8} M] is an effective binding agent and AT_4R improves the functionality of the HUVECs. This suggests that its action is optimal *in vitro* at that same concentration. By using Angiotensin II inhibitors of AT_1R and AT_2R we confirmed the data, further suggesting that AT IV is effective at concentration of this order. Even under conditions similar to those of endothelial dysfunction ***(Yang, 2011)*** the range of Angiotensin IV remains at current levels lower than concentrations studied as effective *in vivo*. The negative effects after induction of endothelial damage may be induced only at concentrations higher[10^{-6} and 10^{-7} M] of Angiotensin IV on the processes of oxidative stress and lipid peroxidation. Overall Angiotensin IV tested at lower concentrations enhances the functionality of HUVEcs while at higher concentrations appears to induce effects similar to those of Angiotensin II, in fact, in literature the affinity constant of inhibitors of Angiotensin II is higher than the Angiotensin IV one in several human cells.

The way AT_4R is activated by Angiotensin IV [10^{-8}M] can not be assigned to the function of collateral pathways in the mechanisms of AT_1 and AT_2 receptors in the regulation of oxidative metabolism in target cells, but this effect happens only at higher concentrations

Publications and Abstracts

Carino A.R., Costanzo S., Bruno R and G. Martino. *" Endothelial Dysfunction : ROS/RNS production, Oxidative Stress and Catechins in Hyperthermia on HUVECs".* ***Oral Presentation of 62° Congresso della Società Italiana di Fisiologia, 23-25 Settembre 2011, Sorrento (NA);***

Carino A.R., Procopio A., Bruno R., Mazzulla S. and Martino G. *" The ADMA/Angiotensin II pathways in endothelial dysfunction".* ***Contributo a Fifth Meeting of Young Researches in Physiology, Sestri-Levante, 8-10 Giugno 2011 ;***

Carino A.R., Ranieri G.,Bruno R.,Miano A., Mazzulla S., Martino G. *" Effect of the Cathechins in the mechanism of production/degradation of ROS and RNS in hypothermia on HUVECs".* ***Atti del Convegno "61° Congresso Nazionale della Società Italiana di Fisiologia", Varese, 15-17 Settembre 2010, Vol. 200, pp 84;***

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"** Natural reductants protect red blood cell membranes in psoriasis patients". **Atti del Convegno "61° Congresso Nazionale della Società Italiana di Fisiologia", Varese, 2010, pp 97-97;

Mazzulla S. , Nicoletta V. , Costabile A. , Esposito G. , Mosello A. , Cipolla F. , Carino A. R. and Martino G. , " Peroxide increase, haemolytic radicalic damages induced by smoke habit of CALABRIA resident population. Effect of nutritional Mediterranean habits.". Atti del convegno "59° Congress of the Italian Physiological Society", Cagliari, 17-19 September, 2008, ACTA PHYSIOLOGICA ,Wiley Blackwell Publishing: Oxford, 2008, Vol. 194, pp. –;

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Nicoletta V. , Carino A. R. , Mazzulla S. , " Evaluation of Oxidative stress in pregnant rat". Atti del convegno "60° National Congress of the Italian Physiological Society", SIENA, 23-25 September, 2009, ACTA PHYSIOLOGICA Wiley - Blackwell: Oxford, 2009, Vol. 197, pp. 87- ;

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Martini A., Oro Serena, Floccari M., Pucci E., <u>Carino A.R</u>. and Martino G. *"Effect of CNP on Human Umbilical Vein Endothelial Cell Migration".* ***Contributo a 62° Congresso Società Italiana di Fisiologia, 25-27 Settembre 2011, Sorrento (NA);***

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LIST OF ABBREVIATIONS

ADMA: asymmetric dymethilarginine

ACD: acil-coenzyme dehydrogenase

Ach: acethylcholine

ADP: adenosine diphosphate

ADP:adenosine diphosphate

Ang II :angiotensin II

BH4: tetrahydrobiopterin

CaM: Calcium/Calmodulin

CAMs: cell adhesion molecules

Carb P: Carboxypeptidase

COX-2: Cyclooxygenase 2

CRP: C-reactive protein

CVD: cardiovascular disease

DDAH dimethylarginine-dimethylaminohydrolase

EDRF: endothelium derived relaxing factors

EDNO : endothelium derived nitric oxide

EGCG: epigallocatechin gallate

eNOS endothelial nitric oxide synthase

EPC: endothelial progenitor cell

ERK: extracellular signal regulated kinase

FAD: flavin adenine dinucleotide

FMN: flavin mononucleotide

GM-CSF: granulocyte-macrophage colony

ICAM-1: inter cellular adhesion molecule-1

IL-1: Interleukin-1

IRAP: insulin – regulated aminopeptidase

LDL: low density lipoprotein

LOX-1: LDL receptor 1

MCP-1 macrophage chemoattracnt peptide 1

MMPs: metalloprotease

NADPH: nicotinamide adenine dinucleotide phosphate

NADPH:dinucleotide adenin phosphate

NO: nitric Oxide

PAF: platelet-activating factor

PAI-1: plasminogen activator inhibitor-1

PGI2: prostacyclin

PRMT: methyltransferasy arginin protein

P-selectin : platelet adhesion molecule

RNS: nitrogen reactive species

ROS : reactive oxygen species

SOD: superoxide dismutase

TFPI: tissue factor pathway inhibitor

TNF-α : tumor necrosis factor α

t-PA:plasminogen activator

VCAM: vascular adhesion molecule-1

vWF : von Willebrand factor

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