UNIVERSITÀ DELLA CALABRIA
CAMPUS DI ARCAVACATA

Università della Calabria

FACOLTÀ DI SCIENZE MATEMATICHE FISICHE E NATURALI

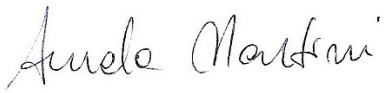
Dipartimento di Biologia Cellulare
Laboratorio di Fisiologia Cellulare

Dottorato in Biologia Animale - XXI Ciclo

L'Angiotensina II modula il calcio intracellulare attivando AT1R, tramite la via di trasduzione dell'IP3 e i canali per il calcio di Tipo T (TCC).

Tesi di Dottorato: BIO/09 - Physiology

Dott. Aurela Martini



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FACULTY OF MATHEMATICAL, PHYSICAL AND NATURAL SCIENCES

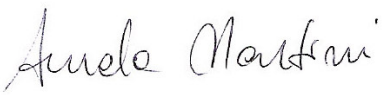
Department of Cell Biology
Laboratory of Cell Physiology

Ph.D. Animal Biology - XXI Cycle

Angiotensin II modulates HUVECs calcium concentration via AT1R involving IP3 and T-Type Calcium Channel pathways.

Ph.D. Thesis: BIO/09 - Physiology

Dr. Aurela Martini



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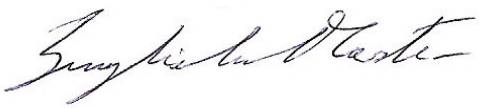

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"Sapere non vuole dire altro se non delimitare le isole della propria ignoranza; chi più sa, più isole vede nella laguna dell'ignoto e più netti ne scorge i confini"

ABSTRACT

Introduzione: L' Angiotensina II è il maggiore effettore del sistema renina-angiotensina. L'Angiotensina I, formata in seguito all'attività enzimatica della renina, interagisce con l'ACE plasmatico e con quello dell'endotelio polmonare convertendosi in Ang II.[1] Successivamente l'Ang II è veicolata dal circolo ai suoi organi bersaglio regolando la pressione sanguigna, il bilancio idrico e il tono muscolare. Gli effetti indotti dal peptide sono mediate principalmente da due tipi di recettori di membrana, AT1 e AT2.[2] I recettori AT1 stimolano un elevato numero di sistemi di trasduzione del segnale all'interno della cellula, quali fosfolipasi A (PLA), fosfolipasi D (PLD), fosfolipasi C (PLC), le MAP kinasi e la mobilizzazione del calcio intracellulare; infatti è ben nota la modulazione della concentrazione intracellulare di questo ione dall'idrolisi di fosfatidilinositolo-4,5-difosfato. Un' ulteriore quantità di Ca^{2+} entra nella cellula anche dall'esterno grazie all'apertura dei canali del calcio. Recenti studi hanno dimostrato l'esistenza dei canali per il calcio di tipo T a livello delle cellule endoteliali, ma il loro vero ruolo non è ancora del tutto chiaro.[3]

Scopo dello studio: Esaminare il coinvolgimento dell'Ang II nell'incrementare la $[Ca^{2+}]_i$ anche attraverso l'attivazione dei canali del calcio di tipo T nelle cellule HUVEC e determinare quale dei due recettori, AT1 o AT2, è coinvolto della attivazione di questi canali.

La prima tappa di questo studio è stata la messa a punto del protocollo per la determinazione della vitalità cellulare, mediante l' Arancio di Acridina, della concentrazione del Calcio, NO e ROS utilizzando rispettivamente le sonde: Fluo-3AM, DAF-2DA e HDCFH-DA.

Metodo: Le HUVEC utilizzate al terzo passaggio, sono state mantenute e cresciute in coltura mediante mezzo specifico per cellule endoteliali EGM® Bullet Kit (Lonza) contenente 10% FBS. Le cellule sono state trattate con Ang II alle concentrazioni: 10^{-9} M o 10^{-7} M o 10^{-6} M in presenza o meno degli antagonisti dei recettori AT1 o AT2 per la messa a punto del protocollo. Nel secondo studio le cellule sono state trattate con Ang II alle suddette concentrazioni in presenza degli antagonisti e in presenza o meno dell' inibitore della via IP3 o del TCC. Le cellule trattate con le sonde sono state osservate dopo 3, 6 e 9 ore. Le immagini sono state catturate col microscopio Olympus utilizzando ProImagePlus 4.0 ed analizzate col programma NIH ImageJ.

Risultati: La valutazione dell'effetto dell'Ang II sulla vitalità e sulla modulazione della concentrazione del Calcio, NO e ROS a livello delle cellule endoteliali, ha evidenziato che la concentrazione ottimale per la valutazione degli effetti intracellulare di questo octapeptide nelle cellule endoteliali è 10^{-7} M; le cellule HUVUC rappresentano un buon modello per valutare l'azione dell'Ang II sull' endotelio capillare.

Il secondo studio ha dimostrato che l' Ang II induce un alterazione del calcio intracellulare attraverso l'interazione col recettore AT1 stimolando la via IP3 per rapidi effetti fisiologi mentre attiva i canali del calcio di tipo T per tempi maggiori o uguali a 9 ore.

Tali risultati suggeriscono che i T-Type Calcium channels regolano direttamente la permeabilità al calcio delle membrane plasmatiche di cellule HUVEC, mediante canali selettivi e non solo tramite la via IP3. Questo risultato mette in risalto il ruolo dei TCC nella regolazione metabolica e strutturale diretta delle cellule endoteliali, accanto a quella delle cellule muscolari lisce dei capillari.

"Truth in science can be defined as the working hypothesis best suited to open the way to the next better one".

Konrad Lorenz (1903-1989)
The Nobel Prize in Physiology and Medicine 1973

ABSTRACT

Background: Angiotensin II (AngII) is a multifunctional peptide, that is the main effector of the Renin- Angiotensin system. Angiotensin I (AngI), which is generated by renin action on angiotensinogen, is converted to Ang II by angiotensin converting enzyme (ACE).[1] Ang II plays an important role in the regulation of blood pressure, fluid balance and vascular smooth muscle tone. Subsequent data showed a role for Ang II in long-term effects on cardiovascular structure and vascular remodelling. The effects of Ang II are mediated by two types of plasma membrane receptors, AT1 and AT2.[2] The AT1R is shown to mediate most of the physiological actions of this hormone. These effects are mediated via complex, interacting signalling pathway, involving activation of phospholipase A (PLA), phospholipase D (PLD), phospholipase C (PLC), MAP kinase and Calcium mobilization; in fact the role of intracellular calcium by inositol 3 phosphate (IP3) pathway is now well established, but the role of the interactions with T type calcium channels (TCCs) is not well understood. These latter are shown to be expressed in vascular endothelial cells.[3] The TCCs are characterized by low threshold activation.[4]

Aims: Examine whether Ang II induces an increase of free intracellular Ca^{2+} concentration ([Ca^{2+}]i), for instance through TCCs in cultured human umbilical vein endothelial cells (HUVECs) and characterize whether the receptors pathway, either AT1 or AT2, stimulates TCCs. The first step was the setting up of the cell culture protocol through the determination of the HUVEC viability, using the fluorescent label Acridin Orange and the determination of the intracellular parameters: Calcium, Nitric Oxide (NO) and Reactive Oxygen Species (ROS), used the label Fluo-3AM, DAF-2DA and HDCFH-DA, respectively.

Methods: HUVEC were obtained in their passages 3-8, grown in EGM2® Bullet Kit (Lonza) containing 10% FBS and growth factors. The cells were treated with Ang II at 10^{-9} M, 10^{-7} M and 10^{-6} M either with or without receptor antagonist in the presence or absence of Wortmannin and Mibefradil. The cells are treated with the label, Fluo-3AM,[5] Diaminofluorescein diacetate (DAF-2DA)[6] and 2,7-dichlorofluorescein diacetate (HDCFH-DA),[7] for a period of 2 hours before the observation to determine Ca^{2+}, NO e ROS levels. The cells were observed after 3, 6 and 9 hours. The images were captured by ProImagePlus on Olympus LX 50 microscope and their analysis was performed by NIH ImageJ program.

Results. The evaluation of Acridin Orange, Calcium, NO and ROS, shows that Ang II 10^{-7} M is the optimal concentration to study the effect of the hormone in EC. The HUVECs model is usefully analysed by our method to evaluate the acting mechanism of Ang II on isolated endothelial tissues.[8]

The second research demonstrates that Ang II induces alterations of intracellular free calcium through AT1 receptor pathway by the activation of IP3, for rapid physiological effects, and TCCs pathway for long term effects and this is also through antihypertensive therapy. These results suggest that TCCs regulate calcium permeability function in endothelial cells.

Significance: The study points out the role of T-Calcium Channels on the regulation of the intracellular calcium levels independently from IP3 mechanism and the relevant role of calcium channels not only on vascular smooth muscle but also on the endothelium functions; in fact the TCCs proved to play a relevant role in the development of vascular disorders linked to derangements of intracellular calcium levels.

CONTENTS

List of abbreviations

Ang II, Angiotensin II

Akt/PKB, Protein Ser/Thr kinase/protein kinase B

ANP, Atrial natriuretic peptide

Arg, Arginine

AT1a, Antagonist of AT1 receptor

AT2a, Antagonist of AT2 receptor

BH4, Tetrahydrobiopterin

$[Ca^{2+}]_i$, Intracellular free calcium

CAGE, Chymostatin- sensitive Ang II-generating enzyme

CaM, Calmodulin

CNP, C-type natriuretic peptide

COX, Cyclooxygenase

DAG, Diacylglycerol

DAF-2DA, Diaminofluorescein diacetate

DHFR, Dihydrofolate reductase

EC, Endothelial cell

EDHF, Endothelium-Derived Hyperpolarising Factor

EDRF, Endothelium-Derived Releasing Factor

EGFR, Epidermal growth factor receptor

ELAM-1, Endothelial leukocyte adhesion molecule-1

ERK, Extracellular signal-regulated kinase

ET-1, Endothelin-1

FAK, Focal adhesion kinase

Fluo-3AM, Fluo-3 acetossimeti

GC, Guanylate cyclase

HDCFH-DA, 2,7-Dichlorofluorescein diacetate

HUVECs, Human umbilical vein endothelial cells

ICAM-1, intracellular adhesion molecule-1

IGFR, Insulin growth factor receptor

InsP3, Inositol 1,4,5-trisphosphate

IP3, Inositol triphosphate

JAK, Janus kinase

JNK, c-Jun N-terminal protein kinase

MAPK, Mitogen-activated protein kinase

MEGJs, Myoendothelial gap junctions

MEK, MAPK/ERK kinase

MKP-1, MAPK phosphatase 1

NEP, Neutral endopeptidase

NO, Nitric oxide

NOS, Nitric oxide synthase

O^{2-} , Superoxide anion

oxLDL, oxidized low-density lipoprotein

p70S6K, p70 S6 kinase

PDGF(R), Platelet-derived growth factor (receptor)

PEP, Prolyl endopeptidase;

PG, Prostaglandin

PGES, PGE synthase

PGH_2, Prostaglandin H_2

PGI_2, Prostaglandin I_2

PGIS, PGI_2 synthase

PI3K, Phosphatidylinositol 3-kinase

PKC, Protein kinase C

PLA, Phospholipase A

PLC, Phospholipase C

PLC, Phospholipase C

PLC, Phospholipase C

PLD, Phospholipase D

PPARα, Peroxisomal proliferator-activated receptor α

RAS, Rennin – Angiotensin system

ROS, Reactive oxigen species

RTK, Receptor tyrosine kinase

S.E.M, Standard error on the mean

SAPK, Stress-activated protein kinases

SMCs, Smooth muscle cells

SOD, Superoxide dismutase

STAT, Signal transducers and activators of transcription

TCC, T type calcium channels

t-PA, Tissue plasminogen activator.

TXA_2, Thromboxane A_2,

VCAM-1,Vascular cell adhesion molecule-1

VPI, Vasopeptidase inhibitors

VSMC, Vascular smooth muscle cell.

"To know that we know what we know, and to know that we do not know what we do not know, that is true knowledge".

Nicolaus Copernicus (1473-1543)

BACKGROUND

1.1 Endothelium and vascular structure

The concept of endothelium as a passive barrier lining the inner side of blood vessels is completely out of date. However, the last three decades have witnessed a dramatic reconsideration of the role played by this cell in the biology and pathology of the cardiovascular system. Endothelial cells have, indeed, a large synthetic capacity and they represent a large mass of the body 720 g in humans. Therefore, these cells form a large secretory and regulatory organ completely exposed to blood.[9] The endothelium is strategically located between the wall of blood vessels and the blood stream. It senses mechanical stimuli, such as pressure and shear stress, and hormonal stimuli, such as vasoactive substances. In response, it releases agents that regulate vasomotor function, trigger inflammatory processes, and affect haemostasis. Among the vasodilatory substances produced by the endothelium, there are nitric oxide (NO), prostacyclin (PGI_2), different endothelium-derived hyperpolarizing factors (EDHF) and C-type natriuretic peptide (CNP). Vasoconstrictors include endothelin-1 (ET-1), angiotensin II (Ang II), thromboxane A2 (TXA_2), and reactive oxygen species (ROS).[10]

Lapses in this complex physiological machinery, or endothelial dysfunctions as they are generically named, are found in every pathology presenting vascular projections such as atherosclerosis, hypertension, infarctus, diabetes. The precise etiological relationship between the endothelial dysfunctions and the pathologies in question might still remain to be defined but, in spite of this lag, a considerable interest had been focused on projects aiming to correct the defective endothelial responses. Some of these attempts have led to therapeutic interventions such as the use of angiotensin converting enzyme inhibitors in the treatment of hypertension.[11]

1.2 Vasodilatators

1.2.1 Nitric Oxide

The physiological properties of EDRF were first described by Furchgott and Zawadzki in 1980. They demonstrated the ability of endothelial cells to release a potent diffusible relaxing factor that caused vasorelaxation in rabbit aortic rings in vitro. The actual chemical structure of the compound had already been identified, and it was not until several years later that two independent groups, those of Louis Ignarro and Salvador Moncada, established that EDRF was in fact nitric oxide (NO).[12] Biosynthesis of NO by NOS starts from the amino acid L-arginine and involves the incorporation of molecular oxygen (O_2) into the unstable intermediate N-hydroxy-L-arginine and subsequently into L-citrulline. The process is an oxidative reaction that consumes reducing equivalents in the form of NADPH. The final products of the reaction are $NADP^+$, L-citrulline and nitric oxide. There are three main isoforms of the enzyme NOS, named neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). The activities of these three enzymes are quite different.[13] In endothelial cells, gene expression of NO synthase, although constitutively

activated, can be upregulated by shear stress and estrogens.[14] The majority of the physiological actions of NO occur through activation of the soluble form of guanylyl cyclase in target cells (e.g. platelets and vascular smooth muscle cells), by binding to the group of this enzyme. This in turn leads to accumulation of the second messenger, cGMP, which in turn activates cGMP-dependent protein kinase; in the case of vascular smooth muscle cells, this leads to relaxation as a result of the subsequent decrease in intracellular Ca^{2+} concentration.[15]

The biological effect of NO in the control of vascular tone is attenuated by the presence of oxygen-derived reactive species, in particular superoxide anion (O_2^-), and to a smaller degree, hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-). It is also well known that nitric oxide reacts with O^{2-} to produce (peroxynitrite) $ONOO^-$, which is recognized as a damaging species. In fact, $ONOO^-$ can oxidize/nitrate other molecules or decay and produce other even more damaging species (e.g., the hydroxyl radical OH^- and NO_2). In particular, oxidation chemistry is likely to be implicated in the detriment effects induced by $ONOO^-$, which readily oxidizes flavins and thiols which may represent the putative biological targets. However, $ONOO^-$ may also have beneficial effects acting, for example, on protein kinase C thus triggering a cascade of events which lead to protection against ischemic stress.[16] $ONOO^-$ can react with a number of compounds such as deoxyribose sugars, sulfydryl groups and cellular lipids and also with tyrosine residues in proteins and peptides, generating 3-nitrotyrosine. Nitrotyrosine production can indeed be taken as a biomarker of peroxynitrite generation with increased nitrotyrosine levels having been linked with atherosclerosis.[17]

1.3 Prostacyclin

Prostacyclin, otherwise known as prostaglandin I_2 (PGI_2), is a member of the prostaglandin family of lipid mediators. PGI_2 was first discovered by Vane and colleagues in the mid-1970s and is produced primarily from vascular endothelial cells and the tunica intima with production showing a gradual decrease towards the tunica adventitia.[18] PGI_2, one of the major prostaglandins, is derived from arachidonic acid by the action of the cyclooxygenase (COX) system coupled to PGI2 synthase (PGIS). The presence of the COX-2/PGIS at the nuclear and endoplasmic reticular membrane suggests differential signalling pathways of PGI_2 actions involving both cell surface and nuclear receptors. Although the signalling of PGI_2 via its cell surface receptor, prostacyclin receptor (IP), is well documented in vascular biology. Stimulation of the IP receptor activates the intracellular enzyme adenylyl cyclase, which in turn leads to an increase in intracellular cAMP levels. cAMP then activates protein kinase A which in turn decreases MLCK activity, thereby inhibiting contraction (hence causing vasodilatation).[19]

1.4 Endothelium-Derived Hyperpolarising Factor (EDHF)

EDHF is a "holy grail," a "third factor" that provides local control of vascular homeostasis in addition to nitric oxide (NO) and prostacyclin.[20]

Taylor and Weston[21] first described this factor in 1988, which caused an increase in membrane potential of the vascular smooth muscle cells. Thus, it was named endothelium-derived hyperpolarising factor (EDHF). Most evidence supports the concept that 'EDHF' activity is dependent on contact-mediated mechanisms. This involves the transfer of an endothelium-derived electrical current, as an endothelium-derived

hyperpolarization (EDH), through direct heterocellular coupling of endothelial cells (ECs) and smooth muscle cells (SMCs) via myoendothelial gap junctions (MEGJs).[22]

The precise nature of EDHF has not been determined yet and remains a subject of great debate. Indeed, EDHF may represent a collection of different factors. Cytochrome P450 2C has been tentatively identified as an EDHF synthase in coronary arteries. It has been shown that cytochrome P450 plays a central role in the production of EDHF mediated responses. However, very little evidence exists to indicate that products of cytochrome P450 epoxygenase such as epoxyeicosatrienoic acids are actually able to diffuse from the endothelium to the underlying smooth muscle.[23] Other candidates for EDHF include the endogenous cannabinoid anandamide (another arachidonic acid product), isoprostanes (metabolites of arachidonic acid generated when endothelial cells are exposed to oxygen-derived free radicals), K^+ ions[24] and C-type natriuretic peptide (CNP) itself released from the endothelium.[25] It has also been proposed that endothelium-dependent vasorelaxation associated with hyperpolarisation of vascular smooth muscle cells may be mediated by myoendothelial cell gap junctions[26]

1.5 C-Type natriuretic peptide

The natriuretic peptides, atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) are a family of polypeptide mediators exerting numerous actions in cardiovascular homeostasis. In 1990, CNP became the third member of the natriuretic peptide family to be discovered. CNP is the most highly conserved natriuretic peptide and has been identified in primitive species.[27] Recent studies considerate C-type natriuretic peptide such as a new candidate for endothelium-derived hyperpolarising EDHF.[28]

Endothelial cells express the transcript of the CNP receptor gene, synthesize and constitutively release CNP.[29] CNP mediates its actions by binding to the cell surface natriuretic peptide receptors (NPR), of which there are three subtypes, NPR-A, NPR-B and NPR-C: and all of which are found on VSM. NPR-A is selectively activated by ANP and BNP, whereas CNP appears to be the only endogenous ligand for NPR-B. NPR-A and B possess an extracellular natriuretic peptide binding domain, and intracellular kinase and particulate guanylate cyclase catalytic domains. NPR-C acts as a clearance receptor and is believed to internalise the bound peptide, delivering it to the lysosomes for degradation.[30]

CNP-induced relaxation has variously been associated with an opening of KCa or KATP channels. It has also been suggested that CNP can activate a non-guanylyl cyclase-coupled NPR-C receptor that opens Kir channels through a pertussis toxin-sensitive G protein-linked mechanism that contributes to EDHF-type relaxations in rat mesenteric arteries but the generality of this mechanism, nevertheless, remains to be established.[31]

1.6 Vasoconstrictors

1.6.1 Endothelin-1

Endothelin (ET) is a potent vasoconstrictive peptide that was initially isolated from the conditioned medium of cultured endothelial cells.[32] Endothelin-1 is synthesised from a prepropeptide of 212 or 202 amino acids. Cleavage of preproendothelin-1 by endopeptidases generates a 38 or 39 amino acid peptide known as ‘big endothelin-1’. Big endothelin-1 is then converted to endothelin-1 by means of an endothelin-converting enzyme.[33] Two main endothelin receptors have been identified in humans. These receptors were designated the ET_A receptor and ET_B receptor, respectively and although different from each other, they were both shown to belong to the family of heptahelical G-protein-coupled receptors. The

ET_A receptor binds ET-1 and ET-2 with greater affinity than ET-3 does, whereas the ETB receptor binds all three isoforms with equal affinity. These two receptors are both distributed in various tissues and cells but with different levels of expression, suggesting the presence of a multifunctional ET system.[32] Vasoconstriction by endothelin-1 is reported to be associated with an intracellular rise in Ca^{2+}. The binding of endothelin-1 to its receptors causes activation of phospholipase C, which in turn leads to the generation of the two second messengers inositol trisphosphate (IP3) and diacylglycerol (DAG). The increase in intracellular Ca^{2+} is brought about both by release from intracellular stores, a process regulated by IP3 and by influx of Ca^{2+} through voltage-dependent Ca^{2+} channels in the membrane. Activation of the ET_A receptor by endothelin-1 stimulates the release of arachidonic acid via the activation of cytosolic phospholipase A_2.[34] Now many questions are to be established concerning the mechanisms of ET function in vascular homeostasis and vascular diseases.

1.7 Prostaglandin H_2 and Thromboxane A_2

Production of prostaglandin H_2 (PGH_2) and thromboxane A_2 (TXA_2), like the other eicosanoids, occurs through arachidonic acid metabolism. The biosynthesis of eicosanoids depends on the availability of free AA. When tissues are exposed to diverse physiological and pathological stimuli, such as growth factors, hormones or cytokines, AA is produced from membrane phospholipids by the action of phospholipase A2 (PLA2) enzymes and can then be converted into different eicosanoids. AA can be enzymatically metabolized by three main pathways: P-450 epoxygenase, cyclooxygenases (COXs) and lipoxygenases (LOXs). The P-450 epoxygenase pathway produces hydroxyeicosatetraenoic acids (HETEs) and epoxides. The COX pathway produces PGG_2 and PGH_2, which are subsequently converted into PGs and thromboxanes (TXs).[35] The PGI_2 is the dominant prostaglandin

produced by endothelial cells, smaller amounts of PGH_2 and TXA_2 are also generated. These can diffuse to subjacent vascular smooth muscle cells, where they can stimulate endoperoxide/thromboxane receptors to elicit an increase in cytosolic $[Ca^{2+}]_i$ and hence vasoconstriction. PGH_2 and TXA_2 also stimulate such receptors on platelets, to induce aggregation. Thromboxane A_2 (TXA_2) is an arachidonic acid metabolite with a chemical half-life of about 30.[36] TXA_2, is a prostaglandin derivative (a kind of prostanoid or eicosanoid) with a chemical structure characteristic of prostanoids, consisting of a six-membered ring, which promotes the development of agonists and/or antagonists among prostanoids. TXA_2 has been shown to be involved in allergies, modulation of acquired immunity, atherogenesis, neovascularization, and metastasis of cancer cells. The TXA_2 receptor (TP) communicates mainly with G_q and G_{13}, resulting in phospholipase C activation and RhoGEF activation, respectively. In endothelial cells, TXA_2 accelerates the surface expression of adhesion proteins, such as intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and endothelial leukocyte adhesion molecule-1 (ELAM-1).[37] In addition, TXA_2 is involved in human microvascular endothelial cell migration and angiogenesis.[38] In addition, TXA_2 produces prostaglandin I_2 (PGI_2), which attenuates platelet aggregation and vascular smooth muscle contraction as a negative feedback regulator.[39] The physiological importance, if any, of endothelium-derived PGH_2 and TXA_2 remains uncertain at present.

1.8 Angiotensin II

The renin-angiotensin system is a central component of the physiological and pathological responses of cardiovascular system. Its primary effector hormone, angiotensin II (Ang II), not only mediates immediate physiological effects of vasoconstriction and blood pressure regulation, but is also implicated in inflammation, endothelial dysfunction, atherosclerosis,

hypertension, and congestive heart failure.[40] The effect of this peptide is principally mediated by two types of receptor AT1 and AT2 receptors. AT1 receptor activation leads to cell growth, vascular contraction, inflammatory responses and salt and water retention, whereas AT2 receptors induce apoptosis, vasodilation and natriuresis. Alterations of these highly regulated signalling pathway may be pivotal in structural and functional abnormalities that underlie pathological processes in cardiovascular diseases such as cardiac hypertrophy, hypertrophy, hypertension and atherosclerosis.

1.9 Endothelial dysfunction.

Endothelial dysfunction is characterized by a shift of the actions of the endothelium toward reduced vasodilation, a proinflammatory state, and prothrombic properties. 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 with these conditions exhibit.[41] Mechanisms that participate in the reduced vasodilatory responses in endothelial dysfunction include reduced nitric oxide generation, oxidative excess, and reduced production of hyperpolarizing factor.[10]

The therapy of endothelial dysfunction represents a new strategy to reduce the various forms of cardiovascular disease. Angiotensin receptor blockers and angiotensin-converting enzyme inhibitors have been shown to be especially beneficial.[42] Mechanisms whereby the blockade of the renin-angiotensin system may improve endothelial function include reduction of oxidative excess and inflammation.[43]

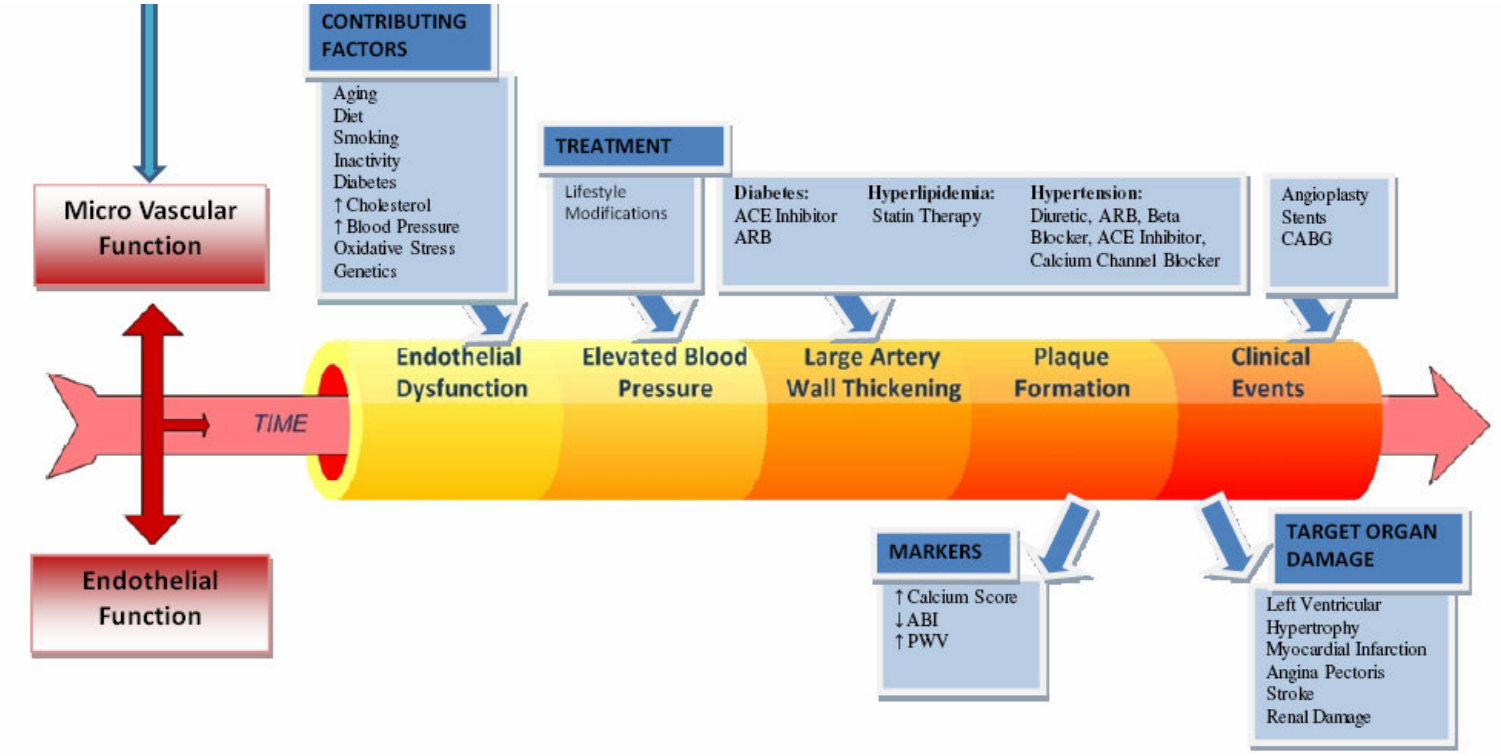

Figure 1: Balance of endothelial function: influence of simultaneous inhibition of angiotensin-converting enzyme (ACE) and neutral endopeptidase (NEP) by vasopeptidase inhibitors (VPI) on production and degradation of vasoactive peptides. Abbreviations are: ET-1, endothelin-1; ECE, endothelin converting enzyme, ANP, atrial natriuretic peptide; CNP, C-type natriuretic peptide; NOS, nitric oxide synthase; Arg, arginine; PLC, phospholipase C; GC, guanylate cyclase; EC, endothelial cell; VSMC, vascular smooth muscle cell.[44]

The best way to get a good idea is
to get a lot of ideas.

Linus Pauling (1901-1994)
The Nobel Prize in Chemistry 1954,
The Nobel Peace Prize 1962

ANGIOTENSIN II AND ENDOTHELIUM

The Rennin – Angiotensin system (RAS) plays a vital role in regulating the physiological processes of the cardiovascular system. Not only does it function as an endocrine system, but it also serves as local paracrine and autocrine functions in tissues and organs. The primary effector molecule of this system is Ang II. Ang II was identified as a hormone that controlled blood pressure based on regulation of renal salt and water metabolism, central nervous system mechanisms (thirst and sympathetic outflow), and vascular smooth muscle cell (VSMC) tone.[45] Later, Ang II was found to exert long-term effects on tissue structure, including cardiac hypertrophy, vascular remodelling, renal fibrosis and cell senescence.[46] Accumulating evidence indicates that the renin-angiotensin system plays an important role in the pathophysiology of vascular thickening and remodelling.[47] Either ACE inhibitors or angiotensin II (Ang II) type 1 (AT1) receptor antagonists significantly prevent neointimal hyperplasia in the balloon-injured rat artery independently from their hypotensive effect.[48] Importantly, recent human studies with ACE inhibitors and ARBs have yielded exciting clinical benefits such as decreased incidence of stroke, diabetes mellitus, and end-stage renal disease.[49]

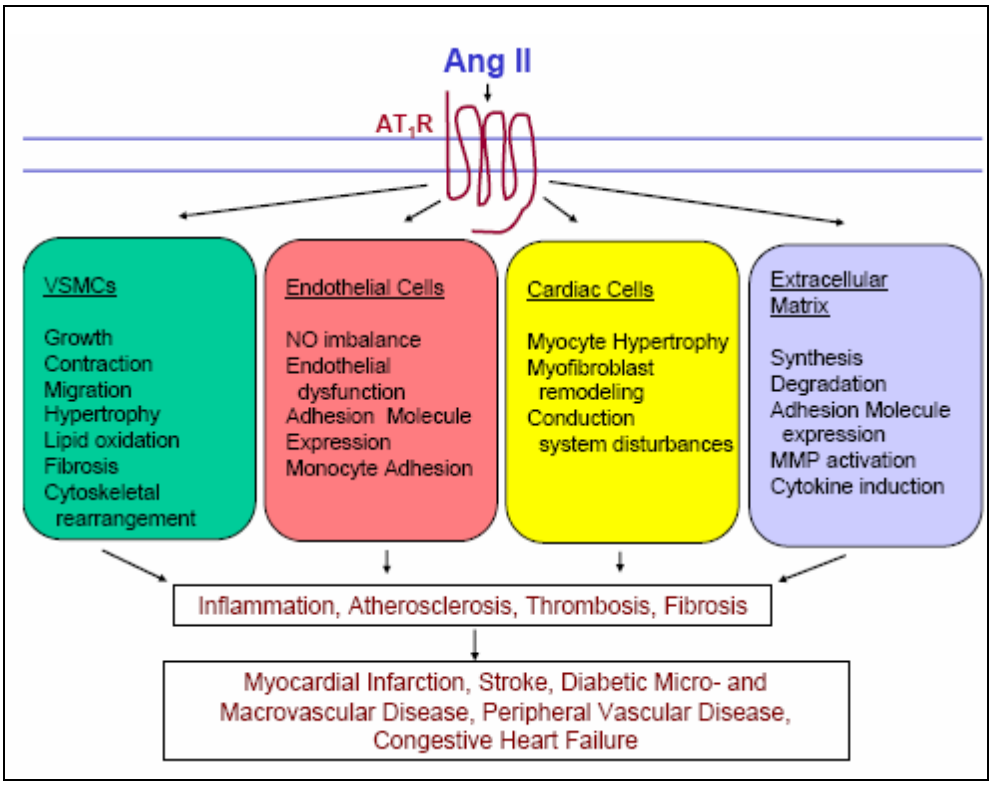

***Figure 2**. Ang II's role in cardiovascular pathology. The octapeptide ANG II exerts its myriad of effects in modulating cardiovascular physiology and pathology by inducing signalling pathways in vascular smooth muscle cells, endothelial cells, and cardiac fibroblasts, and by affecting their interaction with the extracellular matrix. Convergence of these cascades of events, in addition to abnormalities in the coagulation system, ultimately lead to atherosclerosis and thrombosis with the final development of clinically observable signs and symptoms of cardiovascular disease*[50].

The classical RAS is an enzymatic cascade initiated by the cleavage of circulating angiotensinogen by rennin to form the decapeptide angiotensin I (Fig.1). Angiotensin I is further cleaved by ACE, a dipeptidyl carboxypeptidase, to produce the circulating Ang II octapeptide that is the main effector hormone of the RAS. The ectoenzyme, ACE, is expressed at the surface of endothelial cells, where it catalyzes the conversion of Ang I

to Ang II. ACE also cleaves and inactivates bradykinin and thus contributes to the therapeutic effects (and side-effects) of ACE inhibitors.

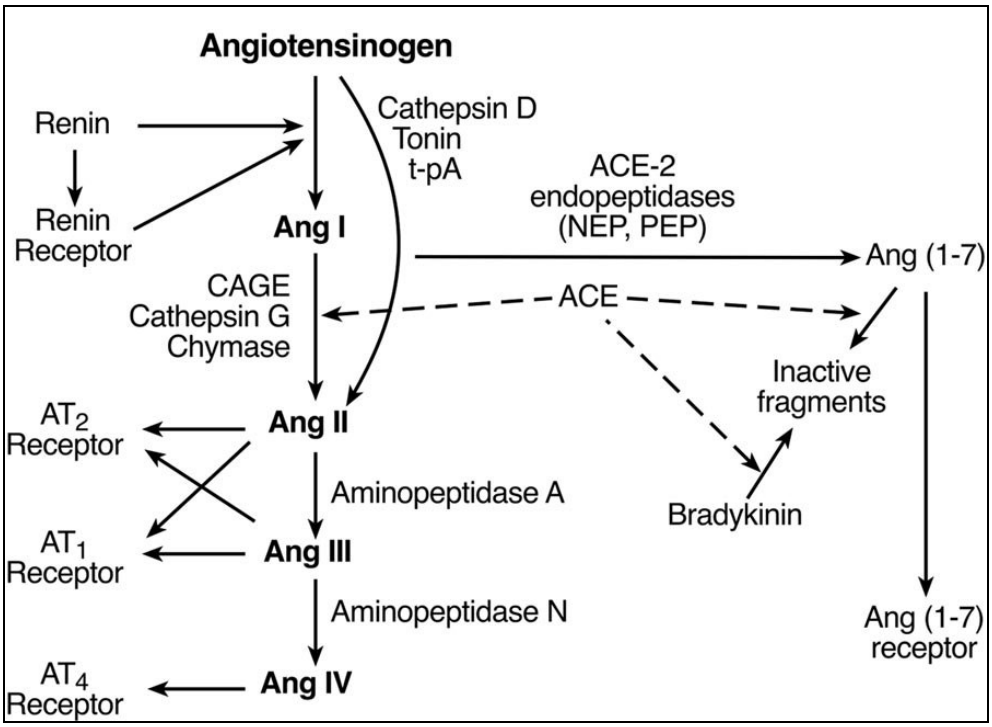

Figure 3. *The Main Components of the RAS. The classical cascade and recently discovered alternative pathways for Ang II generation are shown. CAGE, Chymostatin-sensitive Ang II-generating enzyme; NEP, neutral endopeptidase; PEP, prolyl endopeptidase; t-PA, tissue plasminogen activator.*[51]

The effects Ang II can be further metabolized by aminopeptidase A to form Ang III [Ang(2–8)] and then by aminopeptidase N to Ang IV [Ang(3–8)].[51,52]Ang II and III exert their effects via the AT1R and AT2R. Ang IV is also a biologically active peptide with low affinity for AT1R and AT2R and acts in the brain on insulin-regulated aminopeptidase. This protein is a transmembrane metalloprotease.[53]

The multiple actions of Ang II are mediated via specific, highly complex intracellular signalling pathway that are stimulated following initial binding

of the peptide to its specific receptors: AT1R, AT2R and recently AT4R has also been discovered.[2]

2.1 Effects of Angiotensin Type-1 Receptor

Most of the known physiological effects of ANG II are mediated by angiotensin type 1 receptors (AT1Rs), which are widely distributed in all the organs, including liver, adrenals, brain, lung, kidney, heart, and vasculature. Composed of 359 amino acids, the AT1R (40 kDa) belongs to the seven-membrane superfamily of G protein-coupled receptors.[54] The AT_1 receptor interacts with multiple heterotrimeric G-proteins, including $G_{q/11}$, G_i, G_{12} and G_{13}, and produces second messengers, such as inositol trisphosphate, diacylglycerol and ROS.[55] It also activates various intracellular protein kinases, such as receptor and non-receptor tyrosine kinases and serine/threonine kinases, the MAPK (mitogen-activated protein kinase) family [ERK (extracellular-signal-regulated kinase), JNK (c-Jun N terminal kinase) and p38MAPK], p70S6K (p70 S6 kinase), Akt/PKB (protein kinase B) and various PKC isoforms.[56,57,58]

Endothelial dysfunction is an early event in the pathogenesis of atherosclerosis and a feature of the insulin-resistant condition, including Type 2 diabetes, obesity and hypertension.[59,60] Not much is known about AngII signalling in ECs (endothelial cells); however, recent evidence suggests that AT_1 receptor signalling in ECs induce endothelial dysfunction, possibly through an alternative of NO (nitric oxide) function and induction of vascular insulin resistance.[61] Finally, the C-terminal cytoplasmic domain of the AT_1 receptor appears to associate with novel specific protein members with signal transduction properties. These unique AT_1-receptor-binding proteins may play an important role in Ang II signal transduction pathways leading to cardiovascular remodelling.[55]

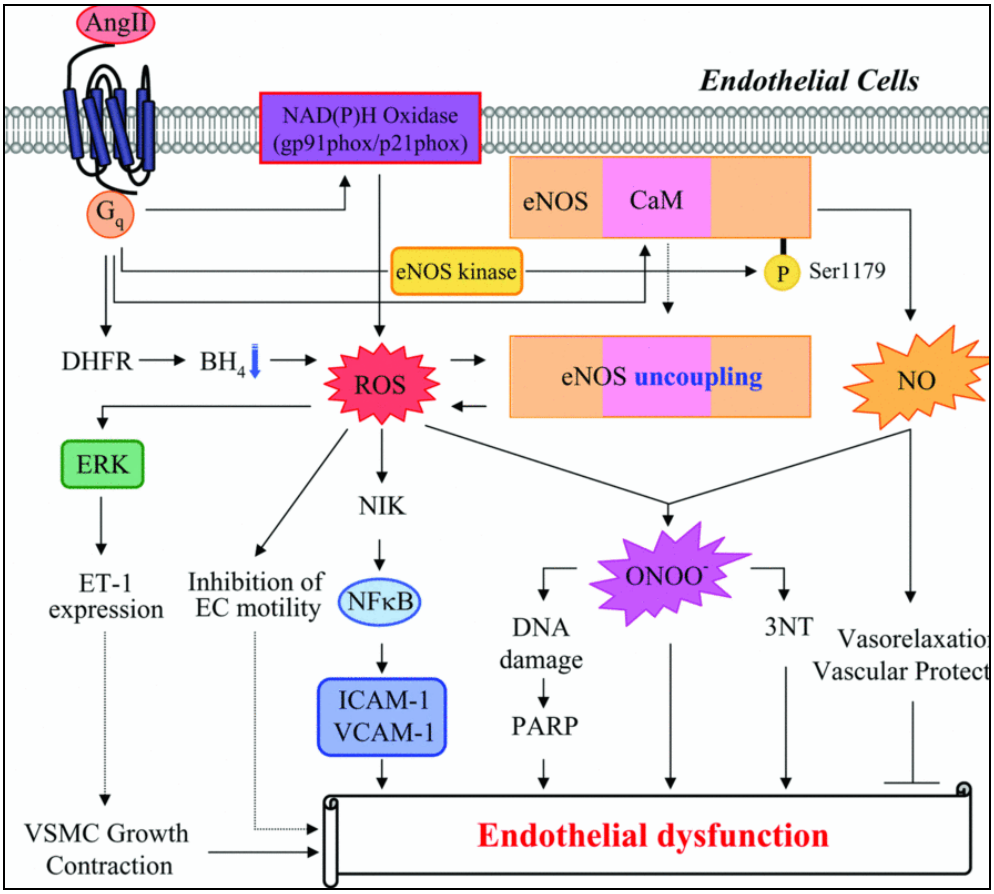

Figure 4. *AngII induces ROS production through NADPH oxidase or eNOS uncoupling which mediates endothelial dysfunction. NO reacts with superoxide anion, resulting in the formation of peroxynitrite ($ONOO^-$). Peroxynitrite induces endothelial dysfunction through the production of 3NT or PARP. Alternatively, the AT_1 receptor stimulates endothelial NO production through eNOS Ser^{1179} phosphorylation to protect endothelial function. In addition, AngII induces ICAM-1 and VCAM-1 through NF-kB in ECs, and this cascade involves ROS and p38MAPK activation. BH4, tetrahydrobiopterin; CaM, calmodulin; DHFR, dihydrofolate reductase; ET-1, endothelin-1.*[55]

It has been shown that Ang II increases and decreases EC apoptosis, suggesting that other factors influence the actions of Ang II (e.g., presence of oxidized low-density lipoprotein [oxLDL]). In fact, it has been suggested that both the AT1R and the AT2R mediate EC apoptosis.[62]

Ang II-mediated EC apoptosis is in part mediated by generating reactive oxygen species, because antioxidants suppress EC apoptosis.[63] Ang II increases NADPH oxidase activity in EC, enhancing superoxide production via AT1R and AT2R. Ang II and ROS also promote EC apoptosis by inhibiting the function of the anti-apoptotic protein Bcl-2. For example, ROS generated by Ang II causes transcriptional downregulation of Bcl-2 and upregulation of pro-apoptotic Bax. In addition, Ang II was shown to inactivate extracellular signal-regulated kinase (ERK) (ERK1/2) by upregulating mitogen-activated protein kinase (MAPK) phos-phatase 3, thereby decreasing Bcl-2.[64] Ang II may indirectly cause EC apoptosis via induction of Fas and LOX-1.6. Finally, the AT1R directly alters endothelial nitric oxide synthase (eNOS) function by binding to membrane-localized eNOS. Marrero et al showed that eNOS was bound and its activity was inhibited by the bradykinin B2 receptor (B2R), the AT1R and the endothelin-1 ETB receptor.[65] The interaction is controlled in part by phosphorylation of serines and tyrosines that interact with eNOS, although the physiological importance and kinases involved remain to be clarified.

In summary, Ang II promotes apoptosis in EC via direct and indirect mechanisms.[2]

In the vessel wall, homeostatic mechanisms balance thrombosis with fibrinolysis. Plasminogen-activator inhibitor type 1 (PAI-1) inhibits tissue plasminogen activator (t-PA) and urokinase, tipping the balance in favor of thrombosis. In VSMCs and ECs, exposure to ANG II leads to increased levels of PAI-1 mRNA.[66] ANG II-mediated inhibition of fibrinolysis and its induction of cell adhesion molecules such as VCAM-1 and ICAM-1 (via NF-$_k$B activation) provide for further mechanisms by which ANG II initiates and causes progression of atherosclerosis. In endothelial cells, ANG II has been shown to induce the LDL receptor which is critical in atherosclerotic lesion formation.[50,63] Thus, ANG II plays a key role in

modulating endothelial function, and its enhanced presence contributes to endothelial dysfunction and inflammation.

2.2 Effect of Angiotensin Type-2 Receptor

Even though most of the vasoactive effects of ANG II occur via AT1Rs, AT2Rs have been shown to exert anti-proliferative and pro-apoptotic changes in VSMCs, mainly by antagonizing AT1Rs. Similar to the AT1R, the AT2R (MW 41 kDa) is a seven transmembrane domain receptor, but it is only 34% identical to AT1R Consisting of 363 amino acids, AT2R is highly expressed in foetal tissue, including foetal aorta, gastrointestinal mesenchyme, connective tissue, skeletal system, brain, and adrenal medulla.[54]

The signalling pathways of the AT1 receptor are largely understood but the elucidation of AT2 receptor-coupled signal transduction turned out to be more complicated, and the 'true' nature of AT2 signalling remains enigmatic; in fact, it is still a matter of intense research efforts, although the AT2 receptor has basic structural features commonly shared by 7- TMRs. It did not reveal any functional features commonly attributed to this class of receptors. With respect to the specific pathways or substrates involved in AT receptor-mediated intracellular signalling, there are still numerous unresolved problems such as GTPgS sensitivity, PTP activation, or cGMP production. The current concept for AT signalling encompasses that the AT2 receptor does not modulate cytosolic Ca2q or cAMP, which are sensitive indicators of Gq-protein-coupled phospholipase Cb activation and Gs- or Gi-coupled activation or inhibition of adenylyl cyclise.[45]

Furthermore, the AT2 receptor has been reported to mediate sustained arachidonic acid release in isolated pure cardiac myocytes, an effect that is completely blocked by the AT-specific antagonist, PD123317. Kohout and Rogers[67] found evidence in myocytes indicating that the AT2 receptor-

mediated release of arachidonic acid may contribute to the activation of Na^+/HCO^-symporter system, which regulates intracellular pH. In addition, AT2 receptor-dependent production of prostacyclin was reported in differentiated adipocytes in culture.[45]

Overall, diverse putative AT2 receptor signalling pathways were unveiled, which include activation of serine/threonine phosphatase PP2A and subsequent opening of the delayed rectifier K^+-channel, activation of cytosolic PTPs which may lead to closing of the T-type Ca^{2+}- channel, inactivation, but in some cases, also transient activation of MAP kinase and the activation of PLA_2.[68]

2.3 Effects of AT4R

Ang IV [Ang (3– 8)] is an important angiotensin degradation peptide that binds to the AT4R, which is primarily expressed in EC. Upregulation of AT4R was detected in the re-endothelialized cell layer after balloon injury.[69] Ang IV has been shown to increase PAI-1 expression via the AT4R.[70] An AT4R blocker, but not AT1R or AT2R blockers, inhibited Ang IV–induced PAI-1 expression.[71] In addition, an aminopeptidase inhibitor suppressed Ang II-induced PAI-1 expression, suggesting a key role for metabolism of Ang II to generate Ang IV.

Ang IV has been reported to mediate cerebral and pulmonary artery vasorelaxation via NO release and pulmonary microvascular EC proliferation.[72] Ruiz-Ortega et al. suggested that Ang IV is proinflammatory because Ang IV activates NF-κB.[73,74] To define the role of Ang IV in vascular disease, identification of the AT4R will be necessary.

The important thing in sciences is not so much to obtain new facts as to discover new ways of thinking about them".

William Bragg (1862-1942)
The Nobel Prize in Physics 1915

AIMS OF THE STUDY

Modulation of cytoplasm $[Ca^{2+}]_i$ is a signalling system involved in the regulation of several processes, including transepithelial transport, learning and memory, muscle contraction, membrane trafficking, synaptic transmission, secretion, motility, membrane excitability, gene expression, cell division and apoptosis.[75] A ubiquitous mechanism of modulating $[Ca^{2+}]_i$ involves the activation of PLC by a wide variety of stimuli including ligand interaction with either G-protein or tyrosine kinase linked receptors. PLC hydrolyzes the membrane lipid phosphatidylinositol 4,5-bisphosphate, generating inositol 1,4,5-trisphosphate (InsP3).[76] Many studies demonstrate that Ang II-AT1R binding mediated with complex, induces calcium mobilization;[77] in fact the role of intracellular calcium by IP3 pathway is now well established, but the role of the interactions with T type calcium channels (TCCs) in endothelial cells is not well understood. Recent studies suggest that the inhibition of T-type Ca^{2+} channels may enhance the release of vasodilatory factors from endothelial cells. It has been reported that mibefradil, a T-type Ca^{2+} channel antagonist withdrawn from the market, potentiated endothelium-dependent relaxation in the rings of arteries from animals.[78] Therefore, T-type Ca^{2+} channels may play an important role in

the physiology of the vascular endothelium.[79] In fact, T-type Ca^{2+} channels have

been detected in vascular endothelial cells although their roles have not been clarified.[80]

Therefore, in the present study, the aims of the research are:

- to study in HUVECs the role of Ang II by means of selective calcium channels to modulate the intracellular calcium levels
- to specify the receptor pathways responsive to Ang II, either AT1 or AT2, useful to control calcium levels in EC.

The foundations of our research are the refinement of reliable fluorescence techniques to measure Calcium, NO and ROS intracellular levels in HUVECs. These parameters are used by means of the labels Fluo-3 acetossimetil (Fluo-3AM), Diaminofluorescein diacetate (DAF-2DA) and 2,7-dichlorodihydrofluorescein diacetate (HDCFH-DA).

"I hear and I forget. I see and I remember.
I do and I understand".

Chinese Proverb

MATERIALS AND METHODS

4.1 Cell Culture

Human umbilical vein endothelial cells (HUVECs)[81] were harvested from fresh human umbilical cord veins and cultured until the third passage, as previously described.[8] 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.[82]

Newly confluent cells were lifted by trypsinization and cells were washed in culture medium. Cell suspension was centrifuged (1100 rpm over a period of 7 minutes at + 20°C), the supernatant was removed and HUVECs were resuspended in culture medium (3 mL EGM2® Bullet Kit) (Lonza, Basel, CH) containing 10% FBS, hydrocortisone 0,2 ml, hEGF 0,5 ml, VEGF 0,5ml, hFGF-B 2 ml, R3-IGF-1, ascorbic acid 0,5 ml, heparin 0,5 ml and gentamicin 0,5 ml. 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 flasks coated with collagen. Cells used were at passages 3–8. Cells at different cycles of trypsinization were seeded in multi-well plates at a density of about 1 x 10^4 cells/well and used for the described experiments.

4.2 Chemicals

ZD7155, AT1Rantagonist, (Zeneca AB Gothenburg, Sweden); PD123319, AT2Rantagonist Wortmannin, inhibitor of PI3-K/Akt pathway, and Mibefradil, T-Type Calcium Channel blocker (Tocris, Bristol, UK); Fluo-3AM (Invitrogen Carlsbad, USA); DAF-2DA (Alexis Biochemicals, Lausanne, Switzerland); Acridin Orange; Ang II and HDCFH-DA (Sigma St. Louis, MO, USA); all other reactants are from Sigma.

4.3 Experiment Protocol

4.3.1 Study 1: Setting up of HUVEC's protocol

Study 1.1

Cell viability is assessed by fluorescence microscopy using the label Acridin Orange at 0,2% that are 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 intercalated AO fluoresces green (525nm); 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 G_1 compartments.[83] AO staining may also be useful as a method for measuring apoptosis, and for detecting intracellular pH gradients.[84]

HUVECs are plated in 24 wells and are incubated with the label and EGM2® Bullet Kit at 10^5 cells/well. After 10 minutes the cells are studied by microfluorometry (200x). The data evaluation is after 3, 6 and 9 hours of culture in the presence of Ang II either with or without ZD 7155 or PD 123319 at 10^{-7} M.

Study 1.2

The determination of $[Ca^{2+}]_i$ levels in HUVECs was performed using the label, Fluo-3AM at 10^{-7} M. Fluo-3 acetoxymethyl (AM) ester was used as the fluorescent indicator. Fluo-3 is a fluorescent chelator excited by visible light (488 nm) and it emits a yellowish green fluorescence (525 nm) when bound to a calcium ion.[86] The intensity of fluorescence depends on the free calcium concentration. 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 larded into the cell across the cell membrane. Once introduce 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.[87]

The cells are treated with the label for a period of 2 hours before observation to determine Ca^{2+}. The cells were observed after 3, 6 and 9 hours (Fig.5).

Study 1.3

DAF-2 DA is a newly developed indicator for the measurement of NO.[88] Diaminofluorescein –2 Diacetate (DAF-2DA) is a non-fluorescent 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 triaole derivative, DAF-2T. Thus, in the present study, the DAF-2T intensity will reflect the NO concentration in EC. 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). Then the cells were incubated in EGM2 culture buffer containing DAF-2DA at 10^{-7}M. To this medium was added Ang II either 10^{-9} M or 10^{-7} M or 10^{-6} M. Two more experimental series were performed with either AT1Ra or AT2Ra 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 II was studied on Olympus LX 50 fluomicroscope calibrated for excitation at 495 nm and fluorescence emission at 515 nm.[90]

Study 1.4

The generation processes of reactive oxygen species can be monitored using the luminescence analysis or also fluorescence methods. The intracellular ROS generation of cells can be investigated using the 2',7'-dichlorfluorescein-diacetate (DCFH-DA). The conversion of the non fluorescent 2',7' - dichlorfluorescein – diacetate (DCFH-DA) to the highly fluorescent compound 2',7'-dichlorfluorescein (DCF) happens in several steps. First, DCFH-DA is transported across the cell membrane and deacetylated by esterases to form the non-fluorescent 2',7'-dichlorfluorescein (DCFH). This compound is trapped inside the cells. Next, DCFH is converted to DCF through the action of peroxid rated by the presence of peroxidase. The label was excited at 502 nm whereas emitted fluorescence was recorded at 523 nm.[91]

The cells were treated with 10^{-7} M label and Ang II with either AT1a or AT2a at 10^{-7} M for 2 hours before observation.

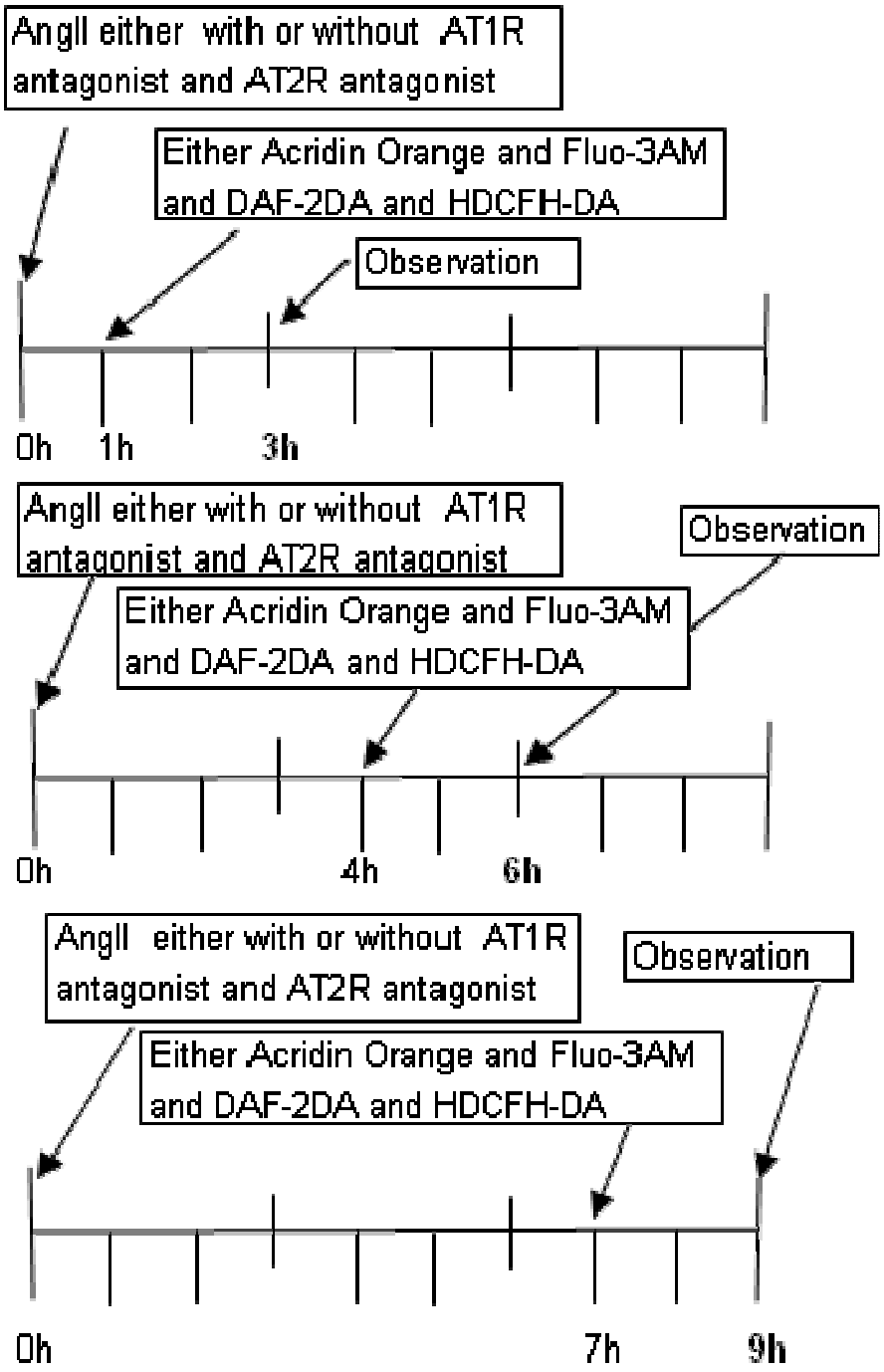

***Figure 5**. Timelines of the experiment. At the beginning, the cells were treated either with or without receptor antagonist. The cells are treated with the label for a period of 2 hours before observation to determine Ca^{2+}, NO e ROS levels. The cells were observed after 3, 6 and 9 hours.*

4.3.2 Study 2: Ang II modulates HUVEC calcium concentration via AT1R involving IP3 and T-Type Calcium Channels.

At the beginning the cells are treated with or without receptor antagonist and one inhibitor between Wortmannin and Mibefradil. The cells are treated with the label for a period of 2 hours before the observation to determine intracellular Ca^{2+} levels. The cells are observed after 3, 6 and 9 hours.

4.4 Fluorescence microscopy.

Cell fluorescence is observed by means of fluorescence microscope Olympus LX 50 with UV source (200x). Emission is acquired by ProImagePlus program and analysed by NIH ImageJ program.

4.5 Statistics

Calcium release is expressed as percentage of fluorescence relative to control (white column). All data are means ± SEM. Tests of significance between curves were accomplished using two-ways ANOVA for multiple comparisons; statistical comparison between two groups of results is carried out by Student t (two-ways). The level of significance was set at $P < 0,05$. Data are shown as means ± SEM. The n values indicate the number of independent experiments.

"The great tragedy of science: the slaying of a beautiful hypothesis by an ugly fact".

Thomas Henry Huxley (1825-1895)
English Biologist.

RESULTS AND DISCUSSION

5.1 Results

5.1.1 Effect of Ang II on cell viability

The observation of the effects of Ang II at 10^{-9} M on cell viability points out that the hormone induces significant increase of cell adhesion only after 9 hours (Fig.6). The treatments with Ang II plus AT1Ra do not modulate the fluorescence intensity after 3, 6 and 9 hours while the treatments with Ang II plus AT2Ra also induce a significant increase of fluorescence intensity only after 9 hours. These data point out that Ang II at 10^{-9} M does not favor cellular adhesion after a short time (fig.6A). The effect of the cell with 10^{-7} M Ang II either in presence or absence of ARBs induces a significant increase of cellular adhesion after 3, 6 and 9 hours (Fig.6B). The effect of AngII at 10^{-6} M is similar to the effect induced by Ang II 10^{-7} M (Fig.6C).

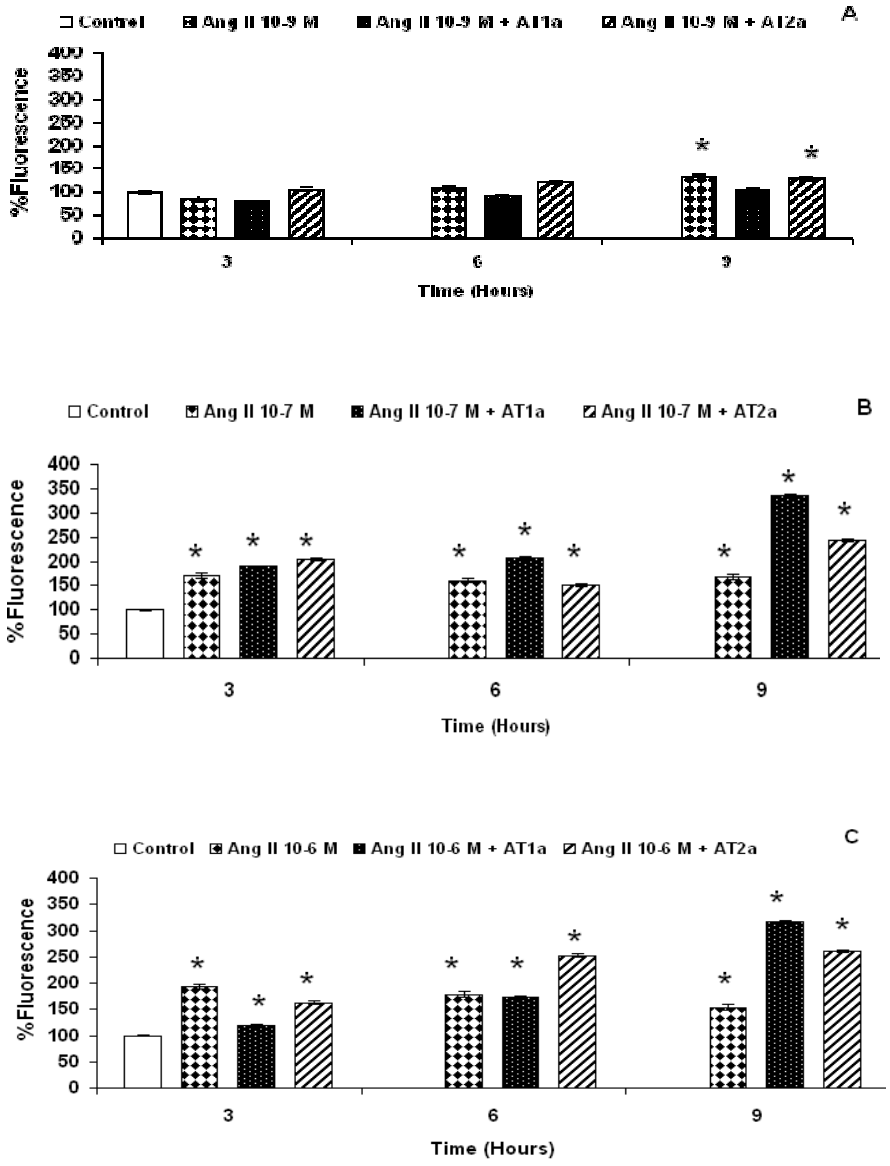

Figure 6. *Effects of Ang II on cell viability either 10^{-6} M or 10^{-7}M or 10^{-9} M with or without treatment with ZD7155 (AT1a,10-7M) or PD123319 (AT2a, 10^{-7} M in HUVECs after 3, 6 and 9 hours. Data are reported as relative % fluorescence (Acridin Orange, 10^{-6}M) in comparison with controls (white columns). Data are shown as means ± S.E.M; n=8. Student's test for differences between two data groups:* P<0.05; significantly different from control; #, P < 0.05 vs Ang II (small checkers columns).*

Ang II at 10^{-9} M induces a significant increase of $[Ca^{2+}]_i$ only after 3h and 6h in comparison with control experiments (Fig.7). The treatment of the cells with AT1Ra decreased the levels of $[Ca^{2+}]_i$ in comparison to Ang II but only after 6 and 9 hours in comparison with the reference (Fig.7A). Ang II at 10^{-7} M either with or without AT2Ra induces a significant increase of the parameter after 3, 6 and 9 hours in comparison with control, confirming the contribution of AT1R to increase the calcium levels. The effect of Ang II plus AT2Ra induces a significant decrease of this parameter in comparison with the octapeptide alone confirming the antagonist action of AT2R (Fig.7B). The effect of Ang II 10^{-6} M is similar to Ang II 10^{-7} M but after a long time the effect of the peptide plus AT1Ra modulates more efficiently calcium levels (Fig.7C)

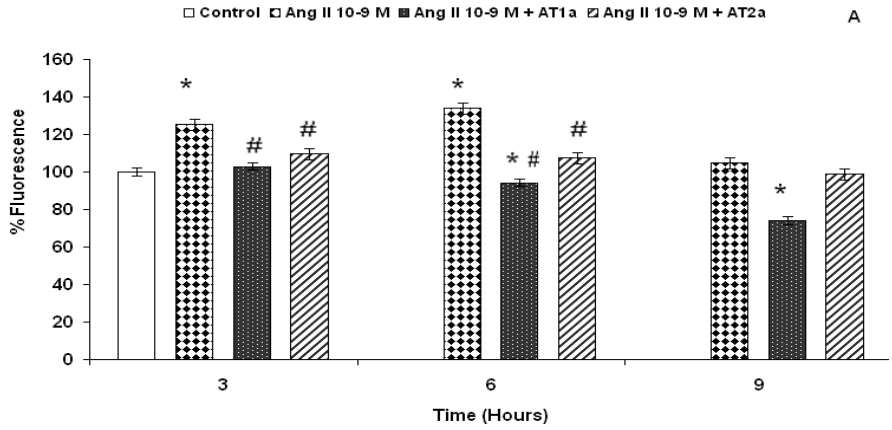

***Figure 7**. Effects of Ang II on $[Ca^{2+}]_i$ at 10^{-9} M with or without treatment with ZD7155 (AT1a,10^{-7}M) or PD123319 (AT2a, 10^{-7} M in HUVECs after 3, 6 and 9 hours. Data are reported as relative % fluorescence (Fluo-3AM,10^{-7}M) in comparison with controls (white columns). Data are shown as means ± S.E.M; n=5. Student's test for differences between two data groups:* P<0.05; significantly different from control; #, P < 0.05 vs Ang II (small checkers columns).*

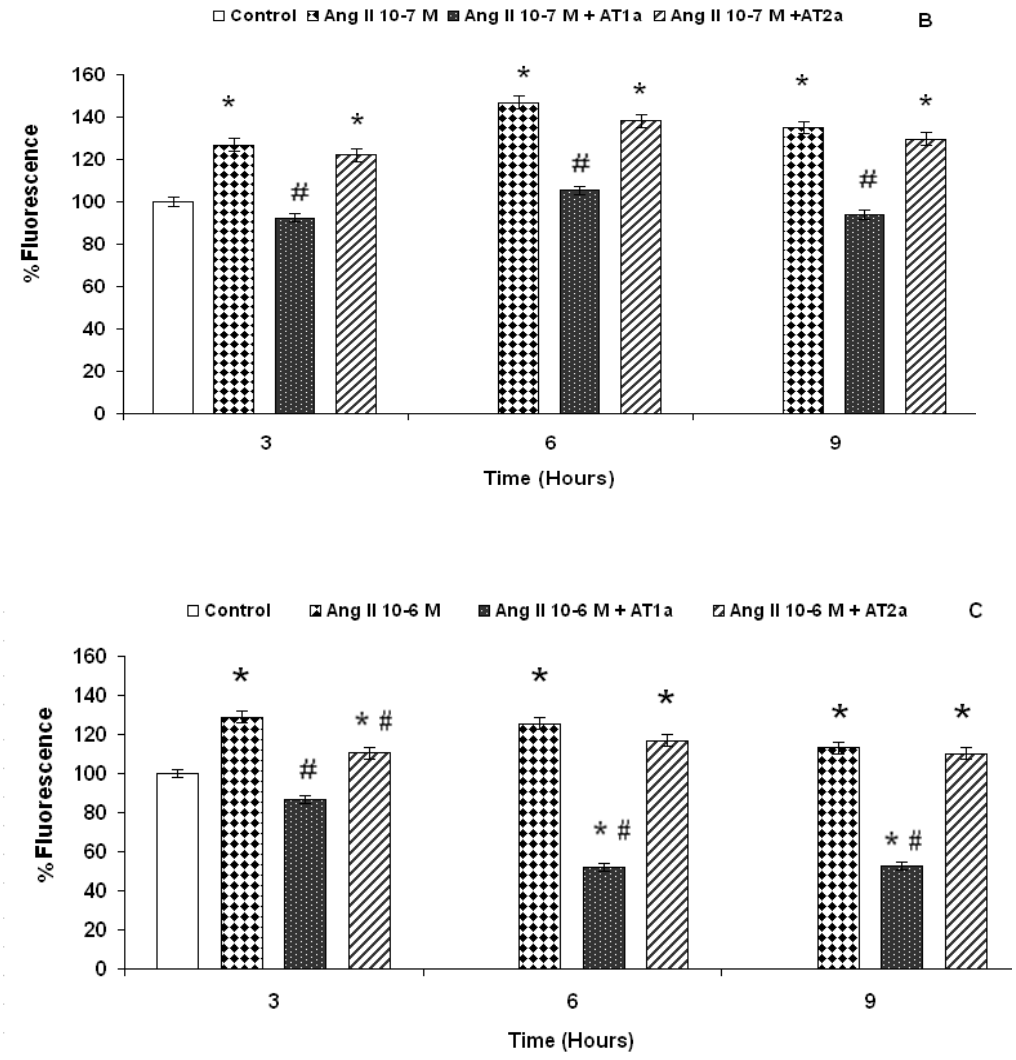

Figure 8. *Effects of Ang II on $[Ca^{2+}]_i$ at either 10^{-6} M or 10^{-7}M with or without treatment with ZD7155 (AT1a,10^{-7}M) or PD123319 (AT2a, 10^{-7} M in HUVECs after 3, 6 and 9 hours. Data are reported as relative % fluorescence (Fluo-3AM,10^{-7}M) in comparison with controls (white columns). Data are shown as means ± S.E.M; n=5. Student's test for differences between two data groups:* P<0.05; significantly different from control; #, P < 0.05 vs Ang II (small checkers columns).*

The treatment of the cell with Ang II 10^{-7} M induces a significant increase of NO levels only after 6 and 9 hours (Fig.9). The treatment with AngII plus AT1a induces a significant increase of the parameter in comparison with control and points out the contribution of antagonist action of AT2R to decrease NO levels after 3, 6 and 9 hours. The treatment with Ang II plus AT2Ra induces a significant decrease of the NO levels, confirming the antagonist action of AT1R to decrease this parameter after 3, 6 and 9 hours.

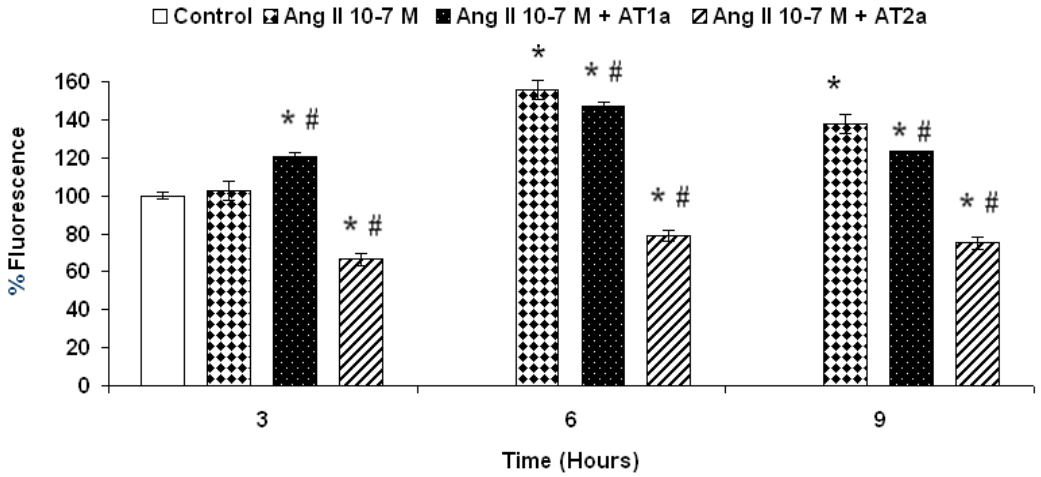

Figure 9. *Effects of Ang II on NO at either 10^{-6} M or 10^{-7}M or 10^{-9} M with or without treatment with ZD7155 (AT1a,10^{-7} M) or PD123319 (AT2a, 10^{-7} M in HUVECs after 3, 6 and 9 hours. Data are reported as relative % fluorescence (DAF-2DA,10^{-6} M) in comparison with controls (white columns). Data are shown as means ± S.E.M; n=5. Student's test for differences between two data groups: * P<0.05; significantly different from control; #, P < 0.05 vs Ang II (small checkers columns).*

Fig.10 shows the activity of Ang II 10^{-7} M in HUVECs after 3, 6 and 9 hours. This peptide induces significant increases of ROS levels both after short and long term observation. The treatment of the HUVECs with Ang II + AT1Ra induces a significant decrease confirming the antagonist effect of AT2R. The treatment of the HUVECs with AngII plus AT2a induces a significant increase of fluorescence in comparison with control after 3, 6 and 9 hours confirming the contribution of AT1R to increase ROS levels.

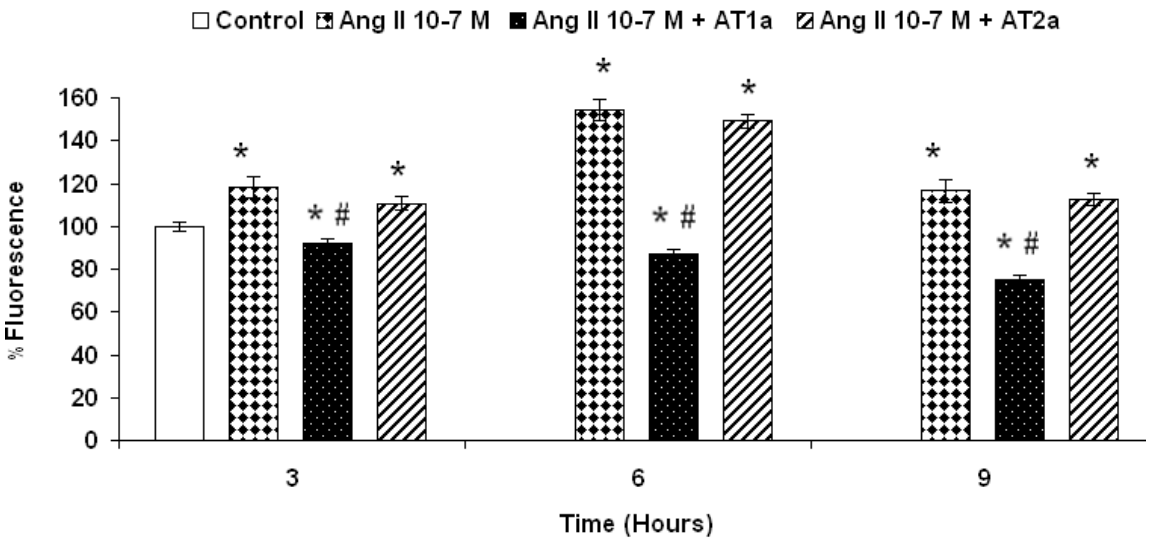

Figure 10. *Effects of Ang II on ROS at 10^{-7}M with or without treatment with ZD7155 (AT1a,10^{-7}M) or PD123319 (AT2a, 10^{-7} M in HUVECs after 3, 6 and 9 hours. Data are reported as relative % fluorescence (HDCFH-DA,10^{-6} M) in comparison with controls (white columns).Data are shown as means ± S.E.M; n=5. Student's test for differences between two data groups: * P<0.05; significantly different from control; #, P < 0.05 vs Ang II (small checkers columns).*

ANOVA of the timecourses of NO, ROS, and Ca^{2+} intracellular levels under the action of AngII 10^{-7} M are co-ordinated by the hormone (Fig.11).

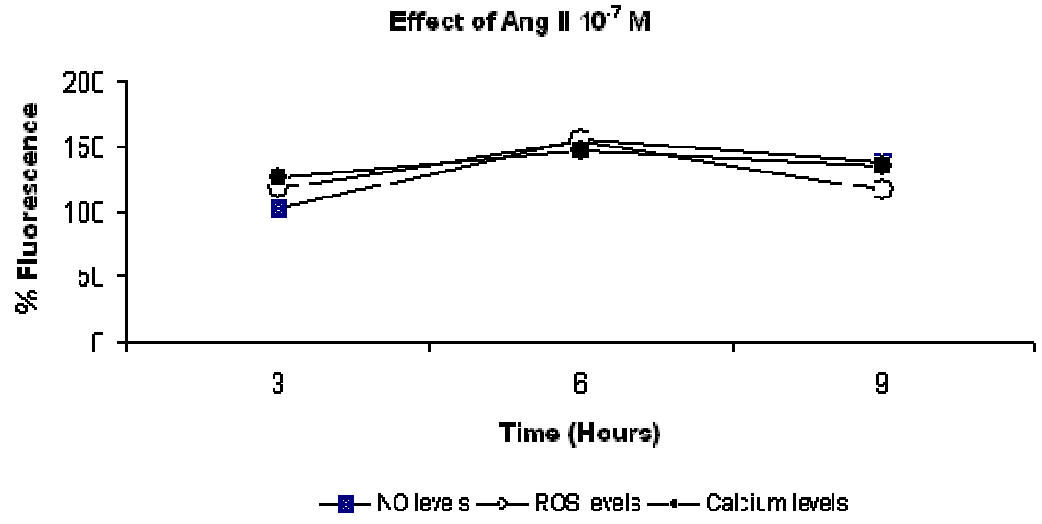

***Figure 11**. Timecourses of cytosolic [Ca $^{2+}$]i, NO and ROS production in HUVECs cultures added with Ang II (10^{-7} M) after 3, 6 and 9 hours. Data are reported as relative % fluorescence. Data are present as mean ± S.E.M; n = 5, and analyzed by two-ways ANOVA: *; P<0.05.*

The evaluation of Ang II activity on ECs calcium after 3, 6 and 9hours points out that the contribution of this peptide in calcium level increases (Fig.12). The treatment of the HUVECs with Ang II either with Wortmannin or CCB points out the involvement of both IP3 and TCCs pathways in the modulation of intracellular calcium; these treatments indeed induce significant decreases of this parameter in comparison with Ang II alone after all 3 observations (Fig.12: A-C)

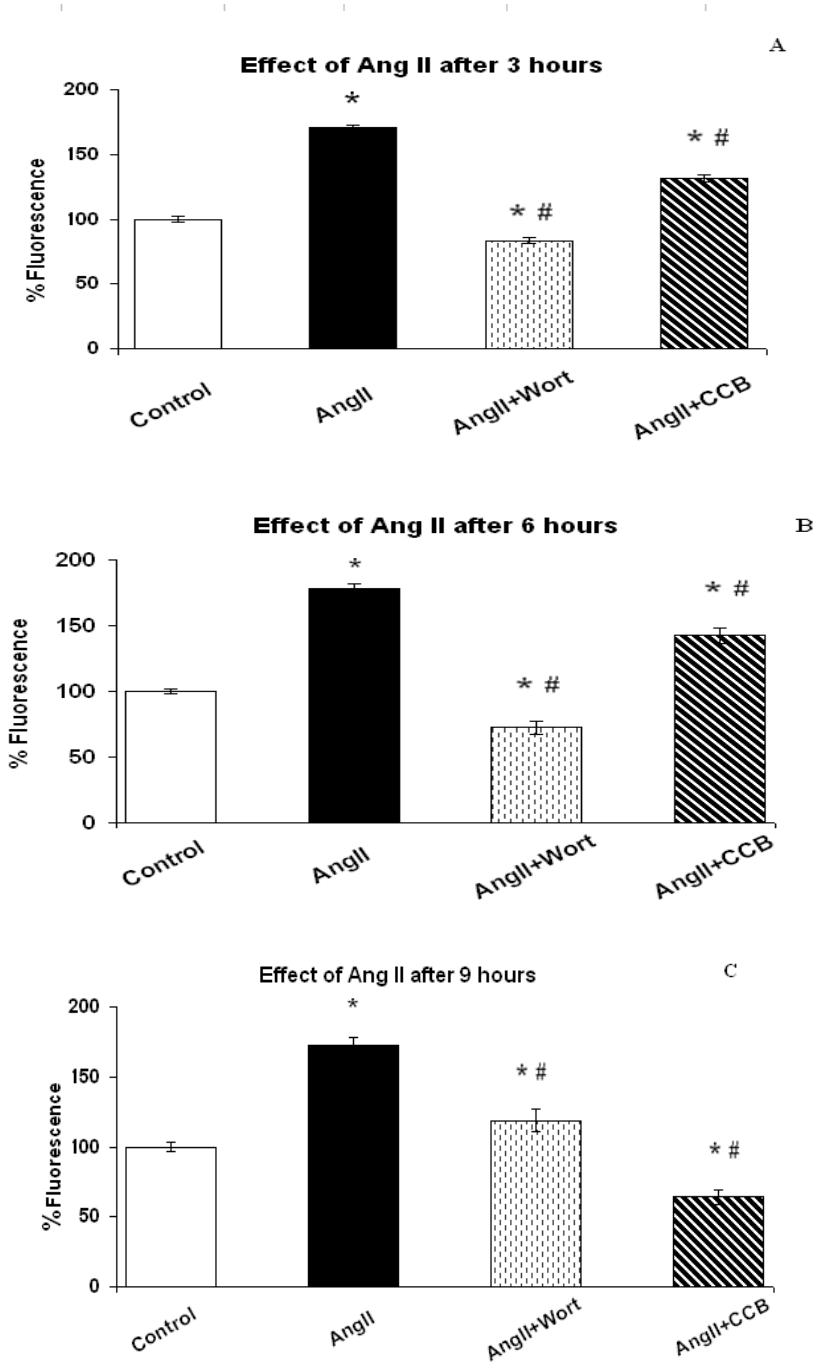

Figure 12. *Effects of AngII on* $[Ca^{2+}]_i$ *at* 10^{-7} *M in presence or absence of Wortmannin (Wort) or Mibefradil (CCB) after 3, 6 and 9 hours. Data are reported as relative % fluorescence (Fluo-3AM) in comparison with controls (white columns). Data are shown as means ± S.E.M; n=6. * P<0.05; significantly different from control; #, P < 0.05 vs Ang II (small checkers columns).*

Fig.13 shows the effect of Ang II. The octapeptide induces a significant increase of $[Ca^{2+}]_i$ in comparison with the reference. The treatments of the cells with Ang II plus AT1Ra with either Wortmannin or CCB do not modulate the parameter in comparison with control but induce significant decreases of this parameter in comparison with Ang II alone, confirming the antagonist action of AT2R and this effect does not involve the IP3 or TCCs pathway.

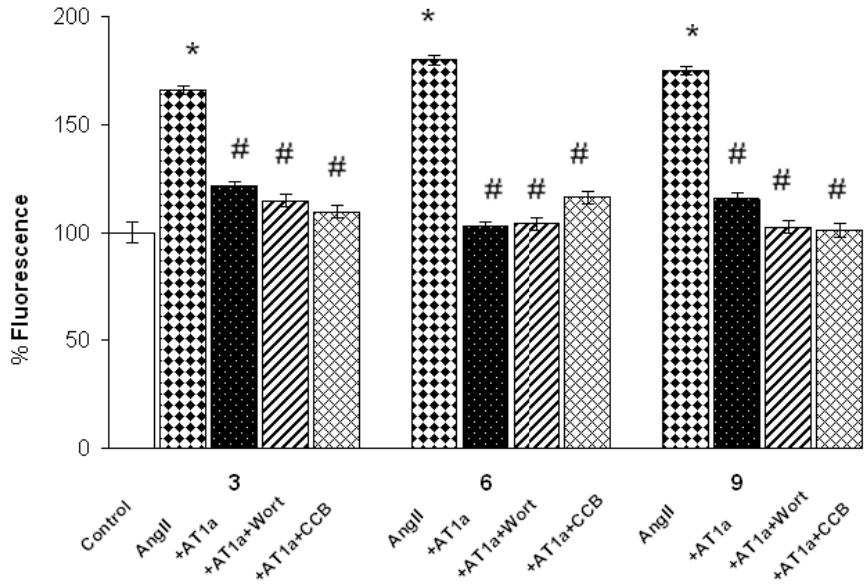

***Figure 13**. Effects of AngII on $[Ca^{2+}]_i$ at 10^{-7}M with or without treatment with ZD7155 (AT1a,10^{-7}M)) in HUVECs either in presence or absence of one inhibitor between Wortmannin (Wort) and Mibefradil (CCB) after 3, 6 and 9 hours. Data are reported as relative % fluorescencein (Fluo-3AM, 10^{-7} M) in comparison with controls (white columns). Data are shown as means ± S.E.M; n=6. Student's test for differences between two data groups: * P<0.05; significantly different from control; #, P < 0.05 vs. Ang II (small checkers columns); §, P < 0.05 vs Ang II+AT1a or AngII+AT2.*

Fig.14 shows the effect of Ang II plus AT2Ra. The treatments induce a significant increase of calcium levels in comparison to control, pointing out the contribution of AT1R to increase the intracellular free calcium. The treatments of the cell with Ang II + AT2Ra + Wortmannin induce a significant decrease of this parameter confirming the contribution of AT1R and IP3 pathway to modulate this parameter after 3, 6 and 9 hours. The effect of co-incubation of Ang II + AT2Ra + CCB induces a significant decrease of this parameter in comparison with Ang II alone, pointing out the contribution of TCCs in the intracellular calcium modulation after all 3 observations.

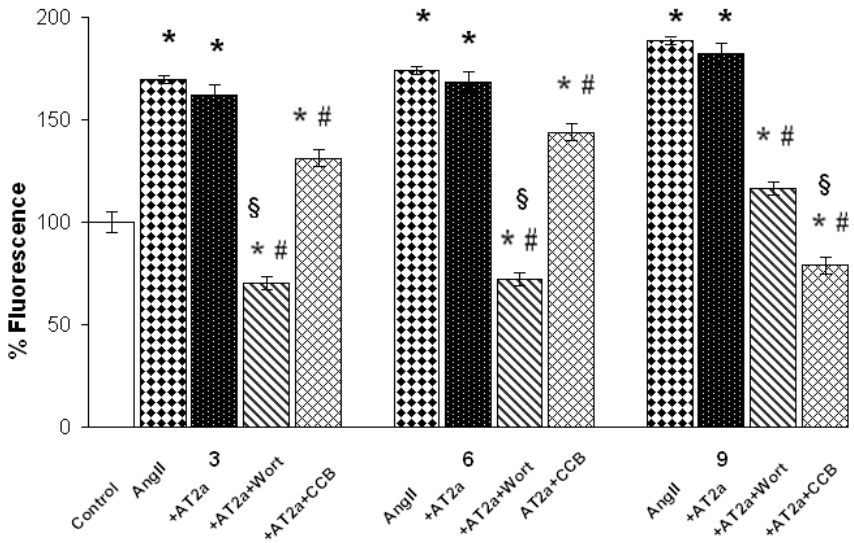

Figure 14. *Effects of AngII on $[Ca^{2+}]_i$ at 10^{-7}M with or without treatment with PD123319 (AT2a, 10^{-7}M) in HUVECs either in presence or absence of one inhibitor between Wortmannin (Wort) and Mibefradil (CCB) after 3, 6 and 9 hours. Data are reported as relative % fluorescencein (Fluo-3AM, 10^{-7} M) in comparison with controls (white columns). Data are shown as means ± S.E.M; n=6. Student's test for differences between two data groups: * P<0.05; significantly different from control; #, P < 0.05 vs. Ang II (small checkers columns); §, P < 0.05 vs Ang II+AT1a or AngII+AT2.*

"In theory, there is no difference between theory and practice. But in practice, there is".

Albert Einstein (1879-1955)
The Nobel Prize in Physics 1921

5.2 Discussion

5.2.1 Setting up of HUVEC's protocol and Ang II modulates HUVEC calcium concentration via AT1R involving IP3 and T-Type Calcium Channels.

The concept of endothelium as a passive barrier lining the inner side of blood vessels is completely out of date. Nowadays, endothelial cells (EC) are actually considered constituents of a metabolic tissue that regulates a variety of biological responses and physiological functions.[92] Endothelial dysfunction is characterized by a shift of the actions of the endothelium toward reduced vasodilatation, a pro-inflammatory state and pro-thrombic properties. It is associated with most forms of cardiovascular disease, such as hypertension, coronary artery disease, chronic heart failure, peripheral artery disease, diabetes, and chronic renal failure.[93]

Therefore, the endothelial cells represent well a means to study and develop new approaches for both the prevention and therapy of cardiovascular diseases.

The use of human EC rather than of animal cells is usually preferred.

Species source	***Tissues sources***
Human Pig Bovine Rat Rabbit	Pulmonary artery and vein[94] Saphenous vein [95] Umbilical artery and vein[96] Heart: microvascular coronary EC[97] Liver: sinusoidal and liver EC [98] Lungs: microvascular EC [99] Kidney: microvascular EC[100] Brain: microvascular EC[101] Dermis: microvascular EC[102]

Table 2. ***Species and sources for isolating EC***

Ang II is an important vasoactive peptide that physiologically regulates vascular tone and maintains normal vessel structure and function, but it has also been claimed to induce several detrimental endothelial effects;[103] in fact increased levels of Ang II have been implicated in pathophysiological processes that include atherosclerosis, cardiac hypertrophy, nephropathy, vascular injury, and remodelling.

Therefore, the first study performed was the setting up of HUVEC's protocol for the determination of the cell viability through the label Acridin Orange under the action of Ang II either at 10^{-9} M or 10^{-7} M or 10^{-6} M and the determination of the intracellular parameters: Calcium, Nitric Oxide (NO) and Reactive Oxygen Species (ROS) levels, using selective labels.

The cell viability results point out the contribution only of Ang 10^{-7} M and 10^{-6} M, with or without treatment with ARBs, to induce significant increases of label fluorescence intensity. The effect is not observed in the presence of Ang II 10^{-9} M after short term but only after 9 hours. These

observations point out the necessity of the Ang II 10^{-9} M concentration to increase viability at long term.[104]

In the bases of such data, it was useful to confirm that results by the study of someone else's parameter, for instance intracellular free calcium levels.

The results regarding the treatment of the cells with increasing concentrations of either Ang II 10^{-9} M or 10^{-7} M or 10^{-6} M, highlight the effect of Ang II at all concentrations to induce significant increase of that parameter in comparison with the reference after all terms, except for the action of Ang 10^{-9} M after 9 hours.

The AT2 antagonist PD 123319 induces a significant increase of that parameter in comparison with control only in the presence of Ang II either 10^{-7} M or 10^{-6} M.

The AT1R antagonist ZD 7155 induces a significant modulation of intracellular calcium levels in comparison with the Ang II alone, confirming the antagonist action of AT2R. In fact, in literature, the effect of AT2R is known to modulate vasodilatation by both NO synthesis and calcium depletion.[105] These results show that the $[Ca^{2+}]_i$ mobilization occurs through the AT1R and not through the AT2R to induce vasoconstriction. The present study shows that an increasing concentration of Ang II at 10^{-9} M, 10^{-7} M and 10^{-6} M increases the intracellular calcium levels.

The peak release of $[Ca^{2+}]_i$ was found at 10^{-7} M Ang II concentration and this response is in agreement with the scenario of agonist-induced increases in $[Ca^{2+}]_i$ for G-protein–coupled receptors. There is a peak in $[Ca^{2+}]_i$ caused by Ca^{2+} mobilization. One of the major acute functions of ANG II is vasoconstriction, which is mediated by "classical" G protein-dependent signalling pathways. Evidence shows that when activated by an AT1Rs agonist, the receptor binds to $G\alpha_{q/11}$, $G\alpha_{12/13}$, and $G_{\beta}y$ complexes, which activate downstream effectors including phospholipase C. Activation of PLC produces inositol-1,4,5-triphosphate and within a few seconds. IP3

binds to its receptor on ER opening a channel that allows calcium efflux into the cytoplasm.[106]

On the basis of the study on HUVECs viability and the evaluation of intracellular free calcium the optimal and reliable Ang II concentration is 10^{-7} M.

The type of endothelial receptor implicated in the ANG II-induced NO production was determined using selective antagonists for each ANG II receptor type, ZD7155 and PD123319. HUVEC treatment with Ang II at 10^{-7} M induced NO production either in the presence or absence of AT1Ra, suggesting that this effect is mediated by the AT2 receptor while the treatments with AT2Ra suggest an antagonistic function of both receptor subtypes. Ang II regulates NO levels, mediated by both AT1 and AT2 receptors. Ang II, via the AT1 receptor, decreases NO bioavailability by stimulating superoxide production as well as blocking NO signal transduction. For example, Ang II reduces sGC activity/expression, induces cGMP hydrolyzing PDE expression, and reduces PKG activity. Ang II, via the AT2 receptor, results in a kinin-dependent stimulation of NO production. Several studies have shown that the endothelium can modulate the ANG II-induced contraction of blood vessels and that the octapeptide even elicits vasodilatation in some vascular beds.[107,108] These studies suggested that ANG II stimulates the release of vasodilators such as NO and vasoconstriction through calcium mobilization.[109]

Incubation of intact HUVECs with Ang II also increased the superoxide formation in endothelial cell and this effect was mediated by AT1 receptors, in fact, in the presence of PD123319, we have significant increase of this ROS after 3, 6 and 9 hours. AT2 receptor does not seem to affect the increase of ROS levels but this receptor induces significant decrease when the cells are treated with ZD 7155 confirming the antagonist action of both receptors. These results are in agreement with previous studies in EC[110]

In conclusion, Ang II receptor subtypes differentially modulate endothelial superoxide formation. While AT1 activates superoxide formation, AT2 receptors appear to attenuate this AT1R effect.

ANOVA of the timecourses of NO, ROS, and Ca^{2+} intracellular levels under the action of Ang II 10^{-7} M, described in fig. 5, shows that the three intracellular parameters are co-ordinated by the octapeptide and these parameters are interrelated among each other.[111]

As previously described, Ang II is involved in the development of atherosclerosis, vascular remodelling and cardiac hypertrophy. Recent studies indicate that cardiac T-type Ca^{2+} channels (TCCs) increase in hypertrophied ventricular cells.[112] Laurent Ferron et al., show that Ang II, via AT1, activates MEK pathway upregulating T-type channel expression in cultured myocytes.[113]

The T-type Ca^{2+} channels have properties different from those of the L-type, such as more negative voltage range of activation and inactivation of standard Ca^{2+} blockers. T-type Ca^{2+} channels are expressed throughout the body, including nervous tissue, heart, kidney, smooth muscle, many endocrine organs and vascular endothelial cells.[114,115]

The role of the TCCs in endothelial cell function up to now is not well understood. In the present study, we showed that Ang II 10^{-7} M regulates $[Ca^{2+}]_i$ by both IP3 pathways for rapid physiological effects and T-Type Calcium Channels for long term effects.

Fig.13:A-C describe the effect of Ang II with or without simultaneous incubation with either Wortmannin or Mibefradil. Such treatment points out the involvement of either IP3 or TCC pathways in the modulation of intracellular calcium. Such pathways induce significant decreases of $[Ca^{2+}]_i$ in comparison with Ang II alone after 3, 6 and 9 hours.

The next step is the determination of the receptor type involved in the stimulation of the TCCs. The effect of the co-incubation with Ang II, AT1Ra and either Wort or CCB confirms the antagonist action of AT2R to decrease the $[Ca^{2+}]_i$ and surely does not involve the IP3 pathway and TCCs. Probably the Ang II binding with AT2 receptor increases intracellular cGMP production and in turn activates PKG. PKG inhibits the IP3 receptor and stimulates the plasma membrane calcium/ ATPase (SERCA) to decrease intracellular calcium concentrations. These results are in agreement with David A. Powis, et al., which showed that in bovine chromaffin cells, Ang II causes Ca^{2+} entry by pathways activated by internal store mobilization; the entry through these pathways forms the majority of the sustained Ca^{2+} influx evoked by Ang II.[116] When the ECs were incubated with Ang II + PD123319, we can see the significant increase of this parameter in comparison with control by AT1R. The use of the inhibitor of IP3 pathway shows that Ang II, via the AT1 receptor bound to G protein, activates PLC and in turn produces IP3, followed by stimulation of Ca^{2+} mobilization from the IP3 receptor.

The effect of Calcium channel blockers on ECs point out the contribution of this pathway to modulate the intracellular free calcium. The significant decrease in comparison with Ang II alone after 9 hours points out that Ang II through AT1 receptor also activates the calcium channel in HUVECs. Our results could explain the Dahai W. et al. data, who showed that Ang II preferentially increases the expression of α1G, a T-type Ca^{2+} channel subunit, via AT1 receptors, Ras and MEK in endothelial cells.[117]

In summary, Ang II induces alterations of intracellular free calcium through AT1 receptor pathway by the activation of IP3, for rapid physiological effects, and TCCs pathway for long term effects. These results suggest that TCCs regulate calcium permeability function.[118]

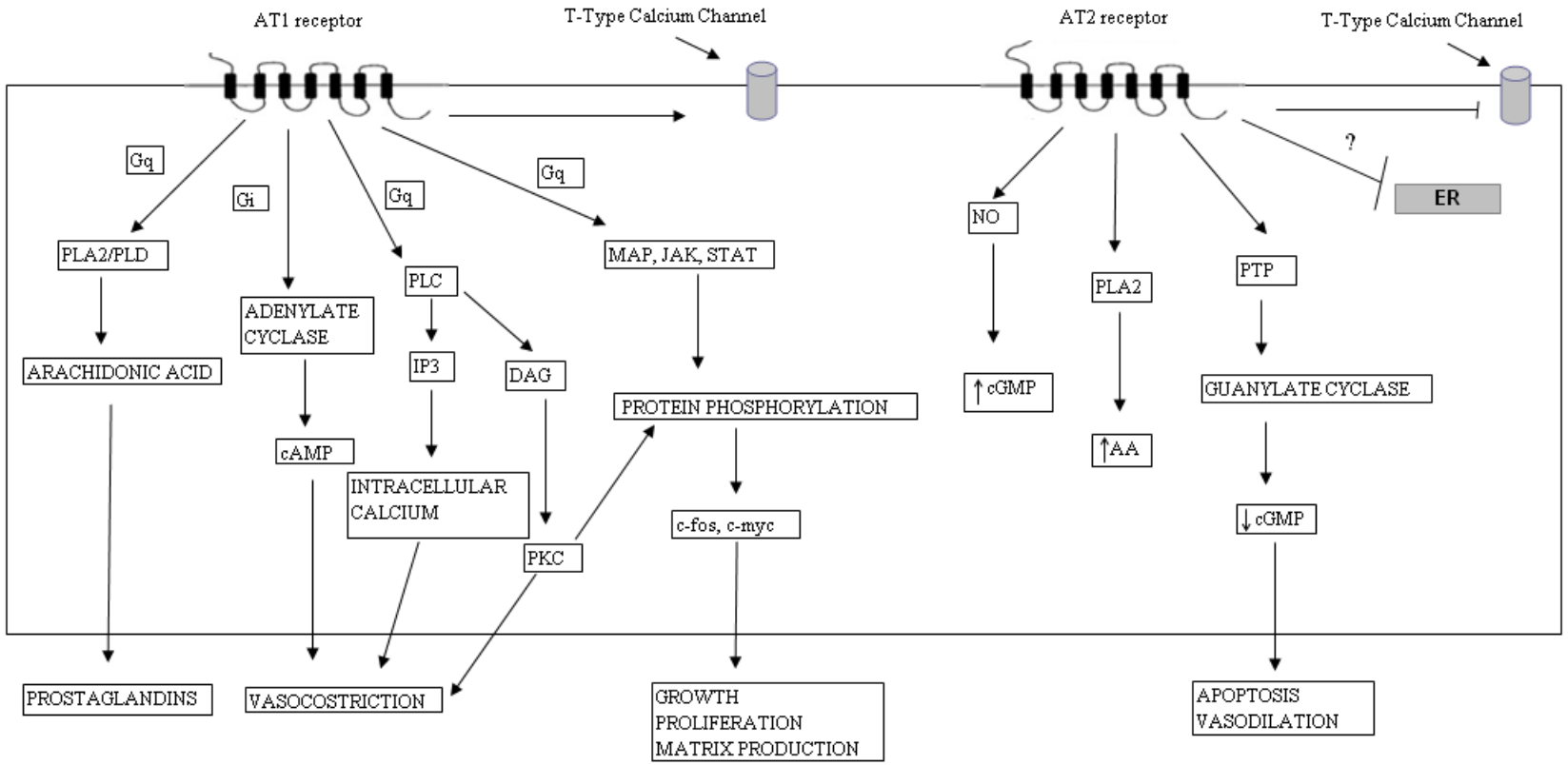

Figure 15. Putative model of signal mechanisms induced by angiotensin II (Ang II) in endothelial cells. AT1 and AT2, angiotensin II receptor subtype; DAG, diacylglycerol; IP_3, inositol-1,4,5-triphosphate; JAK, janus kinase; MAPK, mitogen-activated protein kinase; MAPK kinase kinase; MKP-1, MAPK phosphatase-1; NO, nitric oxide; PKC, protein kinase C; PLC, phospholipase C; PP2A, protein phosphatase 2A; PTK, protein tyrosine kinase; PTPase, protein tyrosine phosphatase; STAT, signal transducer and activator of transcription

"A conclusion is simply the place where someone got tired of thinking".

Albert Einstein (1879-1955)
The Nobel Prize in Physics 1921

CONCLUSIONS

The evaluation of $[Ca^{2+}]_i$, NO and ROS levels in HUVECs under the action of Ang II demonstrates that:

Intracellular calcium levels indeed are well related to both NO and ROS levels as determined by selective fluorescent labels, by Fluo-3AM, DAF-2DA, HDCFH-DA and validated by ANOVA. Consequently, HUVECs model is usefully analysed by our method to evaluate the acting mechanism of Ang II on isolated endothelial tissue. The evaluation of Acridin Orange, Calcium, NO and ROS, shows that Ang II 10^{-7} M is the optimal concentration to study the effect of hormone on human umbilical vein endothelial cells.

Our study points out the role of T-Calcium Channels on the regulation of the intracellular calcium levels independently from IP3 mechanism and the relevant role of calcium channels not only on vascular smooth muscle but also on the endothelium functions and structures; in fact the TCCs demonstrated to play an important role in the development of vascular disorders.

REFERENCES

1. THOMAS MC, STEVEN DC: Kidney in Hypertension Guyton Redux. *Hypertension* **51**:811-816, 2008.
2. WATANABE T, BARKER T, BERK BC: Angiotensin II and the endothelium: diverse signals and effects. *Hypertension* **45**: 163–169, 2005.
3. PÉREZ-REYES E: Molecular physiology of low-voltage-activated t-type calcium channels. *Physiol. Rev* **83:** 117–16, 2003.
4. VINET R, VARGAS FF: L- and T-type voltage-gated Ca^{2+} currents in adrenal medulla endothelial cells. *Am. J. Physiol* **276**: H1313–H1322,1999.
5. MONTIEL M, IDOIA GÀMEZ M, GARCIÀ-VALLEJO JS, PÉREZ DE LA BLANCA E, MARTÍN E, QUESADA J, JIMÉNEZ E: Angiotensin II effect on calcium mobilization and mitogen-activated protein kinase activation in human umbilical vein endothelial cells. *Signal Transduction* **5-6**: 201-208, 2003.
6. STRIJDOM H, MULLER C, LOCHNER A. Direct intracellular nitric oxide detection in isolated adult cardiomyocytes: flow cytometric analysis using the fluorescent probe, diaminofluorescein. *J Mol Cell Cardiol* **37(4)**:897-902, 2004.
7. STEPHEN L. HEMPELGR, BUETTNER YQ, O'MALLEY DA, WESSELS DM, FLAHERTY D: Dihydrofluorescein diacatate is superior for detecting intracellular oxidants: comparison with 2,7-dichlorodihydrofluorescein diacetate, 5 (and 6)-Carboxy-2,7-

dichlorodihydrofluorescein diacetate and dihydrorhodamine 123. *FREE RADICAL BIOLOGY & MEDICINE* **27**: 1/2, 146–159, 1999.

8. DESIDERI G, BRAVI M.C, TUCCI M, CROCE G, MARINUCCI M.C, SANTUCCI A, ALESSE E, FERRI C: Angiotensin II Inhibits Endothelial Cell Motility Through an AT1-Dependent Oxidant-Sensitive Decrement of Nitric Oxide Availability. *Arterioscler Thromb Vasc Biol* **23:**1218-1223, 2003.
9. DEANFIELD MB, HALCOX JP, TON J. RABELINK: Endothelial Function and Dysfunction. Testing and Clinical Relevance. *Circulation* **115**:1285-1295, 2007.
10. DIERK HE, and SCHIFFRIN EL: Endothelial Dysfunction: *J Am Soc Nephrol* **15**: 1983–1992, 2004.
11. GHITESCU L, AND MANON R: Diversity in Unity: The Biochemical Composition of the Endothelial Cell Surface Varies Between the Vascular Beds. *Microscopy research and technique* **57**:381–389, 2002.
12. HURAIRAH H, FERRO A: The role of the endothelium in the control of vascular function. *J Clin Pract* **58**: 173–183, 2004.
13. FISHA JE, AND MARSDEN PA: Endothelial nitric oxide synthase: insight into cell-specific gene regulation in the vascular endothelium. *Cell. Mol. Life Sci.* **63:** 144–162, 2006.
14. THOMAS F. LÜSCHER MD, AND BARTON M: Biology of the Endothelium. *Clin. Cardiol* **20 (Suppl. II),** II-3–II-10, 1997.
15. AL SÁDONI H, FERRO A: S-NITROSOTHIOLS: a class of nitric oxidedonor drugs. *Clin Sci (Lond)* **98**: 507–20, 2000.
16. PAGLIARO P: Differential biological effects of products of nitric oxide (NO) synthase: it is not enough to say NO. *Life Sciences* **73**, 2137–2149, 2003.
17. HURAIRAH H and FERRO A: The role of the endothelium in the control of vascular function. *J Clin Pract* **58**: 2, 173–183, 2004.

18. BUNTING S, GRYGLEWSKI R, MONCADA S, VANE JR: Arterial walls generate from prostaglandin endoperoxides a substance (prostaglandin X) which relaxes strips of mesenteric and coeliac ateries and inhibits platelet aggregation. *Prostaglandin*s **12**: 897–913, 1976.
19. HYUNJUNG L, AND SUDHANSU KD: A Novel Pathway of Prostacyclin Signaling—Hanging Out with Nuclear Receptors. *Endocrinology* **143(9)**:3207–3210, 2002.
20. COHEN RA: The Endothelium-Derived Hyperpolarizing Factor Puzzle A Mechanism Without a Mediator? *Circulation* **111:**724-727, 2005.
21. TAYLOR SG, WESTON AH: Endothelium-derived hyperpolarizing factor: a new endogenous inhibitor from the vascular endothelium. *Trends Pharmacol Sci* **9**: 272–4, 1988.
22. SANDOW SL: Factors, fiction and endothelium-derived hyperpolarizing factor - EDH(F). *Proceedings of the Australian Physiological and Pharmacological Society* **34:** 2004.
23. AHLUWALIA A, AND HOBBS AJ: Endothelium-derived C-type natriuretic peptide: more than just a hyperpolarizing factor. *TRENDS in Pharmacological Sciences* **26:** 3 2005.
24. FÉLÉTOU M, VANHOUTTE PM: Endothelium-Derived Hyperpolarizing Factor Where Are We Now? *Arterioscler Thromb Vasc Biol* **26**:1215-1225, 2006.
25. CHAUHAN SD, AND HOBBS, AHLUWALIA AJ: C-type natriuretic peptide: new candidate for endothelium-derived hyperpolarising factor. *The International Journal of Biochemistry & Cell Biology* **36**: 1878–1881, 2004.
26. INMACULADA CV, HOBBS AJ, AND AHLUWALIA A: Sex differences in vascular function: implication of endothelium-derived

hyperpolarizing factor. *Journal of Endocrinology* **197**, 447–462, 2008.

27. SUDOH T, MINAMINO N, KANGAWA K, MATSUO H: Brain natriuretic peptide-32: N-terminal six amino acid extended form of brain natriuretic peptide identified in porcine brain. *Biochem Biophys Res Commun* **155**:726-32, 1988;
28. VILLAR C: Novel cardiovascular roles for CNP. *Endocrine Abstracts* **15:** S35, 2008
29. CAMPBELL WB, AND GAUTHIER KM: What is new in endothelium-derived hyperpolarizing factors? *Curr. Opin. Nephrol. Hypertens* **11**, 177–183, 2002.
30. D'SOUZAA SP, DAVISB M, BAXTERC GF: Autocrine and paracrine actions of natriuretic peptides in the heart. *Pharmacology & Therapeutics* **101:**113– 129, 2004.
31. SCOTLANDA RS, AHLUWALIAB A, HOBBS AJ: C-type natriuretic peptide in vascular physiology and disease. *Pharmacology & Therapeutics* **105**: 85– 93, 2005.
32. MASAKI T, AND SAWAMURA T: Endothelin and endothelial dysfunction. *Proc.Jpn.Acad, Ser.* **B82**, 2006.
33. BROWN CD, BARNES K, TURNER AJ: Functional significance of the isoforms of endothelin-converting enzyme-1. *J CardiovascPharmacol* **36:** S26–7, 2000.
34. LEE TM, SU SF, TSAI CH: Oestrogen attenuates coronary vasoconstriction after angioplasty: role of endothelin-1. *Eur J Clin Invest* 2002.
35. HARIZI H, CORCUFF JB, AND NORBERT GUALDE. Arachidonic-acid-derived eicosanoids: roles in biology and immunopathology. *Trends in Molecular Medicine* **14**,10, 2008.
36. HAMBERG M, SVENSSON J, AND SAMUELSSON B: Thromboxanes: A new group of biologically active compounds

derived from prostaglandin endoperoxides. *Proc Natl Acad Sci U S A* **72**, 2994–2998, 1975.

37. BAYAT H, XU SH, PIMENTEL D, COHEN RA, AND JIANG B: Activation of Thromboxane Receptor Upregulates Interleukin (IL)-1{beta}–Induced VCAM-1 Expression Through JNK Signaling. *Arteriosclerosis, Thrombosis & Vascular Biology* **28(1):**127-134, 2008.
38. NIE D, LAMBERTI M, ZACHAREK A, LI L, SZEKERES K, TANG K, CHEN Y, HONN KV: Thromboxane A_2 Regulation of Endothelial Cell Migration, Angiogenesis, and Tumor Metastasis. *Biochemical and Biophysical Research Communications* **267**, 245-251(7), 2000.
39. NAKAHATA N: Thromboxane A_2: Physiology/pathophysiology, cellular signal transduction and pharmacology. *Pharmacology & Therapeutics* **118:** 18–35, 2008.
40. FERRARIO CM, STRAWN WB: Role of the Renin-Angiotensin-Aldosterone System and Proinflammatory Mediators in Cardiovascular Disease. *Am J Cardiol* **98:**121–128, 2006.
41. SUWAIDI JA, HAMASAKI S, HIGANO ST, NISHIMURA RA, HOLMES DR JR, LERMAN A: Long-term follow-up of patients with mild coronary artery disease and endothelial dysfunction. *Circulation* **101**: 948–954, 2000.
42. SCHIFFRIN EL, PARK JB, INTENGAN HD, TOUYZ RM: Correction of arterial structure and endothelial dysfunction in human essential hypertension by the angiotensin receptor antagonist losartan. *Circulation* **101**: 1653–1659, 2000.
43. SCHIFFRIN EL, TOUYZ RM: Multiple actions of angiotensin II in hypertension: Benefits of AT1 receptor blockade. *J Am Coll Cardiol* **42**: 911–913, 2003.

44. BUDOFF M, NASER A, HARTLEY C, NASIR K, TANAKA H, METCALFE R, HECHT H, KLEIS S: Thermal Monitoring of Vascular Reactivity and Endothelial Function Improves. *Cardiovascular Risk Assessment*.
45. STOLL M, UNGER TH: Angiotensin and its AT receptor: new insights into an old system. *Regulatory Peptides*. **99:** 175–182, 2001.
46. SHAN H, BAI X, AND CHEN X: Angiotensin II induces endothelial cell senescence via the activation of mitogen-activated protein kinases. *Cell Biochem Funct* **26**: 459–466, 2008.
47. BENNDORF R, BÖGER HR, ERGÜN S, STEENPASS A, WIELAND TH: Angiotensin II Type 2 Receptor Inhibits Vascular Endothelial Growth Factor–Induced Migration and In Vitro Tube Formation of Human Endothelial Cells. *Circ **Res*** **93**: 438**-**447**,** 2003**.**
48. SHOKEI K, YASUKATSU I, YASUHIRO I, YUMEI ZH, MASARU T, HIROSHI I: Beneficial Effects of Combined Blockade of ACE and AT1Receptor on Intimal Hyperplasia in Balloon-Injured Rat Artery. *Arterioscler Thromb Vasc Biol* **22**:1299-1304, 2002.
49. KIM S, IWAO H. Molecular and cellular mechanisms of angiotensin II-mediated cardiovascular and renal diseases. *Pharmacol Rev* **52**:11–34,2000.
50. MEHTA PK, AND GRIENDLING KK: Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system. *Am J Physiol Cell Physiol* **292**: C82-C97, 2007.
51. FERREIRA R, AND SANTOS A.S: Cardiovascular actions of angiotensin-(1-7). *A.J. Braz Journal of Medical and Biological Research:* **38 (3)**: 499, 2005.
52. PARSONS KK, AND COFFMAN THM: The renin-angiotensin system: it's all in your head. *J Clin Invest*: **117(4)**: 873–876, 2007.
53. LÁSZLÓ H, AND KEVIN JC: Pleiotropic AT1 Receptor Signaling Pathways Mediating Physiological and Pathogenic Actions of Angiotensin II. Molecular *Endocrinology* **20**: 953–970, 2006.

54. DIEM TD, AND FRAUMAN AG: Angiotensin receptors: distribution signalling and function. *Clinal Science, Rev* **100**: 481-492, 2001.
55. HIGUCHI S, OHTSU H, SUZUKI S, SHIRAI H, FRANK D.S, EGUCHI S: Angiotensin II signal transduction through the AT1 receptor: novel insights into mechanisms and pathophysiology. Clinical Science 112: 417–428, 2007.
56. GRIENDLING KK, USHIO-FUKAI M, LASSEGUE B, AND ALEXANDER, RW: Angiotensin II signaling in vascular smooth muscle. New concepts. *Hypertension* **29**: 366–373, 1997.
57. SUZUKI, H, MOTLEY, ED, FRANK G.D, UTSUNOMIYA H, AND EGUCHI S: Recent progress in signal transduction research of the angiotensin II type-1 receptor: protein kinases, vascular dysfunction and structural requirement. *Curr. Med. Chem. Cardiovasc. Hematol. Agents* **3**: 305–322, 2005.
58. YIN G, YAN C, AND BERK BC: Angiotensin II signaling pathways mediated by tyrosine kinases. *Int. J. Biochem. Cell Bio* **35**: 780–783, 2003.
59. FELETOU M, AND VANHOUTTE PM: Endothelial dysfunction: a multifaceted disorder. *Am. J. Physiol. Heart Circ. Physiol* **291**: H985–H1002, 2006.
60. KIM JA, MONTAGNANI M, KOH KK, AND QUON MJ: Reciprocal relationships between insulin resistance and endothelial dysfunction: molecular and pathophysiological mechanisms. *Circulation* **113**, 1888–1904, 2006.
61. NAKASHIMA H, SUZUKI H, OHTSU, H: Angiotensin II regulates vascular and endothelial dysfunction: recent topics of Angiotensin II type-1 receptor signalling in the vasculature. *Curr. Vasc. Pharmacol* **4**: 67–78, 2006.
62. DIMMELER S, RIPPMANN V, WEILAND U, HAENDELER J, ZEIHER AM: Angiotensin II induces apoptosis of human endothelial cells. Protective effect of nitric oxide. *Circ Res* **81**:970 –976, 1997.

63. LI DY, ZHANG YC, PHILIPS MI, SAWAMURA T, MEHTA JL: Upregulation of endothelial receptor for oxidized low-density lipoprotein (LOX-1) in cultured human coronary artery endothelial cells by angiotensin II type 1 receptor activation. *Circ Res* **84**:1043–1049,1999.
64. DIMMELER S, ZEIHER AM: Reactive oxygen species and vascular cell apoptosis in response to angiotensin II and pro-atherosclerotic factors. *Regul Pept* **90**:19 –25, 2000.
65. MARRERO MB, VENEMA VJ, JU H, HE H, LIANG H, CALDWELL RB, VENEMA RC: Endothelial nitric oxide synthase interactions with G-protein-coupled receptors. *Biochem* j **343**:335–340, 1999.
66. VAUGHAN DE, LAZOS SA, TONG K: Angiotensin II regulates the expression of plasminogen activator inhibitor-1 in cultured endothelial cells. A potential link between the renin-angiotensin system and thrombosi. *J Clin Invest* **95(3)**: 995-1001, 1995.
67. KOHOUT TA, ROGERS TB: Angiotensin II activates the Naqr NCO3 symport through a phosphoinositide-independent mechanism in cardiac cells. *J Biol Chem* **269**: 20432–8 1995
68. NOUET S, NAHMIAS C: Signal transduction from the angiotensin AT2 receptor. *Trends Endocrinol Metab* **11**, 1–6, 2000.
69. MOELLER I, CLUNE EF, FENNESSY PA, BINGLEY JA, ALBISTON AL, MENDELSOHN FA, CHAI SY: Up regulation of AT4 receptor levels in carotid arteries following balloon injury. *Regul Pept* **83**:25–30,1999.
70. KASAHARA TH, SUGANO T, YOSHIZUMI M, SAWADA S, NAKAGAWA M: Angiotensin II increases plasminogen activator inhibitor-1 and tissue factor mRNA expression without changing that of tissue type plasminogen activator or tissue factor pathway inhibitor in cultured rat aortic endothelial cells. *Thromb Haemost* **77**:1189 –1195,1997.
71. MEHTA JL, LI DY, YANG H, RAIZADA MK: Angiotensin II and IV stimulate expression and release of plasminogen activator inhibitor-1 in cultured human coronary artery endothelial cells. *J Cardiovasc Pharmacol* **39**:789 –794, 2002.

72. PATEL JM, MARTENS JR, LI YD, GELBAND CH, RAIZADA MK, BLOCK ER: Angiotensin IV receptor-mediated activation of lung endothelial NOS is associated with vasorelaxation. *Am J Physiol* **275**: L1061–L1068,1998.
73. LI YD, BLOCK ER, PATEL JM: Activation of multiple signalling modules is critical in angiotensin IV-induced lung endothelial cell proliferation. *Am J Physiol Lung Cell Mol Physiol* **283**:L707–L716, 2002.
74. RUIZ-ORTEGA M, LORENZO O, RUPEREZ M, ESTEBAN V, SUZUKI Y, MEZZANO S, PLAZA JJ, EGIDO J: Role of the renin-angiotensin system in vascular diseases: expanding the field. *Hypertension* **38**:1382–1387, 2001.
75. CRIDDLE DN, GERASIMENKO JV, BAUMGARTNER HK, JAFFAR M, VORONINA S, SUTTON R, PETERSEN OH, GERASIMENKO OV: Calcium signalling and pancreatic cell death: apoptosis or necrosis? Cell Death and Differentiation **14**, 1285–1294J, 2007.
76. FOSKETT K, WHITE C, CHEUNG KH, MAK D: Inositol Trisphosphate Receptor Ca^{2+} Release Channels. *Physiol Rev* **87**: 593–658, 2007.
77. TOUYZ RM, BERRY C: Recent advances in angiotensin II signaling. *Braz J Med Biol Res* **35(9)**: 2002.
78. OSHIMA T, OZONO R, YANO Y, HIGASHI Y, TERAGAWA H, MIHO N, ISHIDA T, ISHIDA M, YOSHIZUMI M, KAMBE M: Beneficial Effect of T-Type Calcium Channel Blockers on Endothelial Function in Patients with Essential Hypertension. *Hypertens Res* **28:** 11, 2005.
79. YAZAWA K, ONO K, IIJIMA T: Modulation by mibefradil of the histamine-induced Ca^{2+} entry in human aoristic endothelial cells. *Jpn J Pharmacol* **90**: 125-130, 2002.
80. WU S, HAYNES J, TAYLOR JT: (α1G) T-type Ca^{2+} channels mediate vaso-occlusion of sickled erythrocytes in lung microcirculation. *Circ Res* **93**: 346-353, 2003.
81. MURAKAMI T, TAKAGI H, SUZUMA K, SUZUMA I, OHASHI H, WATANABE D, OJIMA T, SUGANAMI E, KURIMOTO M, KANETO H, HONDA Y, YOSHIMURA: Angiopoirtin-1 attenuates H_2O_2-induced SEK1/JNK phosphorylation through the phosphatidylinositol 3-kinase/Akt

pathway in vascular endothelial cells. *J Biol Chem.* **280(36)**: 31841-9, 2005.

82. JAFFE EA, NACHMAN RL, BECKER CG, MINICK CR: Culture of human endothelial cells derived from umbilical cord veins: identification bymorphologic and immunologic criteria. *J Clin Invest* **52**: 2745–2756, 1973.
83. DARZYNKIEWICZ Z: Differential staining of DNA and RNA in intact cells and isolated cell nuclei with acridine orange. *Methods in Cell Biology* **33**:285-98, 1990.
84. DARZYNKIEWICZ Z. BRUNO S. DEL BINO G. GORCZYCA W. HOTZ MA. LASSOTA P. TRAGANOS F: Features of apoptotic cells measured by flow cytometry. *Cytometry* **13(8):**795-808, 1992.
85. KWON MS, PARK SC, CHOI K, AHNN J, KIM IJ, EOM SH, KAUFMAN SG, SONG WK: Calreticulin Couples Calcium Release and Calcium Influx in Integrin-mediated Calcium Signaling**.** *Molecular Biology of the Cell* **11**, 1433–1443, 2000
86. MARAVALL M, MAINEN ZF, SABATINI BL, SVOBODA K: Estimating Intracellular Calcium Concentrations and Buffering without Wavelength Ratioing. *Biophys J*p **78:** 2655-2667, 2000.
87. LIN YQ, AND BENNETT MR: Varicosity-Schwann Cell Interactions Mediated by ATP in the Mouse Vas Deferens. *J Neurophysiol* **93**: 2787–2796, 2005.
88. KOJIMA H, SAKURAI K, KIKUCHI K, KAWAHARA S, KIRINO Y, NAGOSHI H, HIRATA Y, NAGANO T: Development of a fluorescent indicator for nitric oxide based on the fluorescein chromofore. *Chem Pharm Bull* **46**: 373-375, 1998.
89. KOJIMA H, NAOKI KK, URANO Y, HIGUCHI T, TANAKA J, KUDO Y, NAGANO T: Detection and imaging of nitric oxide with novel fluorescent indicators: diaminofluoresceins: *Neuroreport Anal. Chem* **70**, 2446, 1998.
90. STRIJDOM H, GENADE S, AND LOCHNER A: Nitric Oxide Synthase (NOS) Does Not Contribute to Simulated Ischaemic

Preconditioning in an Isolated Rat Cardiomyocyte Model. *Cardiovascular Drugs and Therapy* **18**:99–112, 2004.

91. HIPLER U, WOLLINA D, DENNING BH: Fluorescence Analysis of Reactive Oxygen Species (ROS) Generated by Six Isolates of Aspergillus Fumigatus. *BMG Labtechnologies* **49**, 781, 2002.
92. BACHETTI T, MORBIDELLI L: Endothelial cells in culture: a model for studying vascular functions. *Pharmacological Research* **42**: 1, 2000.
93. DIERK HE, AND SCHIFFRIN EL: Endothelial Dysfunction. *J Am Soc Nephrol* **15**: 1983–1992, 2004.
94. JOHNSON AR: Human pulmonary endothelial cells in culture. Activities of cells from arteries and cells from veins. *J Clin In¨est* **65**: 841-50, 1980.
95. WATKINS MT, SHAREFKIN JB, ZAJTCHUK R, MACIAG TM, D'AMORE PA, RYAN US, VAN WART H, RICH NM:Adult human saphenous vein endothelial cells: assessment of their reproductive capacity for use in endothelial seeding of vascular prostheses. *J Surg Res* **36**: 588-96, 1984.
96. GIMBRONE MA JR, COTRAN RS, FOLKMAN J. Human vascular endothelial cells in culture. Growth and DNA synthesis. *J Cell Biol* **60**: 673-84,1974.
97. PIPER HM, SPAHR R, MERTENS S, KR¨UTZFELD A, WATANABE H: Microvascular endothelial cells from heart. In: Cell culture techniques in heart and vessel research. *Piper HM, ed. Berlin Heidelberg: Springer-Verlag* 158 – 77, 1990.
98. BROUWER A, BARELDS LJ, DE LEEUW AM, BLAUW E, PLAS A, YAP SH, VAN DEN BROCK AMWC, KNOOK DL: Isolation and culture of Kupffer cells from human liver: Ultrastructure, endocytosis and prostaglandin synthesis. *J Hepatol* **6**: 36-49,1988.
99. RYAN US: Microvascular endothelial cells from the lungs. In: Cell culture techniques in heart and Vessel research: *Piper HM, ed. Berlin Heidelberg*: *Springer- Verlag,*: 130-9, 1990.
100. GIBBS VC, WOOD DM, GAROVOY MR: The response of cultured human kidney capillary endothelium to immunologic stimuli. *Hum Immunol* **14**: 259-69,1985.

101. GERHART DZ, BRODERIUS MA, DREWES LR: Cultured human and canine endothelial cells from brain microvessels. *Brain Res Bull* **21**: 785-93,1988.
102. DAVISON PM, BENSCH K, KARASEK MA: Isolation and long-term serial cultivation of endothelial cells from the microvessels of the adult human dermis. *In vitro***19**: 937-45,1983.
103. DZAU VJ: Tissue angiotensin and pathobiology of vascular disease: a unifying hypothesis. *Hypertension* **37**:1047–1052, 2001.
104. KOU B, VATISH M, SINGER DRJ: Effects of Angiotensin II on human endothelial cells survival signalling pathways and its angiogenic response. *Vascular Pharmacology* **47**: 199–208, 2007.
105. RUISHENG L, PERSSON AG: Angiotensin II Stimulates Calcium and Nitric Oxide Release From Macula Densa Cells Through AT1 Receptors. *Hypertension* **43**:649-653, 2004
106. USHIO-FUKAI M, ALEXANDER RW, AKERS M, LYONS PR, LASSEGUE B, GRIENDLING KK: Angiotensin II receptor coupling to phospholipase D is mediated by the betagamma subunits of heterotrimeric G proteins in vascular smooth muscle cells. *Mol Pharmacol* **55**: 142–149, 1999.
107. CHEN Y, DONGSOO K, TORU A, AND BERK BC: Functional Interplay Between Angiotensin II and Nitric Oxide: Cyclic GMP as a Key Mediator. *Arterioscler. Thromb. Vasc. Biol* **23**:26-36, 2003.
108. VALLEGA GA, CANESSA ML, BERK BC, BROCK TA, ALEXANDER RW: Vascular smooth muscle Na^+/-H^+ exchanger kinetics and its activation by angiotensin II. *Am J Physiol Cell Physiol* **254**: C751–C758, 1988.
109. SCHENA M, MULATERO P, SCHIAVONE D, MENGOZZI G, TESIO L, CHIANDUSSI L, VEGLIO F: Vasoactive hormones induce nitric oxide synthase mRNA expression and nitric oxide production in human endothelial cells and monocytes. *Am J Hypertens* **12**: 388–397, 1999.
110. HAE YS, ULRIKE R, ANKE H, TORSTEN G, HEERMEIER K, GALLE J AND ULRICH P: Differential role of angiotensin II receptor subtypes on

endothelial superoxide formation. *British Journal of Pharmacology* **131**: 667 - 672, 2000.

111. FRAME DM, RANDALL J, DONGSOO K, MOHAN A, BERK BC, AND YAN CH: Diminished arteriolar responses in nitrate tolerance involve ROS and angiotensin II. *Am J Physiol Heart Circ Physiol* **282**: H2377–H2385, 2002.
112. MARTINEZ ML, HEREDIA MP, DELGADO C: Expression of T-type Ca^{2+} channels in ventricular cells from hypertrophied rat hearts. *J Mol Cell Cardiol* **31**:1617–1625, 1999.
113. FERRON L, CAPUANO V, RUCHON Y, DEROUBAIX E, COULOMBE A, RENAUD JF: Angiotensin II Signaling Pathways Mediate Expression of Cardiac T-Type Calcium Channels. *Circ Res* **93**:1241-1248, 2003.
114. YUNKERL MRA, AND MAUREENW MC: Low-Voltage-Activated ("T-Type") Calcium Channels in Review. *Journal of Bioenergetics and Biomembranes* **35**: 6, 2003.
115. DAHAI WANG, TETSUAKI HIRASE, TERUO INOUE, KOICHI NODE: Atorvastatin inhibits angiotensin II-induced T-type Ca^{2+} channel expression in endothelial cells. *Biochemical and Biophysical Research Communications* **347**, 394–400, 2006.
116. POWIS DA, ZERBES M, HERD LM, DUNKLEY PR: Angiotensin II Causes Calcium Entry into Bovine Adrenal Chromaffin Cells via Pathway(s) Activated by Depletion of Intracellular Calcium Stores. *Neurochemical Research* **28**: 9, 2003.
117. WANG D, HIRASE T, INOUE T, NODE K: Atorvastatin inhibits angiotensin II-induced T-type Ca^{2+}channel expression in endothelial cells. *Biochemical and Biophysical Research Communications* **347**: 394–400, 2006.
118. BALL C, SAINT D, BELTRAME J: The effect of efonidipine hydrochloride on human subcutaneous microvascular constrictor responses. *Journal of Molecular and Cellular Cardiology* **41**:732– 751, 2006.