# ROLE OF OXIDATIVE STRESS AND VASCULAR RENIN ANGIOTENSIN SYSTEM IN THE DYSREGULATION OF ARTERIOLAR TONE BY ASYMMETRIC DIMETHYLARGININE. ASSOCIATION WITH THE IMPAIRMENT OF VENULAR FUNCTION IN HYPERHOMOCYSTEINAEMIA

**Ph.D. Dissertation**


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## ABBREVIATIONS

| | |
|---|---|
| ACE | angiotensin converting enzyme |
| ACEI | angiotensin converting enzyme inhibitor |
| ACh | acetylcholine |
| ADMA | asymmetric dimethylarginine |
| Ang II | angiotensin II |
| $AT_1R$ | angiotensin 1 receptor |
| cGMP | cyclic guanosine monophosphate |
| CAT | catalase |
| COX | cyclooxygenase enzyme |
| DHE | dihydroethydine |
| DDAH | dimethylarginin dimethylaminohydrolase |
| DPI | diphenyl-iodonium |
| EDHF | endothelium-derived hyperpolarizing factor |
| EDRF | endothelium-derived relaxing factor |
| FMD | flow mediated dilatation |
| GTP | guanosine triphosphate |
| GTN | glyceryl trinitrate |
| GSH | glutathione |
| $H_2O_2$ | hydrogen peroxide |
| Hcy | homocysteine |
| HHcy | hyperhomocysteinemia |
| INDO | indomethacin |
| L-NAME | $N^{\omega}$-nitro-L-arginine methyl ester |
| L-NMMA | N(G)-monomethyl-L-arginine |
| MS | methionine synthase |
| NAD(P)H | nicotinamide adenine dinucleotide phosphate |
| NO | nitric oxide |
| NOS | nitric oxide synthase |
| $PGE_2$ | prostaglandin $E_2$ |
| $PGH_2/TXA_2$ | prostaglandin $H_2$/tromboxane $A_2$ |

| | |
|---|---|
| $PGI_2$ | prostaglandin $I_2$ |
| PRMT | protein arginine *N*-methyltransferase |
| PSS | physiological salt solution |
| PON | peroxynitrite |
| ROS | reactive oxygen species |
| RAS | renin-angiotensin system |
| SAH | S-adenosylhomocystein |
| SAM | S-adenosylmethionine |
| SDMA | symmetric dimethylarginine |
| SNP | sodium nitroprussid |
| SOD | superoxide dismutase |
| TP | thromboxane $A_2$ ($TxA_2$) receptor |
| WSS | wall shear stress |
| XO | xanthine oxidase |

# 1. INTRODUCTION

## 1. 1. GENERAL OVERVIEW

### 1.1.1. ROLE OF ARTERIOLES IN THE BLOOD CIRCULATION

One of the most important homeostatic functions of the cardiovascular system is to provide sufficient blood flow to tissues so they can regulate their blood flow in proportion to their metabolic needs. Due to the limitation of blood volume and cardiac output, the most important role of cardiovascular system is the distribution of blood flow in accordance with metabolic states and functional priorities of the organs and tissues. The so called "resistance" vessels (small distributing arteries and arterioles) are primarily responsible for the regulation of blood flow in various tissues and organs. These vessels provide the greatest resistance to the flow of blood and thus have a crucial role of maintenance of systemic blood pressure, as well. The wall of these vessels consists of vascular smooth muscle cells allowing the vessel to change their diameter and the endothelium, which were shown to regulate the contractile function of smooth muscle.

The tone of arteriolar smooth muscle is under control of several mechanisms: it is controled by the autonomic nervous system, hormones and by local factors released from the parenchyma and that of endothelium. The factors of the extrinsic control serve general circulatory homeostasis primarily by adjusting cardiovascular functions, for instance to maintain a normal arterial blood pressure and a normal blood volume. These factors are also involved in some other homeostatic functions, such as thermoregulation[1] and responses to exercise.[2] Local controls mean mechanisms independent of nerves or hormones by which organs and tissues alter their own arteriolar resistance, thereby autoregulating their blood flow.

In vivo, many of these mechanisms are acting in concert and at the same time thus it is difficult to elucidate the role of individual mechanisms in regulation of changes in diameter. Thus during the present studies we used an in vitro technique, allowing the investigation of endothelial and smooth muscle mechanisms responsible for vasoconstrictions and vasodilations.

### 1.1.2. REGULATION OF VASCULAR TONE BY THE ENDOTHELIUM

Endothelium plays a crucial role in the regulation of vascular resistance through the release of vasoactive factors. Its ability to communicate with smooth muscle is especially important in understanding how circulatory substances, which have specific receptors or those released from the vessel wall, regulate smooth muscle tone. The fact that it has a role in mediating smooth muscle relaxation was recognized when the usual vasodilator response to ACh observed in larger arteries was abolished by denuding the endothelium.[3]

*a.) EDRF - NO*

The search for an endothelium-derived relaxing factor (EDRF) that diffused into smooth muscle resulted in the identification of nitric oxide (NO), generated from L-arginine by nitric oxide-synthase (NOS).[4] The role of NO will be discussed in more detail later.

*b.) Arachidonic acid metabolites*

Among other prostanoids, prostacyclin ($PGI_2$) and thromboxane ($TxA_2$) are importantly involved in the regulation of vascular function.[5] Their production is catalysed by cyclooxygenase (COX) enzymes, of which there are two isoforms COX-1 and COX-2.[6] It seems that both COX-1 and COX-2 are expressed in physiological and pathological conditions, but their roles, levels of activation and affinity to arachidonic acid could be different.[7] In most tissues, COX-1 is constitutively expressed and produces dilator prostaglandins. In contrast, COX-2 is believed to be primarily an inducible enzyme, activated by proinflammatory conditions (e.g. in inflammation, during hyperalgesia, cell proliferation),[7] which produces several prostaglandins, leading to inflammatory processes, thrombogenesis or angiogenesis, although recent studies indicate its expression in normal conditions as well.[8] COXs converts arachidonic acid to prostaglandin H2 ($PGH_2$), which is then synthesised into $PGI_2$ by prostacyclin synthase[9] or $TxA_2$ by thromboxane synthase.[5] $PGI_2$ binds to the prostacyclin receptors (IP),[10] which are located on both platelets and vascular smooth muscle cells.[11] Activation of platelet IP receptors leads to inhibition of platelet aggregation.[12] $PGI_2$ binding to the smooth muscle cell IP receptor induces the

synthesis of cyclic adenosine monophosphate (cAMP), which leads to relaxation of the smooth muscle.[13]

In contrast to $PGI_2$, $TxA_2$ causes platelet aggregation and vasoconstriction.[14] $TxA_2$ mediates its effects by its actions on thromboxane-prostanoid (TP) receptors which are located on platelets and their activation causes platelet aggregation.[8] TP receptors are also found on smooth muscle cells and is involved in increasing intracellular $Ca^{2+}$ levels in the smooth muscle, leading to vasoconstriction.[15]

*c.) EDHF*

Experimental data suggested that beside the AA and the NO pathways, additional endothelial mediator(s) is involved in endothelium-dependent regulation of smooth muscle tone.[16] The specific characteristics of this substance gave the origin of the name, endothelium-derived hyperpolarizing factor (EDHF).[16] However, EDHF is a yet an unidentified vasodilator substance, which hyperpolarises the underlying smooth muscle by making the membrane potential of the cell more negative.[17] A number of pathways have been implicated in causing the hyperpolarisation. Although the exact pathway is still unknown, attention so far has been paid to three factors in particular.[18] Activation of endothelial receptors and the subsequent increase in the intracellular calcium concentration cause opening of calcium-activated potassium channels of small and intermediate conductance and the hyperpolarization of the endothelial cells. [19] The smooth muscle cell responds to changes in the extracellular potassium ion levels and also releases potassium out of the smooth muscle cell causing hyperpolarization.[20] The change in the membrane potential of the smooth muscle cell reduces intracellular $Ca^{2+}$ levels, resulting in relaxation.[19] In some blood vessels, the endothelium releases AA metabolites, such as epoxyeicosatrienoic acids (EET), derived from cytochrome P450 monooxygenases.[21] Although synthesised in the endothelial cell, they act by increasing potassium ion efflux from the smooth muscle cells resulting in hyperpolarisation and relaxation.[22] However, in those vessels where EET activity is inhibited, hyperpolarisation still occurs,[23] suggesting that other mechanisms can be involved in hyperpolarising the smooth muscle cells.

Gap junctions are clusters of transmembrane channels that cross the intercellular gap and allow the transfer of potassium ions and second messengers

between from the endothelial cells to the smooth muscle cells. However, it is difficult to establish exactly what is transferred under normal conditions.[24]

Additionally, the endothelium can produce other factors, such as lipoxygenases derivatives, hydrogen peroxide ($H_2O_2$), and endothelium-derived C-type natriuretic peptide (CNP). These substances have been shown to exert a variety of cardiovascular effects including vasodilatation and hyperpolarization of arteries. These different mechanisms are not necessarily exclusive and can occur simultaneously.[25]

*d.) Endothelin*

After the discovery of EDRF, the vascular activity of a peptide secreted from endothelial cells was described in the mid-1980s and was named endothelin, based on its cellular origin, and it soon turned out that three functionally different isoforms exist.[26, 27] Endothelins are formed by enzymatic cleavage from a larger, inactive precursor and constrict both arterial and venous smooth muscle in all vascular beds, acting through a unique receptor in the media.[18]

*e.) Reactive oxygen species (ROS)*

Endothelial cells are able to generate reactive oxygen species (ROS) including superoxide ($O_2^{\cdot -}$), hydrogen peroxide ($H_2O_2$), NO, peroxynitrite ($ONOO^-$), hydroxyl radicals ($HO^{\cdot}$), and other radicals. More recently, it has become clear that ROS, such as $O_2^{\cdot -}$ and $H_2O_2$ also have several potentially important effects on endothelial function and phenotype and are implicated both in physiological regulation and disease pathophysiology.[28] Potential sources of endothelial ROS generation that are implicated in disease processes include mitochondria, xanthine oxidase (XO), uncoupled NO synthases, cytochrome *P*-450 enzymes, and nicotinamide adenine dinucleotide phosphate (NADPH) oxidases. In addition, enzymes such as lipoxygenases may also generate $O_2^{\cdot -}$.[28]

*f.) Local renin-angiotensin systems*

Endothelial cells, as well as cardiac and renal cells, contain intrinsic renin-angiotensin systems (RAS).[29] Angiotensin II (Ang II) is a powerful vasoconstrictor peptide. It is

formed by the action of the enzyme renin on its precursor, angiotensinogen. The product, angiotensin I, is not vasoactive, but is cleaved to Ang II by a converting enzyme (ACE), for which potent pharmacologic inhibitors have been synthesized.[29]

*g.) Functional importance of endothelium derived vascoactive factors. Regulation of wall shear stress*

In the presence of constants diameter of a vessel increases in flow result in increases in wall shear stress (WSS).[30] It has been found in several experiments that increases in blood flow or perfusate flow elicited, with a delay of 5-15 seconds, increases in diameter (flow-induced dilation), a response that occurs only when the endothelium of arterioles is intact.[31-33] Thus, when flow increases, during delay time when diameter does not change, WSS increases resulting in dilation of arterioles and consequent decreases of WSS.[34]

Thus, endothelium of arterioles regulates WSS in a negative feedback manner: increasing in flow leads to the release of dilator substances, such as NO and dilator prostaglandins ($PGE_2$ and $PGI_2$). Regulation of WSS is an important mechanism for regulating peripheral vascular tone, hence blood flow.[35, 36]

## 1.2. NITRIC OXIDE

Since three scientists won the Nobel Prize in Physiology in 1998 for discovering NO and its role in cell signaling, NO has become one of the most researched molecules and medical topics in recent history. However, our understanding of this molecule has grown from humble beginnings. Nitric oxide was first discovered as a colorless, toxic gas in 1772 by Joseph Priestly. It has been only recently discovered that there is a link between nitric oxide and the noni plant (Morinda citrifolia). Morinda citrifolia has a long tradition as a cure-all plant in India and the Pacific Islands. It has been discovered that there is a correlation between the patients using the noni plant and having NO in the body.[37]

It has been well established that L-arginine is the physiological substrate of a family of enzymes named nitric oxide synthases (NO synthases, NOS).[38] Three different isoforms of NOS have been characterized that are named according to the

cell type from which they were first isolated: neuronal NOS (nNOS, NOS I), inducible NOS (iNOS, NOS II), and endothelial NOS (eNOS, NOS III).[39] nNOS and eNOS are expressed constitutively, their activity is regulated by calcium/calmodulin, and they produce NO at low rates. In contrast, iNOS is induced in inflammatory cell types on cytokine stimulation; its activity is independent of calcium because of tight binding of calmodulin to the enzyme, and it produces NO at high rates. Inactive eNOS is bound to the protein caveolin and is located in small invaginations in the cell membrane called caveolae.[40] When in the endothelium the intracellular levels of $Ca^{2+}$ increase, eNOS detaches from caveolin and is activated. [40] Recently, expressional regulation of eNOS has been observed,[41] so that the simple discrimination between constitutively and inducibly expressed enzymes is no longer correct; however, this nomenclature is still broadly used.

The production of the important signaling molecule NO is regulated and modulated by several physiological and pathological mechanisms for example wall shear stress.[42] Shear stress results from increased blood flow in the vessel and can increase NO production by eNOS phosphorylation and also through stimulating endothelial cell receptors.[43] In particular, WSS activates special $Ca^{2+}$-activated potassium ion channels on the endothelial cell surface, causing potassium ion efflux and $Ca^{2+}$ influx into the cell.[43] The contribution of $Ca^{2+}$ and eNOS phosphorylation to NO production is dependent on the duration of the shear stress. For example, short duration of shear stress results in intracellular $Ca^{2+}$ release,[44] whereas shear stress of longer durations (more than 30 minutes) can deplete intracellular $Ca^{2+}$ stores, and so NO production is dependent on eNOS phosphorylation.[45]

Once synthesized, NO diffuses through the endothelial cell into the adjacent smooth muscle, where it binds to the enzyme soluble guanylyl cyclase (sGC). [46] The activated enzyme increases the conversion rate of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP), which reduce the contraction of smooth muscle.[47] Further, cGMP reduces $Ca^{2+}$ release from the sarcoplasmic reticulum in the smooth muscle cell, and also helps to restore $Ca^{2+}$ to the sarcoplasmatic reticulum.[47] Both actions decrease the tone of smooth muscle.

The mechanisms described above are continuously active and produce NO to maintain basal vasodilator tone. By inhibiting NO activity a dose dependent increase

in blood pressure was found due to the constriction of vessels, which was reversed when NO was administered.[48] These findings highlight the importance of NO in maintaining resting vasodilator tone. However, the vessel is also capable of dilating in the absence of NO. After damage to the endothelium, administration of NO donors, such as glyceryl trinitrate (GTN) or sodium nitroprussid (SNP) can still result in vasodilatation.[49, 50] The mechanism by which GTN or SNP causes vasodilatation is not clear. Several researchers have suggested that GTN undergoes bioconversion to NO.[51, 52] SNP releases NO (and $NO^+$) upon dissolution in aqueous solvents and, in contrast to nitric acid esters, does not require enzymatic reduction or hydrolysis for this process.[51, 53]

Except vasodilatation, NO is an endogeonous modulator of leukocyte adherence and prevents platelet and leukocyte activation and adhesion to the vessel wall.[54, 55] When the endothelium is damaged, the subsequent inflammation causes an increase in leucocytes at the damaged site.[56]

### 1.3. L-ARGININE ANALOGUES AND FUNCTION OF NITRIC OXIDE SYNTHASE

Evidence has been provided that extracellular L-arginine can be rapidly taken up by endothelial cells and facilitate NO production.[57] The role of L-arginine has been studied extensively as a precursor for NO synthesis in humans, as well. A peculiar aspect in these studies was that the early studies were performed with high intravenous doses, and low doses have only recently been adopted in oral supplementation studies. A single dose as high as 30 g of L-arginine administered intravenously during a 30-min period was shown to induce vasodilation in human subjects.[58-60] This vasodilation appeared rapidly after the initiation of the infusion in healthy human subjects, and it was reproducible in patients with arterial disease and in patients with coronary artery disease, but not in patients with primary pulmonary hypertension.[61] L-arginine-induced vasodilation was associated with increased release of NO metabolites, nitrite and nitrate, into urine. L-arginine was shown to increase the synthesis of NO and augments NO mediated arteriolar vasodilation.[62, 63] These data suggested that the reaction was NO-dependent; however, subsequent studies demonstrated that intravenous high doses of L-arginine resulted in a significant

increase in the plasma concentration of growth hormone and insulin, and this endocrine effect of L-arginine was blocked by somatostatin co-infusion, which also partly abolished the vasodilator effect.[64, 65]

Although it is beyond the scope of this dissertation to give a complete overview of all published experimental and clinical studies with L-arginine, it becomes clear even from studying recent publications that L-arginine administration has led to discrepant findings. There are some clinical studies with experimental endpoints that failed to show beneficial effects of L-arginine on vascular function. As an example, Blum and associates[66] found no significant improvement of endothelium-dependent vasodilation, blood flow, or inflammatory marker serum levels by dietary L-arginine at a dose of 9 g/day as compared with placebo, given for a period of 1 month. In another study, 40 patients with coronary heart disease and angiographically proven stenosis of >50% received L-arginine 15 g/day or placebo for 2 week[67] L-arginine supplementation had no significant effect on endothelial function, blood flow, markers of oxidative stress, or exercise performance.

Interestingly, L-arginine can be methylated (for example during homocystein metabolism) which changes their role. There are several methylated forms of L-arginine that occur in animals and humans in vivo. Methylated arginine derivatives were first isolated from human urine in 1970.[68] Methylated L-arginine, $N^{\omega}$-nitro- L-arginine, $N^{\omega}$-monomethyl- L-arginine, and $N^{\omega}$-nitro- L-argininemethyl-ester have been shown to inhibit NOS with the consequent elimination of NO-mediated dilations of vessels. These forms of methylated L-arginine, however, are not readily available in vivo.[69]

Studies performed during the last decade have shown that accumulation of so called endogenous inhibitors of nitric oxide synthase, in particular asymmetric dimethylarginine (ADMA), impair nitric oxide formation in certain pathophysiological conditions.[70] ADMA, is an L-arginine analogue, which is though to compete with L-arginine for binding to NOS and thus antagonizes the enzyme's catalytic activity, giving rise to the hypothesis that L-arginine may be beneficial in patients with elevated ADMA, but have no effects on NO-dependent mechanisms in subjects with low or normal ADMA levels.[71]

However, the key study initiating research in this field was published in 1992 by Vallance et al.[72] The authors identified ADMA in human plasma and urine and demonstrated that ADMA inhibited the isolated NO synthase. In addition, ADMA contracted rat aortic rings in vitro, inhibited endothelium-dependent relaxation in response to acetylcholine, and increased blood pressure when infused into guinea pigs.[72] Local infusion of ADMA into the brachial artery of human volunteers caused a dose-dependent fall in forearm blood flow.[72] Finally, it was shown that ADMA concentration was markedly elevated in patients with chronic renal failure.[72]

Apart from ADMA, two other related compounds, symmetric dimethylarginine (SDMA) and N-monomethyl- L-arginine (L-NMMA) are synthesized endogenously. L-NMMA is as potent as ADMA in decreasing NOS activity but its concentration in plasma is about tenfold lower, however, intracellular concentration of L-NMMA and ADMA may be comparable at least in some tissue,[73] indicating that both are important NOS regulators. SDMA at concentrations in the circulation are comparable to ADMA.[72] Interestingly, recent studies showed that SDMA could also be of clinical significance as an independent cardiovascular risk factor for many reasons.[74-77] For example, Meinitzer and associates showed that serum concentrations of SDMA are independently associated with increased cardiovascular and mortality (from all causes) in patients undergoing coronary angiography. However, the pattern of risk linked to SDMA is different from that linked to ADMA, suggesting different pathophysiological roles of these two methylarginine metabolites.[78] It seems that SDMA indirectly interfere with NO synthesis. SDMA inhibits the y+ transporter that mediates the intracellular uptake of L-arginine[79] and decreases renal tubular arginine absorption,[80] both of which could reduce L-arginine availability.

In contrast, in vitro studies using endothelial cells showed that SDMA dose-dependently inhibits NO synthesis. This effect was associated with an increase in reactive oxygen species, whereas SDMA had no effect on protein expression of NO synthase.[81] Whereas, recently it was found that SDMA stimulates production of reactive oxygen species in monocytes by acting on $Ca^{2+}$ entry via store-operated $Ca^{2+}$ channels. Thus future studies should elucidate the mechanisms - that are not associated directly with the production of NO by eNOS - by which various

methylated L-arginines affect cardiovascular function. Structure of ADMA and related compounds is presented in **Figure 1**.

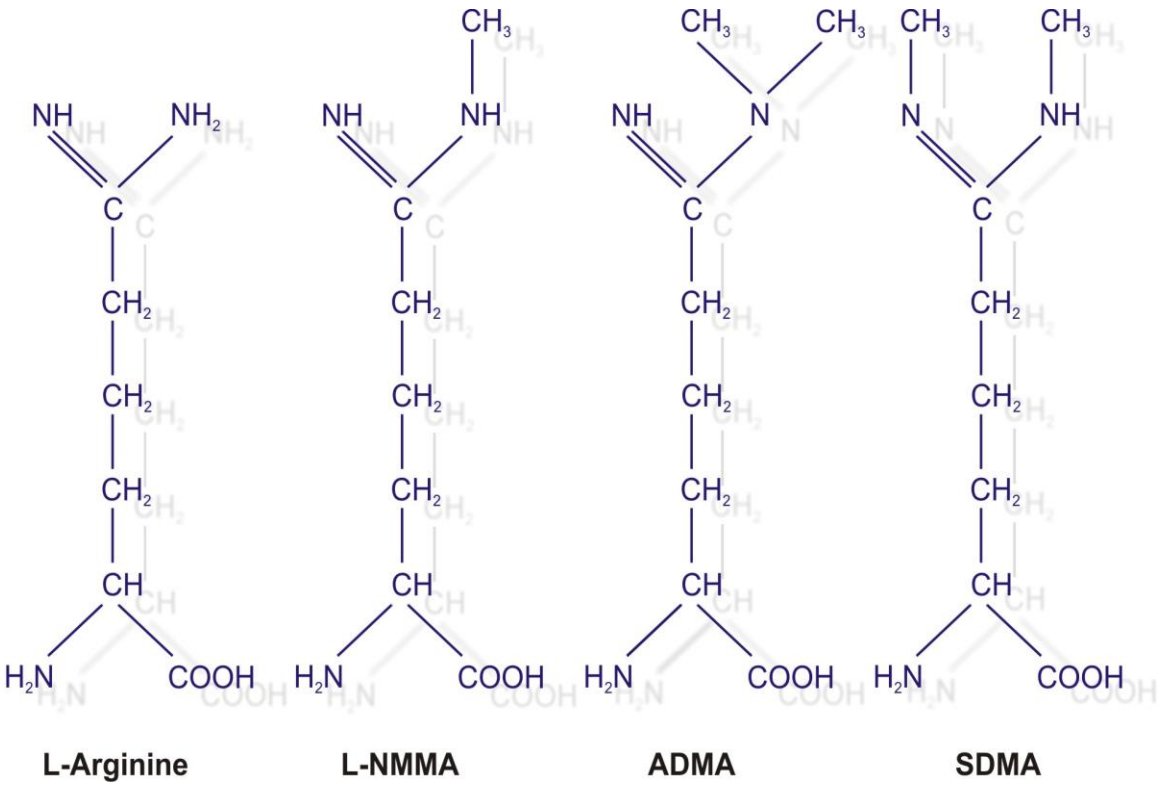


**Figure 1.** L-Arginine analogues: L-NMMA: N(G)-monomethyl-L-arginine, ADMA: asymmetric dimethylarginine, SDMA: symmetric dimethylarginine

## 1.4. ASYMMETRIC DIMETHYL ARGININE (ADMA)

### 1.4.1. METABOLISM OF ADMA

ADMA is synthesized during the methylation of protein arginine residues by S-adenosylmethionine: protein arginine methyltransferases (protein methylases, PRMT). These enzymes transfer the methyl group from S-adenosylmethionine (SAM) to arginine thus forming methylated arginine and S-adenosylhomocysteine (SAH); the latter is subsequently hydrolyzed to homocysteine (**Figure 2**). Two types of PRMT have been identified. PRMT1 methylates histones and nuclear RNA-binding proteins and yields L-NMMA and ADMA, whereas PRMT2 methylates exclusively myelin basic protein and generates L-NMMA and SDMA but not ADMA.[82] Recent studies suggest that multiple isoforms of PRMT1 and PRMT2 encoded by separate genes exist. It is estimated that about 1–4% of arginine residues in nuclear proteins are methylated and this is an irreversible reaction since protein-bound arginine residues cannot be demethylated. Free methylarginines are released during proteolysis and are not incorporated back into proteins. Humans generate approximately 300 μmol/day (approximately 60 mg) of ADMA.[83] Normal plasma level of ADMA is less than 1 μM; it increases up to 10-fold in patients with endstage renal disease and more moderately (2–3 fold) in many other pathologies (see below).[84] ADMA is eliminated by renal excretion, however, more than 90% of ADMA is metabolized by dimethylarginine dimethylaminohydrolase (DDAH), which degrades it to citruline and dimethylamine (**Figure 2**).[85] DDAH exists in 2 isoforms: DDAH1 is predominantly expressed in tissues containing nNOS and DDAH2 mainly in tissues containing eNOS or iNOS.[86] Pharmacological inhibition of DDAH increases ADMA concentration and reduces NO production,[87] whereas transgenic DDAH overexpression has the opposite effect both in vitro[88] and in vivo.[89] DDAH is expressed in many tissues including endothelial cells, brain, pancreas, etc., however, the liver and kidney may be the principal organs responsible for ADMA metabolism. Indeed, only a small portion of ADMA extracted from the blood by the kidney is recovered in urine whereas the rest is metabolized by renal DDAH.[90] The elevation in plasma ADMA that occurs with vascular disease and risk factors is largely due to impaired activity of DDAH.[91]

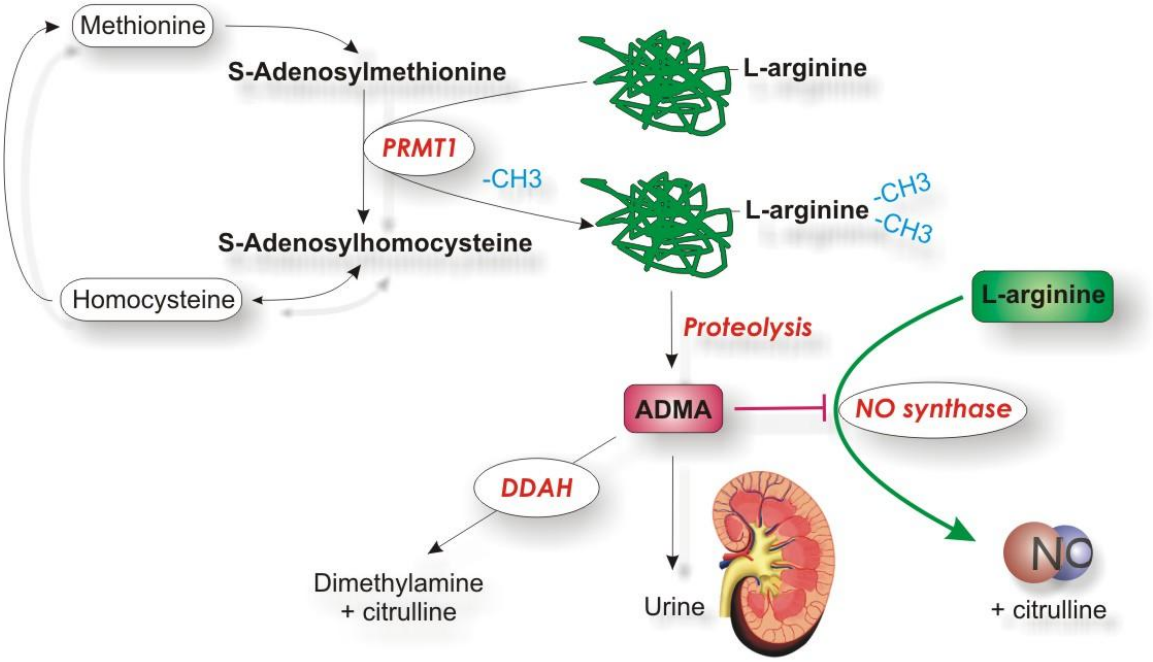


**Figure 2.** Metabolism of ADMA. PRMT1: I type of protein arginine methyltransferase, NO: nitric oxide, ADMA: asymmetrical dimethylarginine, DDAH: dimethylarginine dimethylaminohydrolase. Modified from Böger RH. Asymmetrical dimethylarginine (ADMA): a novel risk marker in cardiovascular medicine and beyond. Annals of Medicine. 2006;38:126-36.

### 1.4.2. DISEASES ASSOCIATED WITH ELEVATED LEVELS OF ADMA

The pathophysiological relevance of ADMA in humans has been demonstrated by administration of ADMA to healthy volunteers.[83, 92, 93] ADMA increases the systemic vascular resistance and arterial blood pressure and decreases cardiac output.[83] It causes endothelial dysfunction in forearm resistance arteries.[92] Due to the considerably high concentrations in patients with renal insufficiency the relationship between ADMA plasma levels and cardiovascular complications were first studied in these high risk patients. Zoccali and colleagues conducted a prospective study and indeed found a significant association between circulating ADMA and future cardiovascular events and mortality.[94] Other studies found elevated ADMA in patients with normal or slightly impaired renal function and an adverse cardiovascular risk profile including patients with peripheral arterial occlusive disease, [95] hypertension,[96] hyperlipidemia,[97] insulin resistance,[98] type 2 diabetes mellitus,[99, 100] type 1 diabetes

mellitus,[101] diabetic nephropathy,[102] hypopituitarism,[103] individuals with metabolic syndrome,[104] or women with previous gestational diabetes.[105] In several other human diseases, such as hyperhomocysteinemia,[106] in coronary artery disease,[107] pulmonary hypertension,[108] as result of smoking[109], migraine[110] and preeclampsia[111] there is also an increase in the serum level of ADMA. It seems that ADMA is an active agent not only in preeclamptic patients, but also in normotensive pregnant women with isolated fetal IUGR and could be a marker of severity of preeclampsia.[112] However, it has to be mentioned that determination of ADMA is very sophisticated and the values obtained by different laboratories diverge considerably. The correlations between available immunoassays and other more complex methods for the determination of ADMA that have been observed are not very encouraging.[113]

Recently, several studies have reported a predictive value of ADMA for cardiovascular events. The occurrence of cardiovascular endpoints in high risk patients has been found to be directly and independently associated with elevated ADMA concentrations in patients with coronary artery disease,[114] peripheral arterial occlusive disease,[115] type 2 diabetes mellitus,[116] type 1 diabetes,[117] chronic heart failure.[118] A particular strong relationship between ADMA and haemodynamic parameters as well as clinical outcome has been observed in patients with pulmonary arterial hypertension[119] and progression of nephropathy in type 2 diabetes.[120]

### 1.4.3. PATHOPHYSIOLOGICAL MECHANISM RELATED TO ADMA

These prospective data from observational studies only describe statistical relationships and do not allow drawing the conclusion that ADMA is causal for future cardiovascular events.[70] It appears possible that elevated ADMA concentrations are only an epiphenomenon in parallel with other alterations. However, results from animal experiments suggest that ADMA represents not only a risk marker but also a risk factor for cardiovascular events.[70] It was shown that continuous infusion of ADMA for 4 weeks led to the development of microvascular lesions in coronary vessels of mice.[121] Overexpression of the ADMA degrading enzyme DDAH reduced ADMA in mice and reduced graft coronary artery disease.[122] Further, ADMA may be involved in glomerular capillary loss and sclerosis, thus contributing to the

progression of chronic kidney disease.[123] Konishi et al showed that transgenic mice with DDAH overexpression exhibited enhanced endothelial cell regeneration and neointima formation after vascular injury.[124] These findings imply that ADMA may directly contribute to vascular organ damage.

These studies suggest a strong correlation between elevated levels of ADMA and vascular diseases associated with reduced release of NO. In vitro experimental studies with isolated arterial segments, cultured murine macrophages and rat cerebellar homogenate, ADMA inhibits vascular NO production at concentrations of 3 to 15 μmol/L.[72, 125-128] Thus, there is indirect evidence that ADMA has a role as an endogenous modulator of NOS activity and could be viewed as an endogenous inhibitor of NOS.[97, 129] Interestingly, there is a significant positive correlation between age and ADMA levels in a random population sample,[130] which was shown to be associated with impaired dilation of the brachial artery after release of occlusions.[131] However, Toth and colleagues have found that elevated levels of exogenous ADMA impair the regulation of arteriolar resistance by interfering with the NO mediation of flow/shear stress-induced dilation.[132] In addition, ADMA elicits the release of reactive oxygen species, primarily superoxide, because superoxide dismutase reversed the ADMA-elicited reduction in basal diameter and ethidium bromide (EB) fluorescence used to detect oxidative stress.[132] Furthermore, Suda et al provide the first direct evidence that ADMA treatment caused superoxide production in both wild-type (WT) and eNOS-deficient mice, suggesting that the primary mechanism of action of ADMA is not related to the inhibition of eNOS, but increased ROS production.[121]

Thus, the exact mechanism(s) by which ADMA interferes with NO production, elicits increased superoxide production and regulates vasomotor function in arterioles remains obscure.

## 1.5. INACTIVATION OF NO BY REACTIVE OXYGEN SPECIES (ROS)

### 1.5.1. REACTIVE OXYGENE SPECIES (ROS)

ROS are metabolites of oxygen that can either strip electrons away from other molecules (oxidize), donate electrons to molecules (reduce), or react with and become part of molecules (ie. oxidative modification).[133] Many ROS possess an unpaired electron in their outer orbital and are, therefore, radicals. A particularly important radical for cardiovascular biology is superoxide ($O_2^{\cdot -}$), which is formed by the one-electron reduction of oxygen. Superoxide is important because it can serve as both an oxidant and as a reductant in biologic systems and is a progenitor for other ROS. Other radicals include the hydroxyl radical ($HO^{\cdot}$), lipid peroxy-($LOO^{-}$) radical, and alkoxy-radicals ($LO^{\cdot}$). Other molecules, including peroxynitrite ($ONOO^{-}$), hypochlorous acid ($HOCl^{-}$), and hydrogen peroxide ($H_2O_2$) are not radicals, but have strong oxidant properties and are, therefore, included as ROS.[133] Another relevant group of molecules are the reactive nitrogen species (RNS) including nitric oxide (NO), the nitrogen dioxide radical ($NO_2$), and the nitrosonium cation ($NO^{+}$). Peroxynitrite is considered both an ROS and RNS and is formed by the near diffusion-limited reaction between $O_2^{\cdot -}$ and NO.[133] RNS are important, because they often react with and modify proteins and other cellular structures and alter function of these targets. Reactive oxygen species or ROS are molecules such as hydrogen peroxide, ions like the hypochlorite ion, radicals like the hydroxyl radical and the superoxide anion which is both ion and radical.[133]

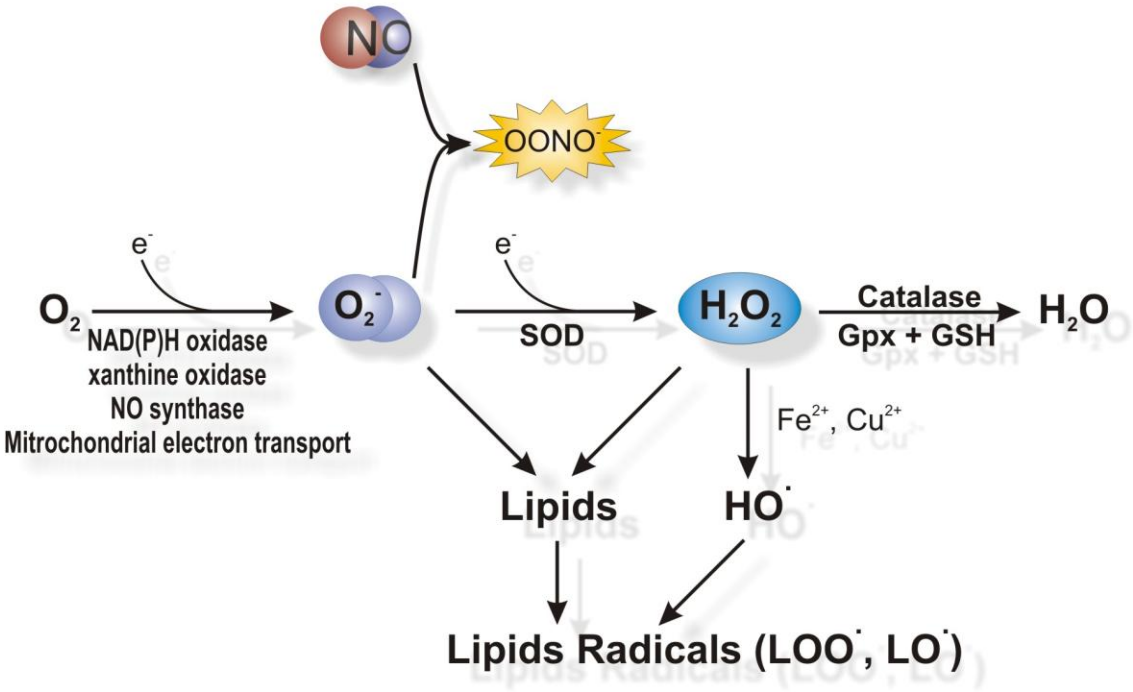


**Figure 3.** Pathways for production of ROS in mammalian cells. Shown are enzymes, which can donate electrons to oxygen to form superoxide ($O_2^{\cdot -}$). A 2-electron of oxygen can form $H_2O_2$. $H_2O_2$ can also be formed by the action of (SOD) on superoxide and is further reduced to water by both catalase or glutathione peroxidases (Gpx) and glutathione (GSH). $O_2^{\cdot -}$ and $H_2O_2$ can undergo reactions with transition metals to form OH. ROS can react with lipids to form biologically active lipid radicals. $O_2^{\cdot -}$:superoxide, Gpx: glutathione peroxidases, GSH: glutathione, $H_2O_2$: hydrogen peroxide, OH: hydroxyl radical, SOD: superoxide dismutase.

In the cardiovascular organs, the most relevant enzyme systems that produce ROS are the NAD(P)H oxidases, the mitochondria, xanthine oxidase (XO), and, under certain conditions, the nitric oxide synthases (**Figure 3**).[134] There are numerous examples of these enzymes being activated in a variety of disease states, including atherosclerosis, hypertension, diabetes, and renal disease. Ang II is well known to activate the NAD(P)H oxidase via its action on the $AT_1$ receptor ($AT_1R$), and many of the pathophysiological effects of angiotensin II have at least in part been attributed to promotion of oxidative stress via this mechanism.[135-137]

Strong oxidants like the various ROS can damage other molecules and the cell structures of which they are a part. Cells have a variety of mechanisms to control the level of ROS. These include two enzymes: superoxide dismutase which converts two superoxide anions into a molecule of hydrogen peroxide and one of oxygen ($2O_2^{\cdot -}$ +

$2H^+ \rightarrow H_2O_2+O_2$) and catalase, which catalyzes the decomposition of hydrogen peroxide into water and oxygen ($2H_2O_2 \dashrightarrow 2H_2O + O_2$), as well as several small molecules that are antioxidants, such as the thiol-containing tripeptide glutathione (GSH) or alpha-tocopherol (vitamin E), uric acid, vitamin C.[138]

### 1.5.2. ROLE OF RENIN-ANGIOTENSIN SYSTEM IN ROS PRODUCTION

Studies within the past several years have revealed that NAD(P)H oxidase family members are major sources of reactive oxygen species that appear to play a pivotal role in the progression of vascular disease. The leukocyte-derived NAD(P)H oxidase (gp91*phox*; now referred to as Nox2) was presumed to be the major source of reactive oxygen species production during inflammatory response.[139] However, Nox2 is now known to be expressed in non-phagocytic cells, such as adventitial fibroblasts, smooth muscle cells from resistance arteries, and endothelial cells.[140, 141] Novel gp91*phox* homologues, termed Nox1, Nox3, Nox4, and Nox5, have been identified in non-phagocytic cells in the vasculature and in the kidney (mesangial cells).[142]

While it is commonly viewed that reactive oxygen species, such as superoxide and $H_2O_2$ elicit their pathologic effects in the vasculature by oxidatively modifying critical biomolecules (*i.e.*, lipids and proteins), it is now clear that these oxygen-derived metabolites play more direct roles as signaling molecules regulating cellular functions as diverse as hypertrophy, proliferation, and cell migration.

Ang II clearly plays a role in altering endothelial function and promotes oxidative injury both in animal models of renal failure[143] as well as in humans with renal vascular disease[144] and is a likely candidate to play a key role in early stages of renal disease. It is now relatively well-established that the hypertrophic and proliferative effects of Ang II on vascular smooth muscle cells and mesangial cells are mediated by oxidants generated from Nox enzymes.[145]

Upregulation of the renin-angiotensin system (RAS) may result in the induction of vascular oxidative stress,[146-149] leading to reduction in the bioavailability of NO. Ang II through activation of $AT_1R$ stimulates the Gq protein causing $Ca^{2+}$ influx into the vascular smooth muscle cells and increases generation of ROS - including superoxide and $H_2O_2$ - in the vessel wall, mainly through activation of

membrane-bound NADH/NAD(P)H oxidase in vascular cells.[135-137] NO produced by vascular endothelial cells can be rapidly scavenged by $O_2^{\cdot -}$ to form the potent oxidant and nitrating species $ONOO^-$ (**Figure 4**). This reaction reduces the amount of bioavailable NO and results in compromised vasodilation.[150, 151] Recent in vivo and in vitro evidence suggested that Ang II could increase intracellular oxidative stress in vascular endothelial cells[152, 153] and ACE inhibitors could enhance tissue antioxidant defense mechanisms.[154]

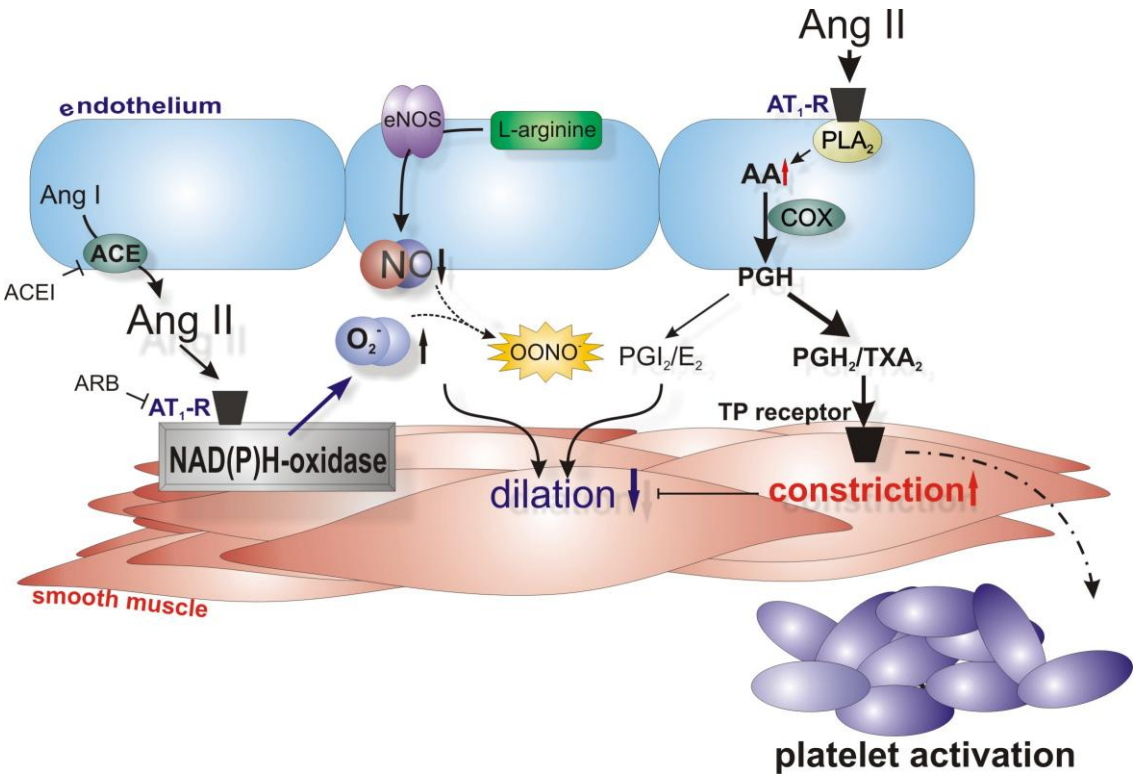


**Figure 4.** Effect of angiotensin II. AngII: angiotensin II, $AT_1$-R: angiotensin type 1 receptor, ARB: angiotensin receptor blocker, ACE: angiotensin converting enzyme, ACEI: angiotensin converting enzyme inhibitor, eNOS: endothelial nitric oxide synthase, NO: nitric oxide, $O_2^{\cdot -}$: superoxide, $ONOO^-$: peroxinitrite, PGI2/E2: prostaglandin $E_2/I_2$, $PGH_2/TxA_2$: prostaglandin $H_2$/thromboxane $A_2$, TP receptor: thromboxane-prostanoid (TP) receptor, $PLA_2$: phospholipase $A_2$ , AA: arachidonic acid; COX: cyclooxigenase

In addition, Ang II stimulates membrane-associated phospholipase $A_2$ ($PLA_2$)-induced releases of arachidonic acid (AA) from tissue phospholipids and generation of AA metabolites, especially $TXA_2$.[155-158] $AT_1R$ blocker significantly counteracts platelet activation, probably via the blockade of $TxA_2$ receptor-dependent signaling (e.g. implying activation of PLA2) rather than acting at the $AT_1R$ itself (**Figure 4**).[156] Furthermore, Ang II increases redox-sensitive and proinflammatory genes such as

vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule (ICAM), which plays a critical role in the initiation and progression of atherosclerosis.[152, 159, 160]

It seems that there is a potential interaction between ADMA and renin-angiotensin system. Several studies have shown that angiotensin-converting enzyme (ACE) inhibitors and angiotensin type I receptor blockers (ARBs) decrease plasma ADMA.[161, 162] It has been shown that the degradation of ADMA could be reduced by downregulating the activity of dimethylarginine dimethylaminohydrolase, the enzyme metabolizing ADMA, in diseased conditions with increased oxidative stress.[91] Furthermore, Suda and co-workers demonstrated that chronic treatment with ADMA caused vascular lesions and superoxide production in both wild-type (WT) and eNOS-deficient mice, and these changes were prevented by either ACE inhibitor or ARB treatment.[121] Recently, Hasegawa et al have also found that ADMA induced ACE protein upregulation in mice cardiac tissues.[163]

### 1.5.3. General Considerations regarding reactive oxygen species in vascular diseases

Although there is an enormous amount of information supporting a role of ROS in various animal models of diseases, it has been difficult to prove a role of these molecules in human diseases. It has been observed that hypertension,[164-166] diabetes mellitus[167, 168] and hyperhomocysteinemia[169-172] are accompanied with the increased formation of ROS in vascular tissues, which can activate vascular signaling mechanisms, resulting in functional and morphological changes of vessels. Hypertension, disorders of carbohydrate metabolism, such as in type I and II diabetes mellitus (T1DM and T2DM) and disorders of certain amino acid metabolism, such as in hyperhomocysteinemia (HHcy) represent an increased risk for the development of cardiovascular diseases. The role of oxidative stress in several cardiovascular diseases is summarized below.

Interestingly, oxidative stress seems to be present in virtually all forms of hypertension,[136, 173] including low-renin hypertension,[174] despite the differences in plasma levels of circulating factors.

A large body of literature has shown that excessive production of ROS contributes to hypertension and that scavenging of ROS decreases blood pressure. In an initial study, Nakazono and colleagues[175] showed that bolus administration of a modified form of SOD acutely lowered blood pressure in hypertensive rats. Membrane-targeted forms of SOD and SOD mimetics, such as tempol lower blood pressure and decrease renovascular resistance in hypertensive animal models.[176-180] There is ample evidence suggesting that ROS not only contribute to hypertension, but that the NAD(P)H oxidase is their major source. Components of this enzyme system are up-regulated by hypertensive stimuli, and NAD(P)H oxidase enzyme activity is increased by these same stimuli. Moreover, both angiotensin II-induced hypertension and deoxycorticosterone acetate (DOCA)-salt hypertension are blunted in mice lacking this enzyme.[181, 182] Importantly, in Ang II-infused rats, reduction of blood pressure with hydralazine or spironolactone (which is unlikely to affect angiotensin levels) normalized aortic superoxide production.[176, 183]

Numerous studies in humans[184] and animals[31, 136, 185, 186] suggest that increased superoxide production contributes significantly to the functional alterations of arteries present in hypertension. In peripheral arteries and arterioles, increased levels of superoxide have been shown to decrease the bioavailability of the endothelium-derived vasodilator nitric oxide (NO) to flow (by forming peroxynitrite anion)[31, 187] thereby contributing to the maintenance of elevated peripheral resistance.

The increased vascular formation of ROS in T1DM,[167, 168, 188, 189] is likely responsible for activating vascular signaling mechanisms, which results in diabetic angiopathy. Oxidative stress could be due to an increased production of ROS (e.g., superoxide anion, hydrogen peroxide, hydroxyl radical) and/or decreased concentration of antioxidants and antioxidant enzymes (e.g., glutathione, vitamin E, ascorbate, glutathione peroxidase, superoxide dismutase, catalase), both of which could play a significant role in the microvascular dysfunction in T1DM. It is well documented that oxidative stress contributes importantly to the development of vascular dysfunction in diabetes.[190-192] In diabetic subjects, NO mediation of vascular responses is impaired primarily by the increased production of reactive oxygen species.[193]

Epidemiological and experimental studies suggest that increased plasma concentrations of homocysteine increases the risk of cardiovascular diseases, such as ischemic heart diseases, cerebrovascular, peripheral vascular diseases, hypertension.[194-198] The Hcy level > 16 μmol/L is defined as hyperhomocysteinemia (HHcy), when the risk for atherothrombotic diseases increases independently from other risk factors.[194, 199-201] Methionine, an essential amino-acid, is metabolized to Hcy by methionine-adenosyl-transferase via the transmethylation pathway. In the reaction S-adenosyl-methionine (SAM) and then S-adenosyl-homocysteine (SAH) - in a methyl-transferase reaction - is formed. The transmethylation pathway is present in most mammalian tissues. SAH is converted to homocysteine by SAH hydrolase. Homocysteine may be remethylated to methionine by either folate-dependent or folate-independent mechanisms. For folate-dependent remethylation, the B12-dependent enzyme methionine synthase (MS) utilizes a methyl group from 5-methyltetrahydrofolate (5-CH3-THF). Betaine-homocysteine S-methyl-transferase (BHMT) catalyzes the folate-independent remethylation of homocysteine using betaine. Alternatively, homocysteine can be catabolized through the transsulfuration pathway to cysteine, beginning with the irreversible conversion to cystathion by cystathion ß-synthase (CBS). HHcy can develop due to genetic (e.g., cystathione-synthase and methyltetra-hydrofolate reductase) and nutritional alterations (deficiency of vitamins, e.g., folic acid, vitamin B6, and B12), factors that participate in the metabolism of homocysteine and methionine.[202] Although the underlying mechanisms responsible for the elevated risks have not yet been fully elucidated, there is increasing evidence to suggest that endothelial dysfunction of vessels contributes to the development of vascular diseases observed in both humans and animals with HHcy.[203, 204] Moderate HHcy (15-30 μM) is associated with a significant impairment of endothelium-dependent relaxation of large vessels,[205, 206] dilation of arterioles,[207, 208] primarily due to the reduced mediation of responses by NO. In an isolated aortic ring preparation of HHcy rabbits impaired endothelium-dependent relaxations could be restored by acute administration of vitamin C to the vessel chamber.[209] Acute in vitro administration of superoxide dismutase (a scavenger of superoxide) or inhibition of ROS-producing enzymes restored flow-induced, NO mediated dilations of coronary or skeletal muscle arterioles of HHcy rats.[210, 211]

Biochemically, homocysteine and ADMA are linked via several ways. First, methylation of arginine to L-NMMA and from L-NMMA to ADMA yields two molecules of homocysteine. Second, homocysteine may enhance protein degradation by destabilizing protein structure or by increasing oxidative stress, resulting in ADMA release. Third, homocysteine inhibits DDAH, the enzyme responsible for the breakdown of ADMA.[212] Homocysteine and ADMA share many of the presumed pathophysiological mechanisms that link these compounds to vascular disease.[213, 214] Most of these mechanisms are related to impaired NO-dependent endothelial function, leading to vasoconstriction, hypertension, platelet activation, proliferation of smooth muscle cells and monocyte adhesion. Mechanisms that are more specific for homocysteine include increased oxidative stress, protein homocysteinylation/acylation, endoplasmic reticulum stress and hypomethylation, although the latter may theoretically also be caused by elevated ADMA levels. More ADMA-specific vascular effects comprise left ventricular hypertrophy, reduced sodium excretion and inhibition of angiogenesis. On the basis of the biochemical links, one would expect a firm relationship between plasma homocysteine and ADMA levels. Tyagi et al showed that Hcy increases oxidative stress by decreasing L-arginine concentration and increasing ADMA concentration in cardiac microvascular endothelial cells (MVEC).[215] Their findings that there was no change in basal levels of NO with different doses of Hcy is consistent with the report of Jin and colleagues, [216] who observed an increase in nitrotyrosine formation in response to Hcy without an alternation in basal NOS activity. It may also be argued that, in addition to inhibiting NO production, ADMA may also be involved in increased production of ROS, which further decreases NO bioavailability. Recently, Rodionov and associates have been found that overexpression of the ADMA-hydrolyzing enzyme DDAH-1 in transgenic mice protects from adverse structural and functional changes in cerebral arterioles in hyperhomocysteinemia. Both homocysteine and ADMA are predictors of cardiovascular events in end-stage renal disease patients.[94, 217-219] Interestingly, in the two studies in which both homocysteine and ADMA were analyzed, it was found that higher ADMA, but not homocysteine, levels were associated with cardiovascular disease.[94, 219] From these and other studies, it has been hypothesized that in HHcy, it is

the high level of ADMA and ROS, which plays an important role in the development of vascular dysfunction.

## 2. HYPOTHESES AND SPECIFIC AIMS

### 2.1. HYPOTHESES

In several diseases affecting the cardiovascular system there is an increased plasma level of ADMA,[84] which is likely indicate even higher levels of intracellular ADMA because it is produced there.[79] Because tissue blood flow is determined primarily by arterioles, contributing to large part of total peripheral resistance (TPR), elucidation of the role of ADMA in the local regulation of arteriolar tone can help us to better understand the role of ADMA in normal and pathophysiological conditions, such as atherosclerosis, diabetes mellitus, hypertension and hyperhomocysteinemia.

Previous studies propose a potential link between ADMA and the vascular RAS, yet its functional consequence on the regulation of arteriolar resistance is not known. Ang II produced locally in the vessel wall has important autocrine and paracrine effects, even in the presence of normal or low circulating renin/angiotensin II levels.[220] Also, it has been well established that Ang II plays an important role in the activation of the vascular NAD(P)H oxidases and, thus superoxide production,[136, 137] whereas recent studies have also shown that exogenous ADMA elicits superoxide generation.[121, 221-225]

Thus, one can suppose that ADMA, apart from the inhibitory effect of NO synthase, may activate other mechanisms contributing to the dysfunction of microvessels, known to be involved in the regulation of tissue blood flow and peripheral vascular resistance.

Thus, on the basis of the aforementioned and results of studies described in the previous sections, we hypothesized that

**In isolated arterioles**,

1. Extraluminal administration of ADMA, by activating the arteriolar RAS, upregulates the activity of NAD(P)H oxidase leading to oxidative stress

2. ADMA by eliciting oxidative stress, interferes with NO released to increases in flow/shear stress or NO donor resulting in vasomotor dysfunction of skeletal muscle arterioles

All of these studies were done in the presence of indomethacin an inhibitor of cyclooxygenases to exclude the contribution of prostaglandins in the vasomotor responses studied.

## 2.2. SPECIFIC AIMS

1. To investigate the effect of ADMA on the changes in diameter of arterioles in the presence of inhibitors of specific cellular mechanisms
2. To investigate the effect of ADMA on flow-induced dilation in arterioles in the presence of inhibitors of specific cellular mechanisms
3. To investigate the effect of ADMA on agonist-induced responses in arterioles in the presence of inhibitors of specific cellular mechanisms
4. To elucidate the mechanisms by which ADMA induces vasomotor dysfunction of arterioles.

## 3. MATERIALS AND METHODS

### 3.1. ANIMALS

Experiments were carried out in isolated arterioles of Male Wistar rats (n=80; weight: ≈ 350 g). Animals were housed separately in an animal care facility, were fed standard rat chow, and had free access to drinking water and treated according to Insitutional Guidelines. All of the protocols were approved by the Institutional Animal Care and Use Committees. Male Wistar rats purchased from Charles River Laboratories (Budapest, Hungary and Wilmington, MA).

Rats were anesthetized with an intraperitoneal (I.P.) injection of sodium pentobarbital (50 mg/kg), and segments of gracilis muscle were removed; animals were then euthanized by an additional injection of sodium pentobarbital (150 mg/kg), followed by performing a bilateral pneumothorax.

### 3.2. ISOLATION OF GRACILIS SKELETAL MUSCLE ARTERIOLES

With the use of microsurgery instruments and an operating microscope, gracilis arterioles (1.5 to 2.0 mm in length) were isolated[226] and transferred into an organ chamber containing two glass micropipettes filled with physiological salt solution (PSS) composed of (in mM) 110 NaCl, 5.0 KCl, 2.5 $CaCl_2$, 1.0 $MgSO_4$, 1.0 $KH_2PO_4$, 5.5 glucose and 24.0 $NaHCO_3$ equilibrated with a gas mixture of 10% $O_2$-5% $CO_2$ balanced with nitrogen, at pH 7.4. Both perfusate and bath solutions were continuously saturated with this gas mixture to mimic in vivo level of $pO_2$. Vessels were cannulated at both ends and micropipettes were connected with silicon tubing to adjustable PSS reservoirs. Inflow and outflow pressures were set to 80 mmHg and measured by pressure servo control system (Living System Instrumentation). Temperature was set at 37°C by a temperature controller *(YSI Tele Thermometer*). The internal diameters at the midpoint of the isolated arterioles were measured by videomicroscopy with a microangiometer (Texas A&M University System, College Station, TX 77843). Changes in arteriolar diameter and intraluminal pressure were continuously recorded with a chart recorder and in digital form with PowerLab system

(ADInstruments Ltd, Castle Hill, Australia) connected to a computer and analysed with PowerLab and Sigma Plot software. Perfusate flow was measured with a ball flow meter (Omega, Stamford, CT)[132] (**Figure 5**).

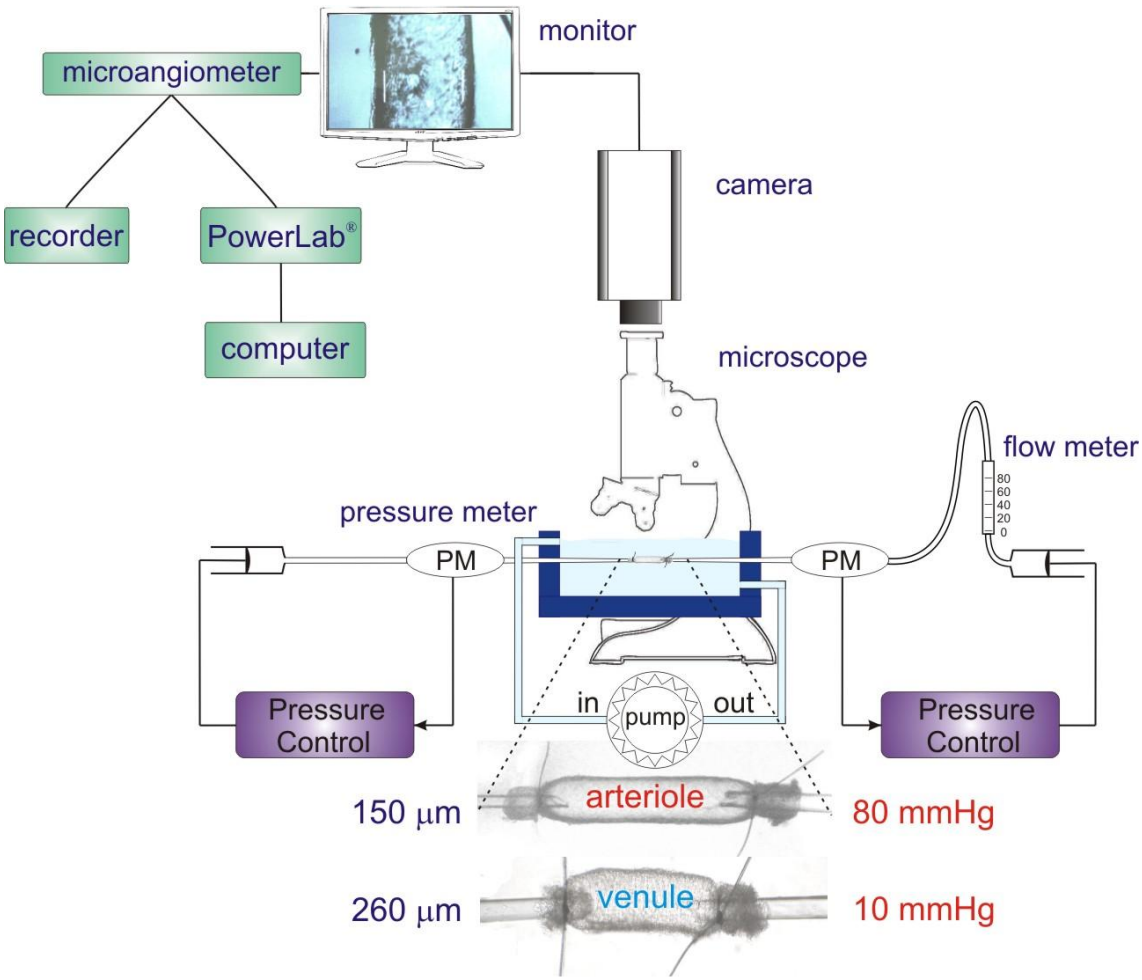


**Figure 5.** Experimental setup: videomicroscopic system for in vitro experiments on isolated gracilis vessels. PM: pressure meter

### 3.3. EFFECT OF ADMA ON BASAL ARTERIOLAR DIAMETER

During 1 hour equilibration period the vessel was allowed to reach stable active diameter in the presence of 80 mmHg perfusion pressure. To exclude the potential contribution of prostaglandins, experiments made on the arterioles were performed in the presence of indomethacin ($2.5\times10^{-5}$ mol/L). The basal arteriolar diameter was measured as a function of time after the administration of ADMA. ADMA-induced change in basal diameter was also assessed in the presence of apocynin, oxypurinol, quinapril or in the absence of the endothelium. The endothelium of the arteriole was removed by perfusion of air for ~1 min at a low perfusion pressure.[226] The arteriole was then perfused with PSS to clear the debris. The intraluminal pressure was then raised to 80 mmHg for ~15 min to reestablish a stable arteriolar tone. The efficacy of endothelial denudation was ascertained by a single dose ($10^{-7}$ mol/L) of acetylcholine. Also, to demonstrate the effect of superoxide on basal arteriolar diameter, arterioles were incubated with pyrogallol ($10^{-8}$-$10^{-6}$ mol/l, for 20 min), which is known to generate superoxide,[4, 227] in the presence or absence of SOD.

### 3.4. EFFECT OF ADMA ON PRESSURE-INDUCED ARTERIOLAR RESPONSES

Basal arteriolar tone was established at 80 mmHg. Changes in diameter of arterioles in response to stepwise increases in intraluminal pressure from 20 to 120 mmHg were then measured before and after ADMA treatment. Each pressure step was maintained for 5–10 min to allow the vessel to reach a steady-state diameter. To obtain passive diameters, arterioles were exposed to $Ca^{2+}$-free PSS containing EGTA ($10^{-3}$ mol/L) and SNP ($10^{-4}$ mol/L), and pressure-induced responses were reassessed.

### 3.5. EFFECT OF ADMA ON FLOW-INDUCED ARTERIOLAR RESPONSES

In the next series of experiments changes in diameter of arterioles were obtained in response to step increases in intraluminal flow (from 0 to 20 μL/min, in 5 μL/min steps) at constant intravascular pressure (80 mmHg) and in the presence of

indomethacin as well.[226] Each flow rate was maintained for 5 to 10 minutes to allow the vessel to reach a steady-state diameter. First, flow-induced changes in arteriolar diameter were measured in control conditions. Then, arterioles were incubated with ADMA ($10^{-4}$ mol/L) for 30 minutes. After incubation arteriolar responses to step increases in intraluminal flow were obtained again in the continuous presence of ADMA in the absence or presence of 120 U/mL superoxide dismutase (SOD) and 80 U/mL catalase (CAT) (a method that was shown to effectively scavenge superoxide [49, 228]) or nitric oxide (NO) synthase inhibitor $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME, $10^{-4}$ mol/L for 30 min) to assess the role of reactive oxygen species and NO contribution in flow-induced responses. Then, changes in arteriolar diameter to flow were obtained in the continuous presence of ADMA in the absence or presence of L-arginine ($5\times10^{-4}$ mol/L, for 30 min). In other experiments, in the presence of ADMA apocynin ($3\times10^{-4}$ mol/L), an inhibitor of NAD(P)H oxidases[173, 229] or xanthine oxidase inhibitor[211, 228] oxypurinol, ($10^{-4}$ mol/L) or quinapril ($10^{-5}$ mol/L), an inhibitor of angiotensin-converting enzyme (ACE) or losartan ($10^{-5}$ mol/L), an angiotensin type 1 ($AT_1$) receptor blocker was administered and flow-induced responses were obtained.

### 3.6. Effect of ADMA on agonist-induced arteriolar responses

In these series of experiments, responses of the arterioles to increasing concentrations of acetylcholine (ACh, $10^{-8}$–$3\times10^{-7}$ mol/L), and the NO donor sodium nitroprusside (SNP, $10^{-9}$–$10^{-6}$ mol/L) were obtained first under control conditions. Then arterioles were incubated with ADMA for 30 min, and then vasomotor responses of arterioles were obtained again in the continuous presence of ADMA. In the presence of ADMA the effect of the free-radical scavengers superoxide dismutase, SOD (120 U/mL) on vasomotor responses of arterioles were assessed. In other experiments arterioles were incubated with pyrogallol ($10^{-6}$ mol/l, for 10 min) and agonist-induced responses were obtained in the presence of pyrogallol in the absence or presence of SOD (120 U/ml) or CAT (80 U/ml).

### 3.7. ASSESSMENT OF VASCULAR SUPEROXIDE PRODUCTION IN THE PRESENCE OF ADMA

Superoxide production was assessed in arterial samples by the dihydroethidium (DHE) fluorescence and lucigenin-enhanced chemiluminescence method. DHE is a cell-permeable compound that can undergo a two-electron oxidation to form the DNA-binding fluorophore ethidium bromide (EB).[230] The reaction is relatively specific for superoxide with minimal oxidation induced by H2O2 or hypochlorus acid.[231] Femoral arteries were removed from rats and were immersed in PSS or $10^{-4}$ mol/L ADMA-containing PSS or $10^{-4}$ mol/L ADMA and $3\times10^{-4}$ mol/L apocynin-containing PSS or $10^{-4}$ mol/L ADMA and $10^{-5}$ mol/L quinapril-containing PSS or $10^{-4}$ mol/L ADMA and $10^{-5}$ mol/L losartan-containing PSS for 30 min. Then DHE ($5\times10^{-6}$ mol/L) was added to the vials and incubated for a further 10 min. After the incubation period, arteries were washed out with ice-cold PSS and immersed in an embedding medium. Frozen sections of femoral arteries were visualized by a digital camera attached to a fluorescence microscope. Intensity of EB fluorescence of the arteriolar wall was measured and quantified by Image J software. Relative EB fluorescence intensity was counted by extracting the intensity of the background from a standard size of the arterial wall. Measurement was repeated five times and relative intensity of EB fluorescence was presented as percentage of control.

In lucigenin chemiluminescence method, as described previously,[132, 232] in to asses the production of ROS we used small femoral arteries of rats, which provided sufficient amount of tissue and signal. Arteries were removed from the rats (n=8), cleared of connective tissue, immersed in PSS or $10^{-4}$ mol/L ADMA containing PSS in the presence or absence of NO donor sodium nitroprusside (SNP) or the angiotensin type 1 receptor blocker, telmisartan and were oxygenated and incubated for 30 min at 37°C. Then arteries were placed in scintillation vials containing HEPES-buffered ($10^{-6}$ mol/L; pH 7.4) PSS solution, and lucigenin ($10^{-6}$ mol/L, Calbiochem) chemiluminescence was measured in a liquid scintillation counter (Beckman LS-6000IC) as we used and described previously.[132, 232] Scintillation counts were obtained 20 min after addition of vessels, and background-corrected values were expressed and the values were normalized to the weight of vessels.

### 3.8. STATISTICAL ANALYSIS OF DATA

Peak constrictions of arterioles in response to ADMA are expressed as a percentage of the baseline diameter at an intraluminal pressure of 80 mmHg and plotted as a function of time. Peak dilations of arterioles are expressed as changes in arteriolar diameter as a percentage of the maximal dilation of the vessel, defined as the difference of the passive diameter (at 80 mmHg intraluminal pressure in a $Ca^{2+}$-free physiological salt solution containing $10^{-3}$ mol/L EGTA and $10^{-4}$ mol/L sodium nitroprusside) and the initial basal diameter of the arterioles (at 0 flow condition, at 80 mmHg). Statistical analyses were performed by 2-way ANOVA for repeated measures followed by the Tukey's posthoc test or Student's *t* test, as appropriate. $P<0.05$ was considered statistically significant. All of the data are expressed as mean±SE.

## 4. RESULTS

### 4.1. EFFECT OF ADMA ON BASAL ARTERIOLAR DIAMETER

Isolated gracilis muscle arterioles developed an active tone in response to the presence of intraluminal pressure of 80 mmHg, without the use of any vasoactive agent (active diameter: 153±4 μm vs. passive diameter: 235±3 μm; P<0.05). Summary data show that, in control conditions, ADMA elicited a significant decrease in the basal diameter of isolated gracilis muscle arteriole as a function of time. The maximum decrease in diameter occurred at ~15 minutes (11 ± 1 %) (**Figure 6**). Compared to control, the basal arteriolar diameters were significantly different in the presence of ADMA (control: 153±4 μm vs. ADMA: 132+-10 μm). The presence of apocynin, quinapril or endothelium removal abolished the constrictor effect of ADMA on basal diameter (**Figure 6**). The previous incubation with oxypurinol did not eliminate the constrictor effect of ADMA on basal diameter (**Figure 7A**).

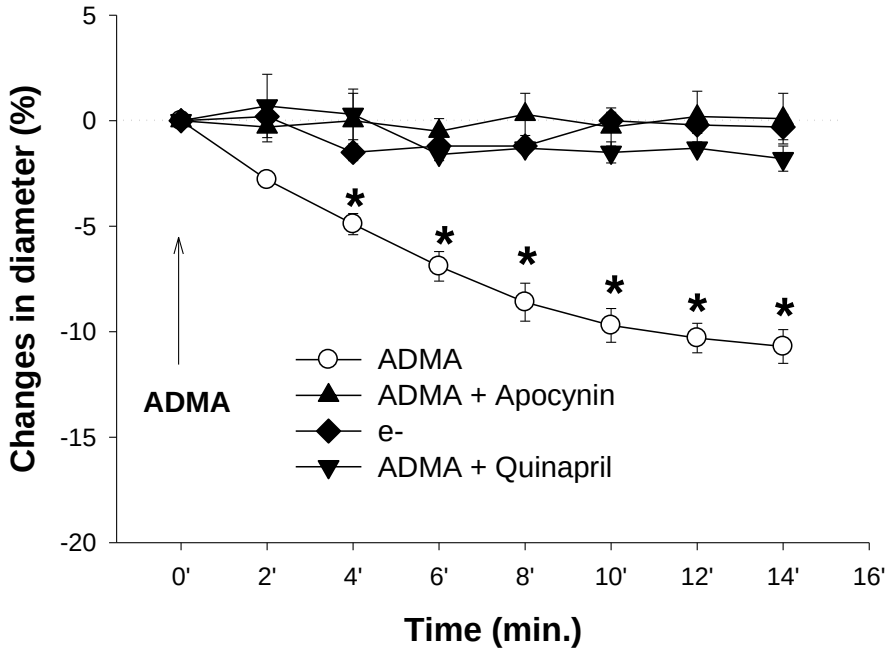


**Figure 6.** Summary data show the effect of ADMA ($10^{-6}$ mol/L) on the basal diameter of skeletal muscle arterioles in control conditions (n=16) and after removing endothelium (e-). Also shown is the effect of ADMA in the presence of NAD(P)H oxidase inhibitor apocynin or the ACE inhibitor quinapril. Data are mean±SEM; *P < 0.05 vs. control.

Increasing doses of pyrogallol elicited significant decreases in arteriolar diameter (maximum 24±6% at $10^{-6}$ mol/L), which was prevented by SOD/CAT (**Figure 7B**).

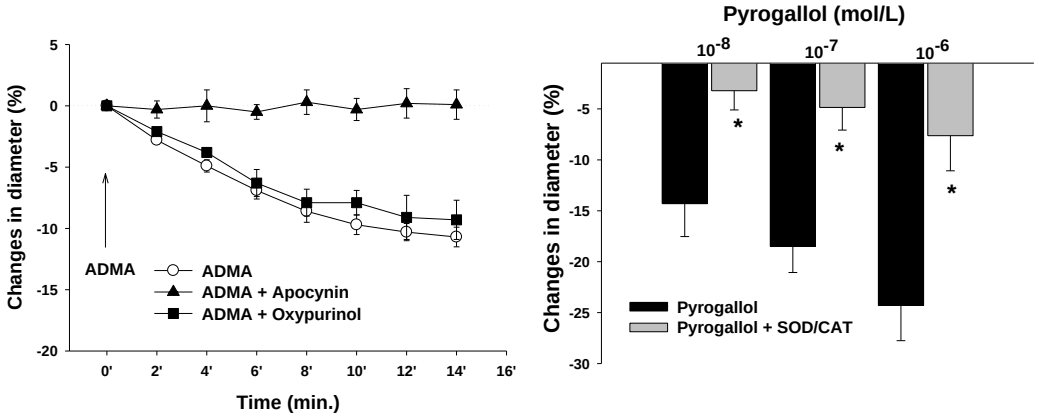


**Figure 7 A**, Summary data show the effect of ADMA (10-4 mol/L) on the basal diameter of skeletal muscle arterioles in control conditions and in the presence of xanthine oxidase inhibitor oxypurinol or NAD(P)H oxidase inhibitor apocynin (n=6, respectively). **B**, Effect of superoxide generator pyrogallol on the basal diameter of skeletal muscle arterioles in control conditions and in the presence of SOD and CAT. Data are mean ± SEM. *P < 0.05 vs. control.

### 4.2. EFFECT OF ADMA ON PRESSURE-INDUCED ARTERIOLAR RESPONSES

There were significant differences between the active arteriolar diameters in control and ADMA-treated arterioles skeletal muscle arterioles isolated from rats (156+-6 and 132+-10, respectively) developed to 80-mmHg intraluminal pressure (**Figure 8A**). Also, there was significant difference in the calculated myogenic tone (**Figure 8B**) developed to stepwise increases in intraluminal pressure from 20 to 110 mmHg in the two groups.

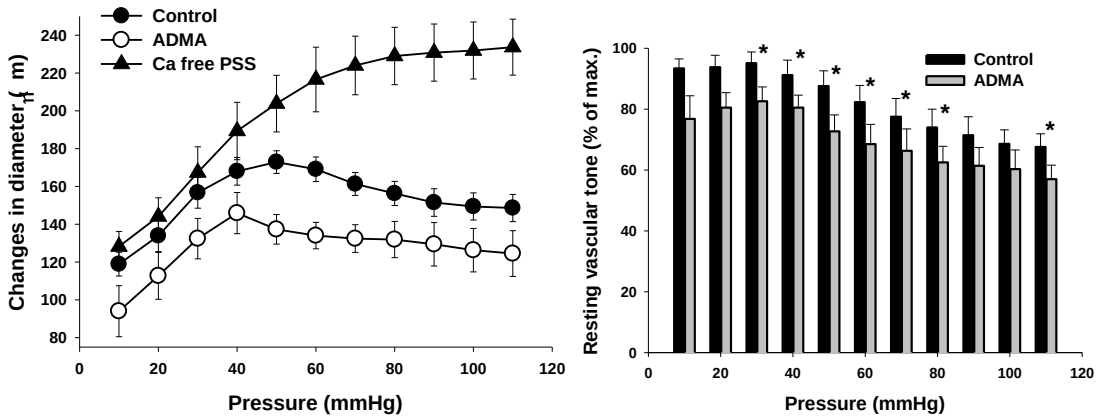


**Figure 8 A,** Changes in diameter of skeletal muscle arterioles in response to step increases (from 20 to 110 mmHg) in intraluminal pressure in control conditions, in the presence of ADMA and in the absence of extracellular $Ca^{2+}$. **B**, Calculated myogenic tone of skeletal muscle arterioles in control conditions, in the presence of ADMA and in the absence of extracellular $Ca^{2+}$ in response to step increases in intraluminal pressure. Data are mean ± SEM. *P < 0.05 vs. control.

### 4.3. EFFECT OF ADMA ON AGONIST-INDUCED ARTERIOLAR RESPONSES

After incubation with ADMA arteriolar dilations in response to cumulative doses of acetylcholine (ACh) were decreased compared with those vessels incubated without ADMA, but it didn't reach significant level (**Figure 9A**). On the other hand, responses to the higher concentrations of NO donor SNP were significantly reduced after ADMA treatment, which were partially restored in the additional presence of SOD and CAT ($10^{-6}$ mol/L SNP in ADMA-treated arterioles from 33±3% to 61±9%; P<0.05; **Figure 9B**).

In similar conditions, arteriolar dilation to ACh and SNP were also measured in the presence of pyrogallol, to reveal possible effect of ROS. Responses to acetylcholine were not different from the control conditions (**Figure 10A**), but SNP-induced dilation decreased, which were partially restored in the additional presence of SOD and CAT ($10^{-6}$ mol/L SNP in pyrogallol-treated arterioles from 29±8% to 51±8%; P<0.05; **Figure 10B**)

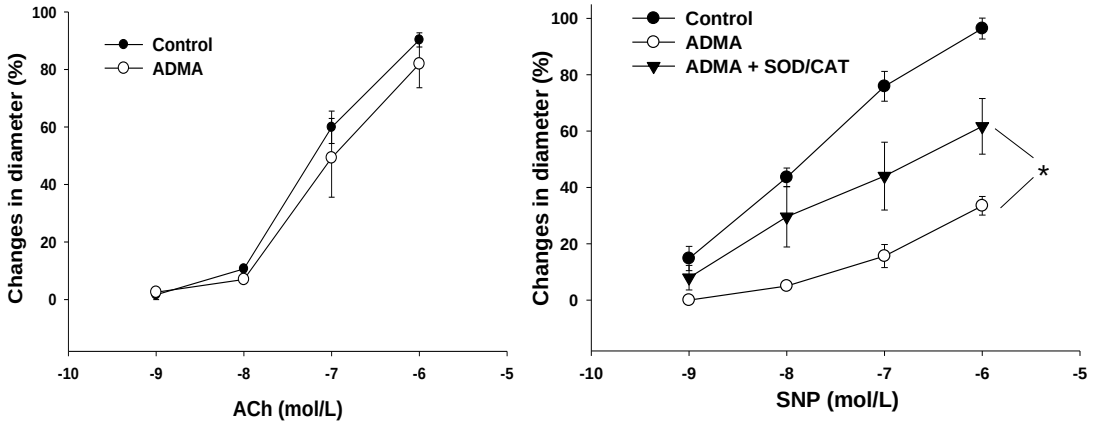


**Figure 9 A**, Summary data show the effect of asymmetric dimethylarginine (ADMA, $10^{-4}$ mol/L) on acetylcholine (ACh)-induced dilations of skeletal muscle arterioles (n=6). **B**, Nitric oxide (NO) donor sodium nitroprusside (SNP)-induced dilations (n=6) in absence or presence of ADMA and SOD/CAT (n=6). Data are mean ± SEM. *P < 0.05 vs. control.

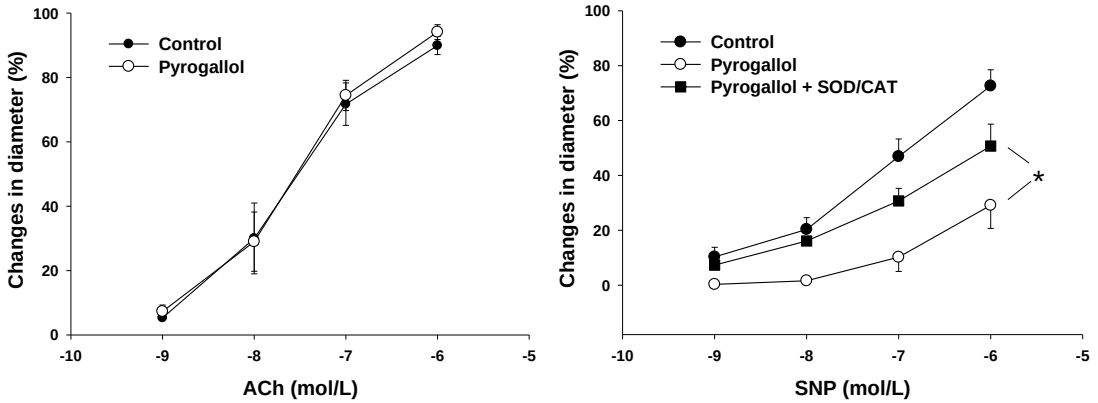


**Figure 10 A**, Summary data show the effect of pyrogallol, a known superoxide donor (pyrogallol, $10^{-6}$ mol/L) on acetylcholine (ACh)-induced dilations of skeletal muscle arterioles (n=6). **B**, Nitric oxide (NO) donor sodium nitroprusside (SNP)-induced dilations (n=6) in absence or presence of ADMA and SOD/CAT (n=6). Data are mean ± SEM. *P < 0.05 vs. control.

### 4.4. EFFECT OF ADMA ON FLOW-INDUCED ARTERIOLAR RESPONSES

Original records of diameter as a function of time shows that in control conditions step increases in intraluminal flow (5, 10, 15, and 20 μL/min) elicited substantial dilations of an isolated arteriole. After returning flow to zero, the diameter of arteriole returned to the control level. However, in the presence of ADMA ($10^{-4}$ mol/L) step increases in flow did not elicit dilations (maximum from 31 ± 2% to 3 ± 1%; **Figures 11A and 11B**). Flow-induced dilations were restored to the control level by the presence of SOD/CAT, and the restored dilations were abolished by the presence of L-NAME (maximum from 3 ± 1% to 28±2% and 1±1%; **Figure 11A**). However, the dilations to increases in flow in the presence of L-arginine and ADMA were not significantly different from the ADMA-treated arterioles (Figure **11B**).

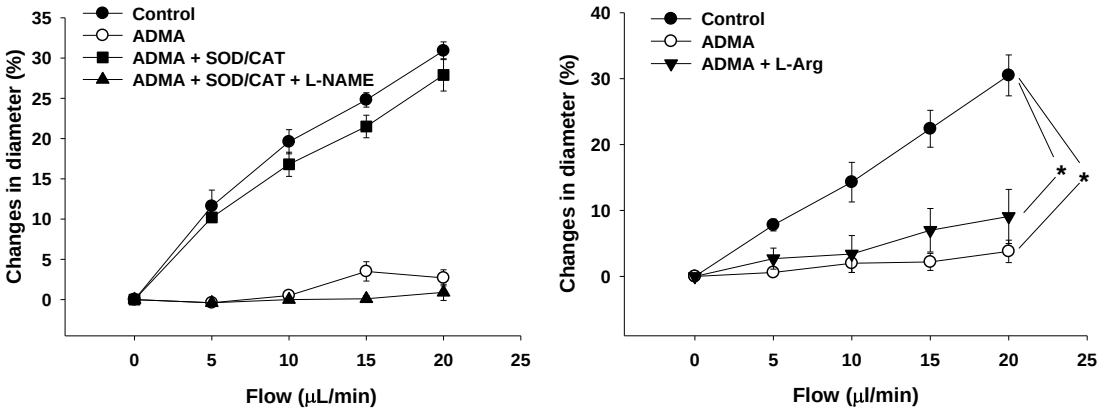


**Figure 11 A,** Flow-induced changes in diameter of skeletal muscle arterioles in control conditions, in the presence of ADMA, in the presence and absence of SOD/CAT and after administration of nitric oxide synthase inhibitor L-NAME (n=9). **B,** Flow-induced changes in diameter of skeletal muscle arterioles in control conditions, in the presence of ADMA and ADMA plus L-Arginine ($5\times10^{-4}$ mol/L, n=8,8,8; respectively). Data are mean ± SEM. *P < 0.05 vs. control.

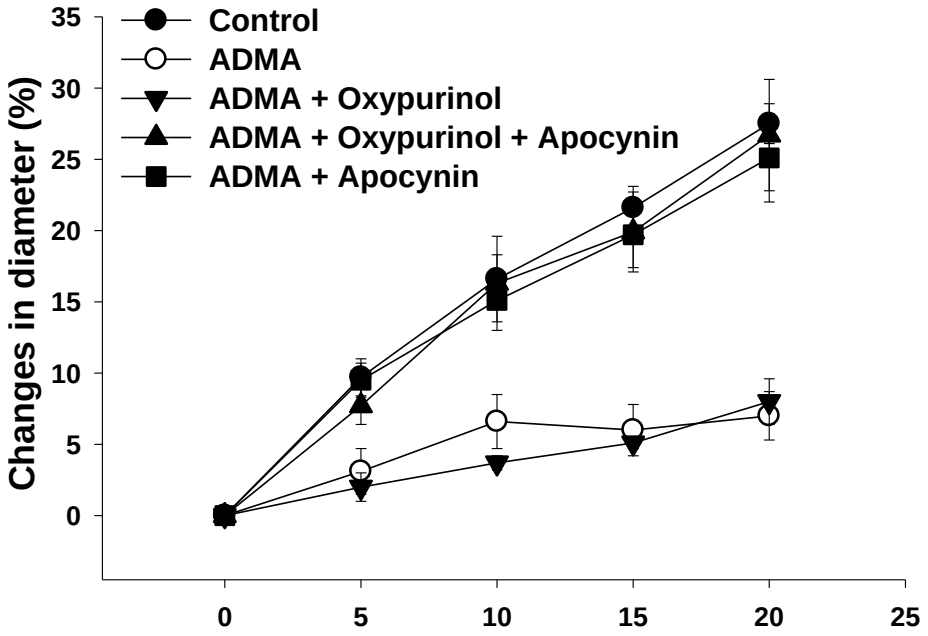


**Figure 12.** Flow-induced changes in diameter of skeletal muscle arterioles in control conditions, in the presence of ADMA and ADMA plus xanthine oxidase inhibitor oxypurinol and ADMA plus oxypurinol plus the NAD(P)H oxidase inhibitor apocynin and ADMA plus apocynin (n=6,6,8; respectively). Data are mean ± SEM.

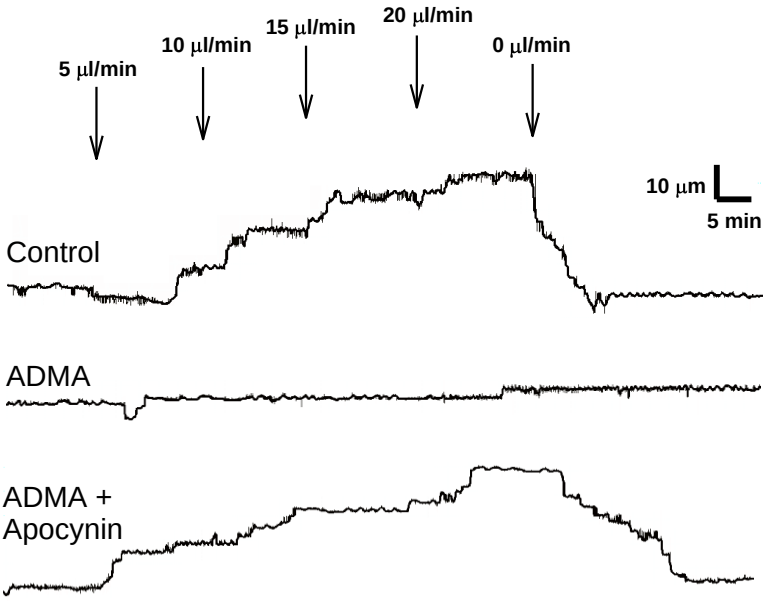


**Figure 13.** Original records showing changes in diameter of skeletal muscle arterioles in control conditions and in the presence of ADMA, in the presence and absence of NAD(P)H oxidase inhibitor apocynin

Also, summary data (**Figure 12**) and original records **(Figure 13**) show that the presence of apocynin significantly restored dilations to increases in flow in ADMA-treated arterioles (maximum from 4 ± 1% to 25 ± 3%), the magnitude of which reached the control levels (control, maximum 28 ± 2%). We have also found that the presence of oxypurinol did not change the flow-induced dilations in the presence of ADMA, whereas additional administration of apocynin restored dilations to flow (**Figure 12**).

In other experiments, we have found that presence of quinapril or losartan also restored flow-induced dilations to the control level (maximum 32 ± 2% and 23±2%, respectively; **Figures 14A and 14B**).

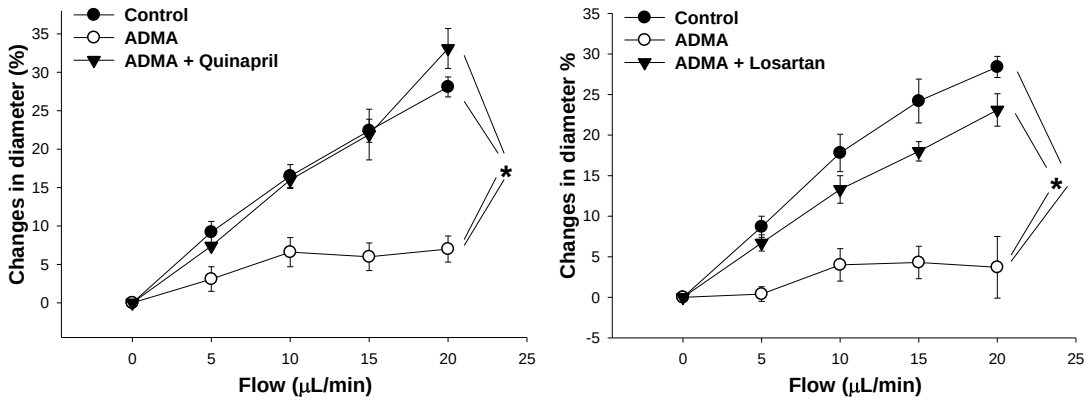


**Figure 14 A,** Flow-induced changes in diameter of skeletal muscle arterioles in control conditions and in the presence of ADMA, in the presence and absence of angiotensin-converting enzyme (ACE) inhibitor quinapril (n=8). **B,** Flow-induced changes in diameter of skeletal muscle arterioles in control conditions, in the presence of ADMA, in the presence and absence of angiotensin type 1 ($AT_1$) receptor blocker losartan (n=6). Data are mean ± SEM. *P < 0.05

### 4.5. ASSESSMENT OF VASCULAR SUPEROXIDE PRODUCTION IN THE PRESENCE OF ADMA

Representative fluorescent photomicrographs of EB fluorescence in control and ADMA- incubated arterial sections (**Figure 15A**) indicate an increased EB fluorescence in the smooth muscle in ADMA- incubated vessels as compared with the control (the enhanced autofluorescence of lamina elastica interna can also be seen). Simultaneous incubation of ADMA with apocynin decreased the fluorescence in the

arterial wall. Summary data show (**Figure 15B**) that EB staining was significantly higher in vessels incubated with ADMA as compared with the control, whereas in the simultaneous presence of ADMA and apocynin, quinapril, or losartan arterial fluorescence intensity was significantly decreased, close to the control levels.

Summarized data show that ADMA elicited an enhanced lucigenin chemiluminescence in arteries, which was significantly inhibited by pre-incubation of the vessels with the NO donor, SNP or the angiotensin type 1 receptor blocker, telmisartan (**Figure 16**).

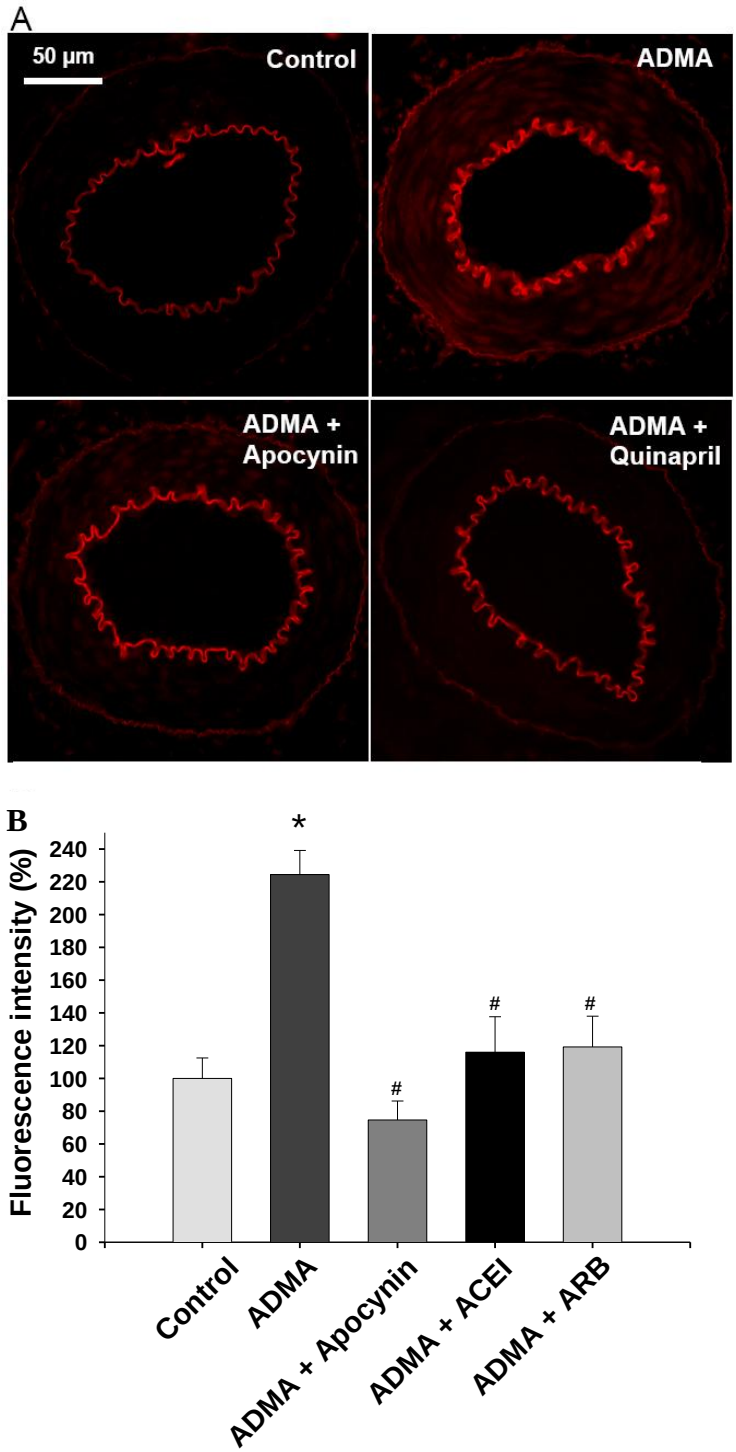


**Figure 15 A,** Ethidium bromide (EB) fluorescence in the smooth muscle of sections of small branches of isolated rat femoral artery in control conditions, in the presence of ADMA ($10^{-4}$ mol/L), in the presence of NAD(P)H oxidase inhibitor apocynin. **B,** Summary data of EB fluorescence are presented as percent change from control of sections of small branches of isolated femoral artery in the presence of ADMA ($10^{-4}$ mol/L), in the presence or absence of NAD(P)H oxidase inhibitor apocynin, the angiotensin-converting enzyme (ACEI) inhibitor quinapril or the angiotensin type 1 ($AT_1$) receptor blocker losartan (ARB) (n=4). Data are mean ± SEM. *P < 0.05 vs. control; #P < 0.05 vs. ADMA-treated group.

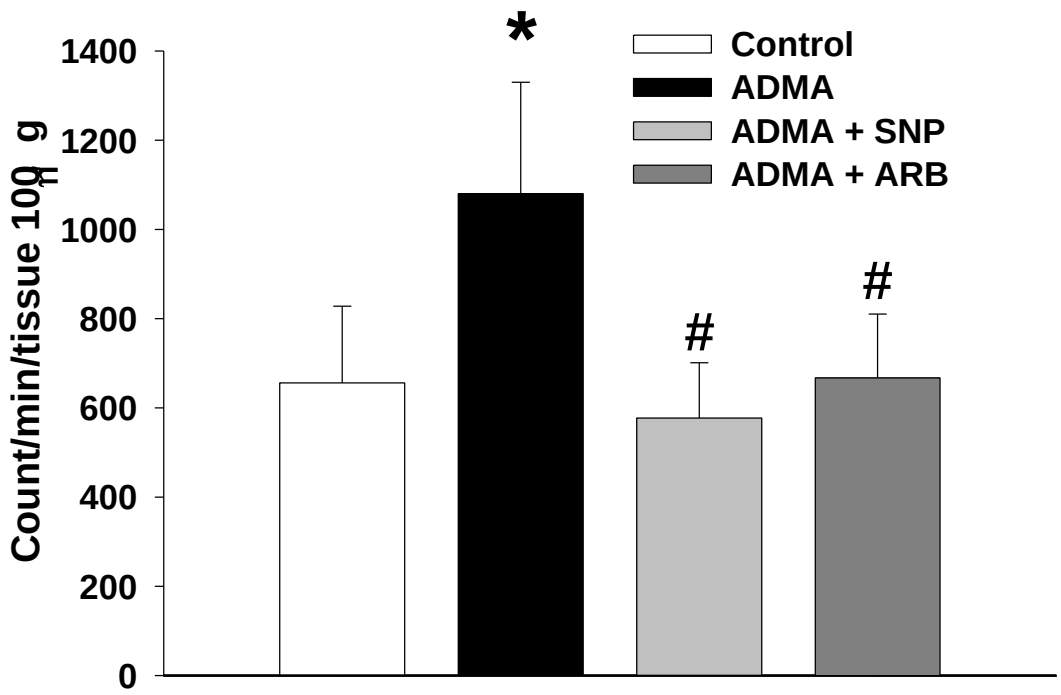


**Figure 16,** Summary data of lucigenin chemiluminescence to detect superoxide anion production in arteries treated with ADMA (10-4 mol/L) before and after incubation with NO donor sodium nitroprusside (SNP) or angiotensin type 1 receptor blocker telmisartan (ARB) (n=8). Data are mean ± SEM. *P < 0.05 vs. control, #P < 0.05 vs. ADMA treated arterioles

## 5. DISCUSSION

The novel findings of the present studies are that, ADMA in gracilis arterioles isolated from rats,

1) reduced the basal diameter, which was reversed by apocynin and ACE inhibitor quinapril and was unaffected by oxypurinol,

2) inhibited flow/shear stress-induced dilations, which were not restored by L-arginine or xanthine oxidase inhibitor, oxypurinol, but were restored by the scavenger of reactive oxygen species superoxide dismutase and catalase or the NAD(P)H oxidase inhibitor apocynin, ACE inhibitor or $AT_1$ receptor blocker, and

3) elicited vascular oxidative stress (indicated by increased EB fluorescence) which were normalized by SOD, apocynin, ACE inhibitor, or $AT_1$ receptor blocker and enhanced lucigenin chemiluminescence, which was inhibited by SNP and the $AT_1$ receptor blocker.

Our findings support the presence of novel mechanisms, responsible for the effects of ADMA on the vasomotor function of microvessels and as such, they can have important consequences on our understanding of microvascular regulation.

***L-arginine, ADMA and NOS function***

It has been shown that L-arginine is the substrate of NOS and that methylated L-arginines, such as $N^{\omega}$-nitro-L-arginine, $N^{\omega}$-monomethyl-L-arginine, and $N^{\omega}$-nitro-L-arginine-methyl-ester, inhibit NOS, with the consequent elimination of NO-mediated dilations of vessels.[42, 233] These forms of methylated L-arginine, however, are not readily available in vivo. Methylations of L-arginine in proteins, however, do occur in vivo, which then released from proteins during proteolysis.[234] ADMA is one of the most important endogenously produced methylated L-arginines.[72] Although in vitro biochemical studies demonstrated that ADMA reduces NO production and likely enhances superoxide production via „uncoupling of NOS activity" in endothelial cells.[221]There are, however, effects of ADMA seemingly unrelated to NOS, which have not yet been clarified. For example, Suda and associates have found in wild-type and endothelial NOS-knockout mice that long-term treatment with ADMA induced coronary microvascular lesions.[121] These changes were not because of the developed hypertension and were not antagonized by administration of L-arginine. Also, increased superoxide production in monocytes, epithelial, endothelial and even in cardiac cells were reported after ADMA incubation[163, 221-225], yet the mechanisms responsible for the enhanced superoxide production by ADMA remain unclear. However, the mechanisms responsible for the enhanced superoxide production by ADMA remain unclear. Previous studies also reported an increased NAD(P)H oxidase activity in most peripheral vascular beds of animals with various forms of hypertension,[136-186] diabetes,[235-237] or hyperhomocysteinaemia.[238] Interestingly, in these human diseases, the serum levels of methylated L-arginines, such as ADMA are increased.[106, 107, 239, 240] Thus, it was logical to hypothesize that the presence of ADMA, in addition to inhibiting NOS, may leads to increased release of superoxide, which is due to activation of NAD(P)H oxidase. Because Ang II is a known activator of NADP(H) oxidase the potential role of RAS in ADMA induced arteriolar

dysfunction could be hypothesized as well. To test these hypotheses, we have used isolated gracilis arterioles to elucidate the effect of ADMA on NO-mediated dilator responses elicited by increasing flow/wall shear stress. Previous studies showed that, in gracilis arterioles, increases in intraluminal flow elicit the release of prostaglandins in addition to NO.[226] In addition, in certain conditions, cyclooxygenases produce reactive oxygen species. Thus, to exclude the potential contribution of these pathways, which may interfere with the interpretation of results, we performed our experiments in the presence of indomethacin an inhibitor of cyclooxygenases involved in the production of prostaglandins.

The normal concentration of ADMA in plasma is in the range of 0.355±0.066 μM [241], which however, could be much higher intracellularly, where it is produced, and then - in part - is transported to the plasma.[79] ADMA becomes elevated in diseases associated with oxidative stress, as well as nitrosative stress because these conditions decrease the activity of the ADMA demethylating enzyme, dimethylarginine dymethylaminohydrolase (DDAH).[242] In rats, intravenous administration of homocysteine (10 mg/kg/day for 4 weeks) increased serum ADMA level (from 1 to 2 μmol/L) [243], whereas in rats with Type 2 diabetes the level of ADMA significantly increases from the control 0.5 μmol/L to 1.5 μmol/L as the disease progresses [240]. In subjects with high body mass index (BMI ≥26 kg/m$^2$) the plasma concentration of ADMA is significantly higher (1.44 compared to 1.31 μmol/L) than in subjects with low BMI (<26 kg/m$^2$), whereas the L-arginine/ADMA ratio is lower (obese: 66 vs. lean: 89). Also, several studies have shown that plasma concentration of ADMA is significantly higher in smokers as compared with non smokers.[244]

Recent studies measuring intracellular levels of ADMA in red blood cells showed that it ranges between 40.61±7.15 μM.[245] Importantly, a 5-fold increase in methylarginine concentration has been shown in endothelial cells when they were exposed to methylarginines added to culture medium.[246] This level of methylarginines is probably attributable to the arginine transport system referred to as the Y+ transporter.[247] In human endothelial cells the $K_m$ for transport of methylated L-arginines is around 70 μM and the $V_{max}$ is in the range of 2 μmol/mg protein/min.[248] It is likely however, that in the intracellular environment ADMA compartmentalizes

reaching high concentrations in localized regions and that removal of ADMA might also be a slow process. Collectively, one can logically assume that ADMA levels can reach high intracellular concentrations under certain pathologic conditions.[84, 248] These concentrations of ADMA can inhibit NOS and can elicit superoxide production resulting in the consequent pathologic regulation of vascular tone.[97, 249] Thus present experiments were performed in the presence of $10^{-4}$ M concentrations of ADMA to mimic potential intracellular conditions and also to be comparable to the findings of other studies using other methylated L-arginine in this concentration.

***ADMA activates NAD(P)H oxidase in arterioles and elicits oxidative stress***

First, we confirmed our previous finding that ADMA elicits significant constriction of arterioles (**Figure 6 and 7A**). Similarly, pyrogallol, known to produce superoxide elicited significant constrictions (**Figure 7B**). This constriction was prevented by previous incubation of arterioles with superoxide dismutase and catalase, suggesting that the decrease in the diameter of arterioles was due to increased oxidative stress. NAD(P)H oxidase has been shown to be a key oxidative enzymes involved in many diseases associated with arteriolar dysfunction.[136, 173, 186] Thus, we have used apocynin, know to inhibit NAD(P)H oxidase.[173, 229] We have found that, in the presence of ADMA, apocynin restored the basal diameter of arterioles. In endothelium-denuded vessels, additional administration of ADMA did not elicit a reduction in the diameter of arterioles. Furthermore, these constrictions were not prevented by prior incubation of xanthin oxidase inhibitor[211, 228] oxypurinol (**Figure 7A**). Collectively these findings suggest that the decrease in diameter of arterioles was due to increased levels of superoxide interfering with NO, but which itself could be a vasoconstrictor agent and that ROS is produced by NAD(P)H oxidase rather than xanthin oxidase.[136, 173, 186]

As mentioned above, biochemical studies utilizing the purified enzyme showed that eNOS may become “uncoupled” in the absence of the NOS substrate L-arginine when electrons flowing from the reductase domain to the oxygenase domain are diverted to molecular oxygen rather than to L-arginine resulting in production of superoxide rather than NO.[250] Previous studies have provided evidence that L-

arginine is the precursor of the formation of nitric oxide and supplementation of L-arginine optimizes the formation of NO.[71] In case of eNOS "uncoupling" the excess formation of superoxide by NOS can be prevented by L-arginine.[71] However, in the present vascular experiments, in which several other enzymes and cellular organs are present L-arginine did not restore flow-induced dilations in the ADMA-treated arterioles (**Figure 11B**).

These findings are supported by a recent report, that ADMA significantly impaired glucose utilization, induced ROS and TNF-alpha production in adipocytes, whereas L-arginine increased NO, but failed to reduce the effects of ADMA.[251] We interpret these findings to mean that the primary effect of ADMA may not (only) relate to eNOS. That is, in the presence of ADMA NO is still produced by eNOS and ROS are not produced by eNOS.

More recently, Korandji and colleagues[252] have found that 2 weeks of high fructose diet increased plasma levels of ADMA and increased vascular oxidative stress markers and later an increased NAD(P)H oxidase activity could be detected.

Thus, it seems that the primary action of ADMA, (in addition to the potential inhibition of NOS, if any) is the activation of an oxidative pathway. Once ROS, such as superoxide anion is produced it chemically interferes with NO (likely producing peroxynitrite), which then results in the reduction of NO bioavailability and thus, reduction of flow dependent dilation. Indeed, we found that scavenger of ROS (SOD plus CAT) and NAD(P)H oxidase inhibitor apocynin restored flow-induced dilation (**Figure 11-13)** in the presence of ADMA supporting our ideas. Furthermore, the NOS inhibitor L-NAME abolished the "SOD/CAT restored" flow-induced dilation in the presence of ADMA. We interpret these findings to mean that the primary effect of ADMA is an increased production of reactive oxygen species, which then interferes with NO released by NOS and, thus, dilation. The findings that presence of xanthin oxidase inhibitor oxypurinol did not change the flow-induced dilations in the presence of ADMA, whereas additional administration of apocynin restored dilations to flow (**Figure 12**), suggest that the primary source of superoxide in the presence of ADMA is likely to be NAD(P)H oxidase..

***ADMA Enhances Myogenic Tone***

Microvessels respond to an increase or decrease in transmural pressure by constriction and dilation, respectively. Because vascular resistance is influenced by myogenic reactivity and enhanced myogenic tone could adversely affect vasodilator function of arterioles, in the present study, responses to increases in intraluminal pressure were obtained arterioles of skeletal muscle. There were significant differences between the active arteriolar diameters in control and ADMA-treated arterioles and between the calculated myogenic tone developed to stepwise increases in intraluminal pressure in the two groups (**Figure 8A and 8B**). These findings indicate that in the diseases with the elevated levels of ADMA enhanced myogenic tone can be responsible for the impaired vasomotor function.

***ADMA and Superoxide effect NO Donor Induced Dilations Similarly***

To further test the hypothesis that ADMA act via superoxide we have used the NO donor, SNP[42, 53] to elicit dilations of isolated gracilis arterioles. We have found that ADMA and ROS producer pyrogallol significantly reduced the NO donor, SNP-induced arteriolar dilations, which were restored by SOD/CAT (**Figure 9B and 10B)**, suggesting that increased level of ROS in the presence of ADMA or pyrogallol interfered with the NO released from SNP is responsible for the reduced dilations.

Endothelium plays an important role in maintaining vascular homeostasis by synthesizing and releasing several mediators of vasodilation, which include $PGI_2$, NO, and EDHF. ACh elicited endothelium-dependent relaxation in the presence of inhibitors of nitric oxide synthase and cyclooxygenase in many types of vessels. In gracilis muscle arterioles ACh-evoked relaxation appears to be mainly mediated by EDHF.[253, 254] First, we have found that endothelium-dependent vasodilator ACh did not change significantly dilations in the presence of ADMA compared to control conditions, as expected (**Figure 9A**). Pyrogallol had the same effect as ADMA on responses to ACh of isolated arterioles during this condition (**Figure 10A**)

Our previous findings that dilations to 8-bromo cGMP (cGMP-dependent protein kinase analog) and the calcium channel blocker nifedipine were not affected by ADMA suggest that ADMA does not affect the signaling pathways downstream from cGMP and - in general - the dilator capacity of arteriolar smooth muscle.[132]

***ADMA activates renin-angiotensin system in arterioles***

Several in vitro and in vivo studies have established an important role for angiotensin II in the activation of NAD(P)H oxidase leading to oxidative stress.[136, 137, 186] Also, previous studies proposed a potential interaction between ADMA and the RAS. [161, 163]

Thus, we hypothesized that the arteriolar RAS is involved in the ADMA-induced oxidative stress. Indeed, we have found that the ACE inhibitor quinapril restored flow-induced dilations in arterioles in the presence of ADMA (**Figure 14A**) and also inhibited a reduction of diameter by ADMA (**Figure 6**). In addition, we have also found that the $AT_1R$ blocker losartan restored flow-mediated dilation of arterioles in the presence of ADMA (**Figure 5**). Collectively, it seems that ADMA, via as yet unknown mechanism(s), activates the microvascular RAS,[255] which leads to an increased level of Ang II in the microvascular wall, and $AT_1$ receptors are involved in the ADMA-angiotensin II pathway producing reactive oxygen species.

The relationship between ADMA and local RAS may also present in chronic conditions, as shown by Hasegawa et al[163] that long-term ADMA administration caused upregulation of local ACE and increased wall:lumen ratio and perivascular fibrosis in coronary microvessels in wild-type mice. Also, overexpression of dimethylarginine dimethylaminohydrolase-2, an ADMA degrading enzyme, in transgenic mice prevented the development of ADMA-induced microvascular lesions and upregulation of ACE.[163] Suda and colleagues[121] also suggested a role for the upregulation of local ACE and increased oxidative stress in the long-term vascular effects of ADMA in vivo.

***ADMA induces oxidative stress via activating vascular renin-angiotensin system***

To provide further evidence for the idea that ADMA induces vascular oxidative stress and that NAD(P)H oxidase and RAS contribute to these processes, we have investigated the effect of ADMA on EB fluorescence and on lucigenin-enhanced chemiluminescence, indicators of oxidative stress, in sections of small branches of femoral artery. We have found that ADMA increased vascular smooth muscle DHE fluorescence (**Figure 15A** and **15B**),[256] which was significantly reduced toward

control levels in the presence of apocynin, quinapril, or losartan. Furthermore, we have also found that ADMA enhanced lucigenin chemiluminescence (**Figure 16**) which was inhibited by the prior incubation with the NO donor, SNP or the $AT_1R$ blocker, telmisartan.

These findings support the hypothesis that the renin-angiotensin system in the arteriolar wall is involved in the ADMA-induced oxidative stress. Indeed, it seems that there is a complex relationship between ADMA and the tissue renin angiotensin system. In our study and observation made by others[163] found that ADMA – in addition to affecting $AT_1$ receptor - upregulates the ACE expression in endothelial cells. Then the increased level of Ang II and $AT_1R$ activates NAD(P)H oxidase and subsequently generates ROS,[257] which interferes with NO released in response to agonists or flow. Recently, Chen and associates[223] found that ADMA in HUVECs increased ROS formation - in part - reduced NO formation, both of which could be restored by losartan. Another group[258] have found that in bovine retinal capillary endothelial cells that ADMA increased intracellular ROS generation, which was markedly inhibited by the angiotensin II receptor-blocker telmisartan, the angiotensin-converting enzyme inhibitor benazepril, the reduced form of NAD(P)H oxidase inhibitor diphenyliodonium (DPI), or the antioxidant and free-radical scavenger N-acetyl-l-cysteine.[258]

Moreover, because ROS is reported to inhibit dimethylarginine dimethylaminohydrolase (DDAH), an enzyme which degrades ADMA,[242] Ang II could elevate ADMA production.[259] Furthermore Ang II increases ADMA production in HUVECs.[223] Thus these pathophysiological mechanisms seem to provide a self-amplifying feedback process keeping ADMA level high and NO level low.

In the present experiments we aimed to investigate the short term, vasomotor effects of ADMA, thus, changes observed were unlikely due to the upregulation of various genes or protein synthesis. Nevertheless, it is likely that the chronic presence of elevated levels of ADMA upregulates several enzymes of microvascular RAS, such as expression of ACE protein, $AT_1R$ and others. This idea is supported by studies of Hasegawa and their co-workers[163] showing that the chronic presence of ADMA enhanced the p38 mitogen-activated protein kinase activity in human coronary artery endothelial cells, which may provide a link between ADMA and RAS, because ACE

protein expression has been show to be regulated by various mechanisms, including p38 mitogen-activated protein kinase.[260] Nevertheless, further studies are needed to elucidate the exact mechanism of action by which ADMA activates RAS in the arteriolar wall.

Because of the data obtained in our studies and those of others it is clear that the mechanisms of actions of ADMA are still not yet clarified. Thus we believe that refering to ADMA only as an endogenous inhibitor of NOS is an oversimplified view. Thus it is important to further explore the mechanisms by which ADMA exerts its deleterious effects on various functions of vascular tissues.

In conclusion, our findings suggest that elevated levels of ADMA by activating RAS in the wall of microvessels elicits increased production of Ang II, which by activating NAD(P)H oxidase results in an increased production of reactive oxygen species. Increased oxidative stress reduces the bioavailability of NO and agonists and flow/shear stress-induced dilations mediated by NO (**Figure 17**), both of which favor the development of increased peripheral resistance.

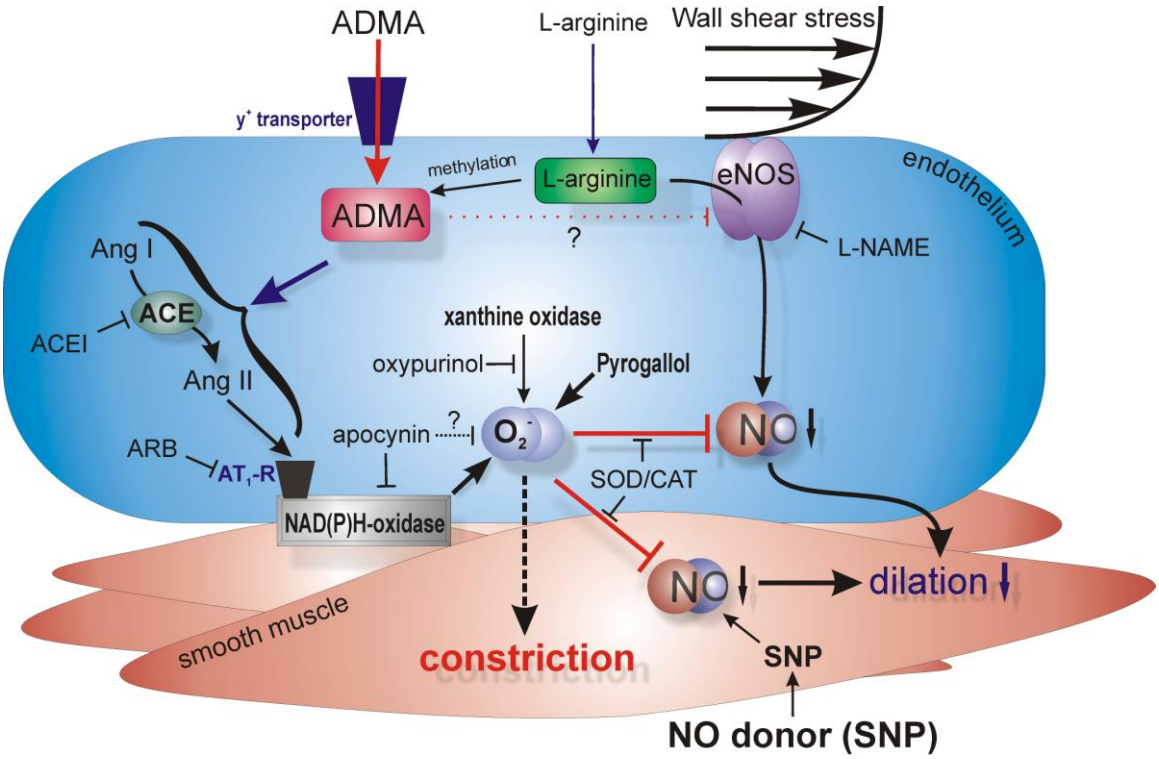


**Figure 17.** Proposed mechanisms, by which ADMA induces enhanced oxidative stress and vasomotor dysfunction of arterioles. Elevated levels of ADMA activate the renin-angiotensin system in the arteriolar wall, leading to increased production of angiotensin II, which then activates NAD(P)H oxidase. The consequent increased level of reactive oxygen species interferes with the bioavailability of nitric oxide donors and NO released to increases in flow/shear stress, resulting in diminished NO donor dilation, inhibition of flow-induced dilation and enhanced arteriolar tone both of which favoring the development of increased peripheral resistance. NO: nitric oxide; eNOS: endothelial nitric oxide synthase; L-NAME: $N^{\omega}$-nitro-L-arginine methyl ester, inhibitor of nitric oxide synthase SNP: NO donor sodium nitroprusside; ADMA: asymmetric dimethylarginine; O2-: superoxide; SOD/CAT: superoxide dismutase/catalase, scavenger of reactive oxygen species; apocynin: proposed inhibitor of NAD(P)H oxidase; pyrogallol, a known superoxide donor; oxypurinol: inhibitor of xanthine oxidase; Ang I: angiotensin I; Ang II: angiotensin II; $AT_1$-R: angiotensin type I receptor; ACEI: angiotensin converting enzyme inhibitor; ARB: angiotensin type 1 receptor blocker.

The present study provide evidence for the idea that ADMA, which levels are elevated in many cardiovascular diseases, activates microvascular RAS leading to the increased production of reactive oxygen species and dysfunction of the vasomotor regulation of resistance arterioles. This is a newly discovered mechanism, because previously it was thought the ADMA - a methylated form of the nitric oxide synthase

substrate L-arginine – exerts its action only via inhibiting NOS. More importantly however, these findings may explain some of the beneficial, pleiotropic effects of $AT_1R$ blockers. This is especially interesting because elevated levels of ADMA may not only affect vasomotor, but other functions of tissues, as well. Among others, ADMA affects pancreatic beta-cell function[261] and serum cholesterol concentrations[262] both are promoting the development of metabolic syndrome. Interestingly, ADMA is produced in relatively high concentrations in the brain.[126] Topical application of ADMA significantly constricted the basilar artery in anesthetized rats using cranial windows, suggesting special role in modulation of cerebral vascular tone under resting conditions and in response to vasoactive stimuli.[126]

***The Pathophysiological and Clinical Importance of Methylation***

It seems that there are interesting links between ADMA and homocystein metabolism and vascular actions. Like many other cardiovascular risk factors, hyperhomocysteinemia (HHcy) produces endothelial dysfunction due to impaired bioavailability of NO. The molecular mechanisms responsible for decreased NO bioavailability in HHcy are incompletely understood, but emerging evidence suggests that ADMA may be a key mediator. Several animal and clinical studies have demonstrated a strong association between plasma total homocysteine, plasma ADMA, and endothelial dysfunction. Again, it is important to emphasize that homocysteine and ADMA are produced intracellularly, where their concetrations are higher than in the plasma. HHcy has been shown to impair the endothelial function of arterial vessels and promote thrombosis. Previous studies have suggested an important relation between elevated levels of plasma homocysteine and venous diseases, such as venous thromboembolism[196] in the lungs,[263] brain,[264] portal and splenic circulation, in the central retinal vein[265-267] Also, there are important links between our previous and present studies on HHCy and ADMA. Our previous studies in skeletal muscle arterioles isolated from HHcy rats showed that increases in flow-induced constrictions, instead of dilations, which were due to the altered function of endothelium, including the impaired bioavailability of NO, the elevated synthesis of

$TxA_2$ and reactive oxygen species.[211, 268] Thus, it was logical to assume that HHcy affects - not only the arterial, but also the venous side of circulation. Thus we aimed to elucidate the effects of HHcy on venular vessels known to provide a large endothelial surface area of the circulation and thus responsible for the release of numerous factors that are involved, not only in the maintenance of rheological properties of blood, but also in the regulation of resistance of venous circulation.

Changes in diameter of isolated gracilis muscle venules (diameter: ~250 μm at 10 mmHg) of control and HHcy rats (induced by methionine diet for 5 weeks) to increases in intraluminal flow were measured. We have confirmed that increases in flow elicit dilations of venules isolated from control animals (at max.: 14±1 %), which were augmented by the presence of SQ 29,548 TP receptor blocker (**Figure 18**) [269] showing that the amount of dilator factors released overcome the constrictor effect of $TxA_2$. The finding that increases in intraluminal flow resulted - instead of dilations - in substantial constrictions (at max.: -24±4 %) in venules isolated from HHcy rats (**Figure 18**), which were then converted to dilations in the presence of TP receptor antagonist (**Figure 18**), suggests that increases in flow/shear stress – in addition to dilator factors - elicit a substantial release of constrictor $TxA_2$ in skeletal muscle venules of HHcy rats. In the presence of TP receptor blockade a role for NO and dilator prostaglandin (likely $PGI_2/E_2$), could be revealed in mediating flow-induced dilations, as shown by the findings that these dilations were inhibited by L-NAME and indomethacin (**Figure 19**). Thus, it seems that in HHcy the dilator effects of NO and prostaglandins are overcomed by the substantial release of $TxA_2$

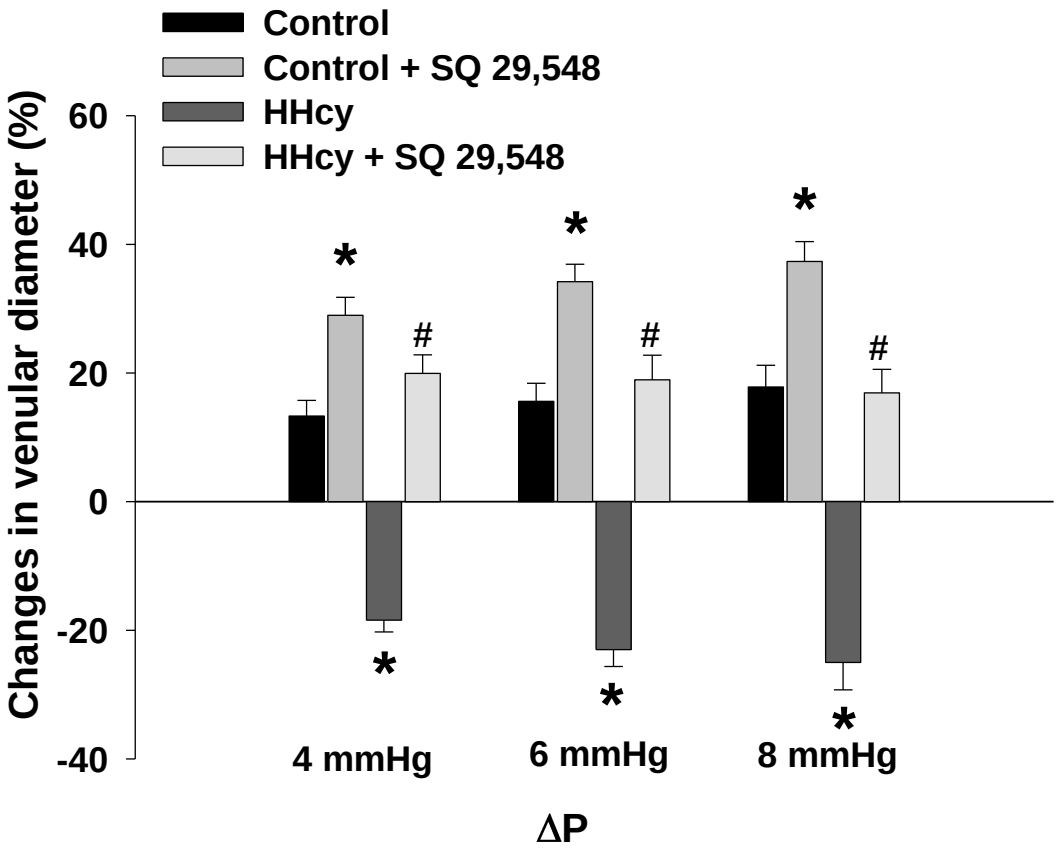


**Figure 18.** Changes in diameter of skeletal muscle venules of control and HHcy rats as a function of intraluminal flow elicited by increasing the pressure difference between the inflow and outflow cannula, in the absence or presence of the TP receptor blocker, SQ 29,548 (n=7, 6, 6). Data are mean ± SEM. *P < 0.05 vs. control, #P < 0.05 vs. HHcy venules.

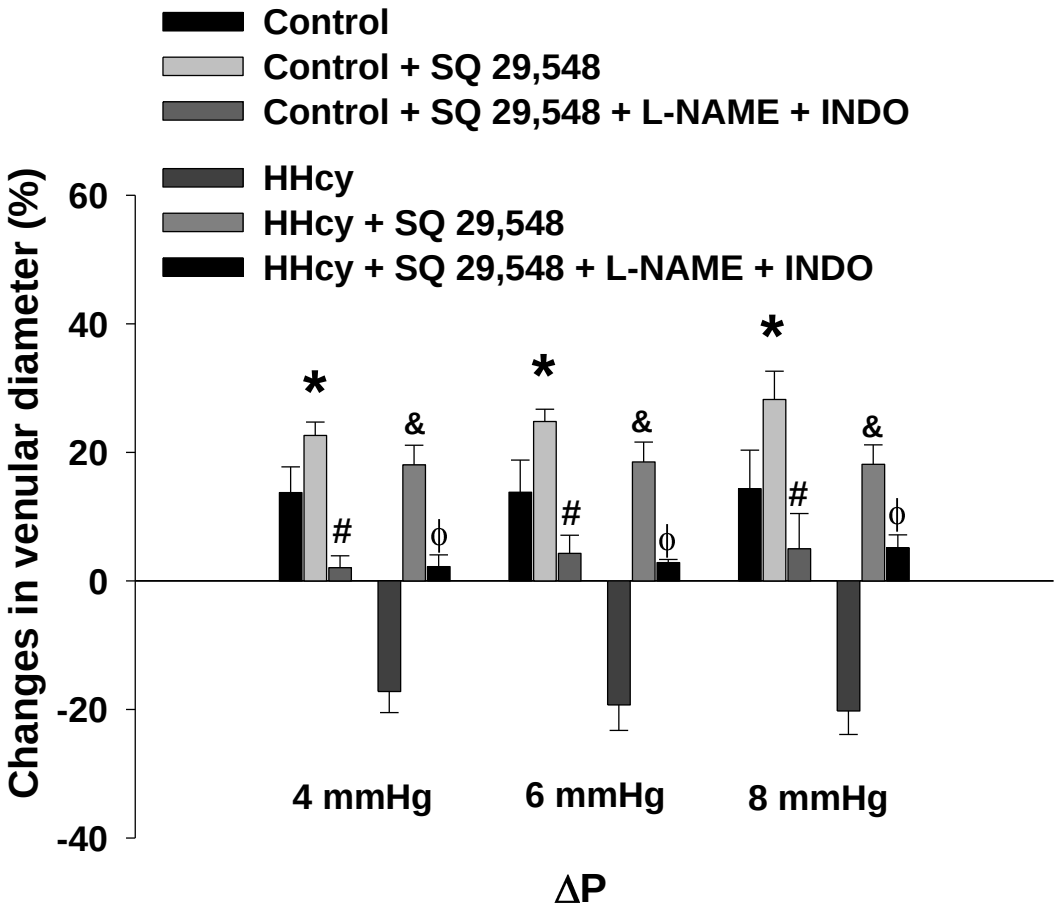


**Figure 19.** Changes in diameter of control and HHcy venules to flow in the presence or absence of the TP receptor blocker, SQ 29,548, or SQ 29,548 + the nitric oxide synthase inhibitor, L-NAME + the non-specific cyclooxygenase inhibitor, indomethacin (n=7, 5). Data are mean ± SEM. *P < 0.05 vs. control, #P < 0.05 vs. SQ 29,548 treated venules, &P < 0.05 vs. HHcy venules, φP < 0.05 vs. SQ 29,548 treated HHcy venules.

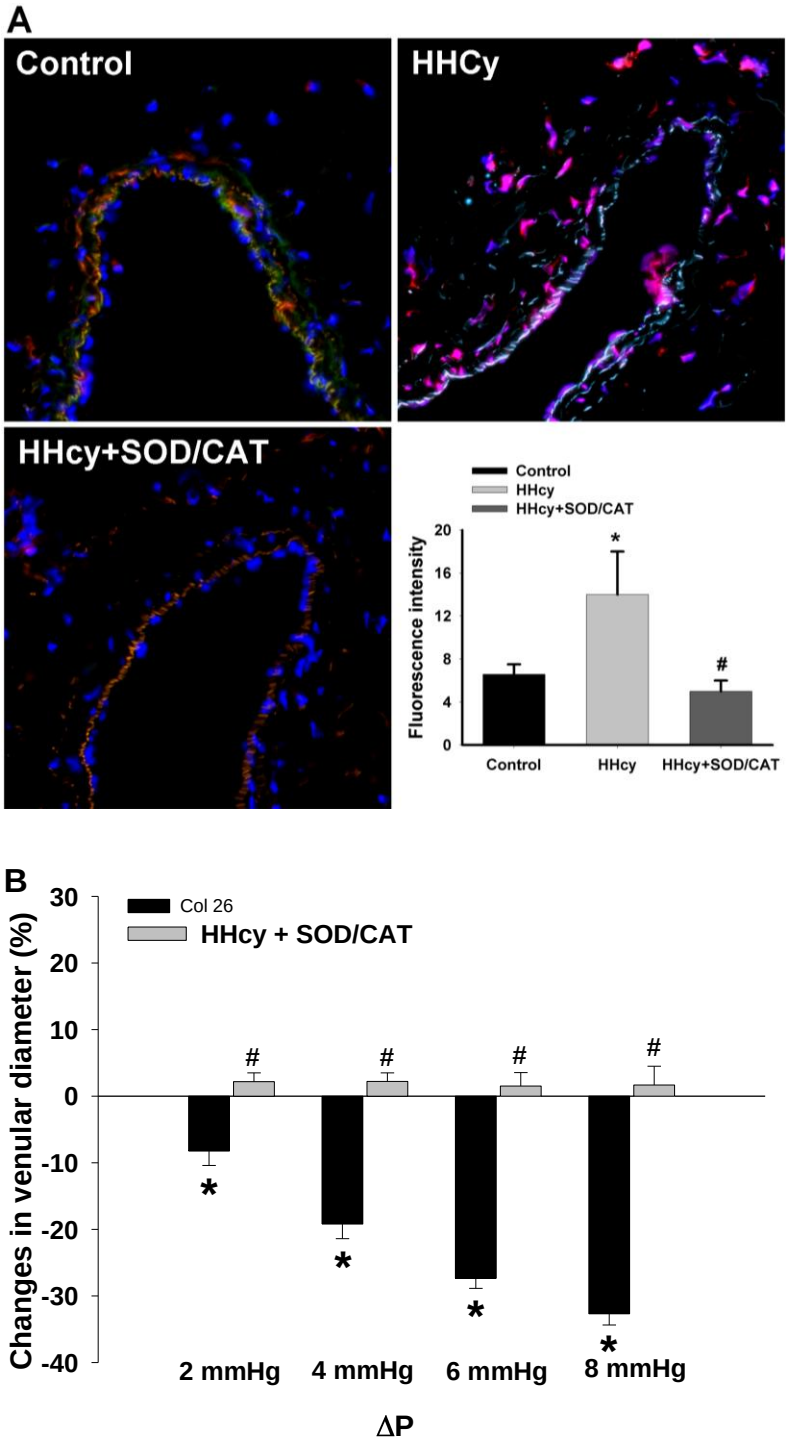


**Figure 20 A,** DHE-staining overlayed with DAPI-staining in saphenous veins in control (top, left), HHcy (top, right) and HHcy plus SOD plus CAT conditions (bottom, left). Blue stains: DAPI-stained nuclei, purple color: ethidium-bromide stained nuclei overlayed with DAPI, red color: ethidium-bromide staining other than nuclei. Summary data showing fluorescence intensity in control, HHcy and HHcy plus SOD/CAT conditions (bottom, right, *P<0.05 vs. control; #P<0.05 vs. HHcy. **B**, Changes in diameter of skeletal muscle venules of HHcy rats in response to increases in flow in the presence or absence of SOD/CAT (n=7). Data are mean ± SEM *P<0.05 vs control, #P < 0.05 vs. HHcy venules

In previous studies in arterioles isolated from HHcy rats the increased level of ROS and their role in the adverse vascular effect of HHcy has been already demonstrated.[211] In vivo studies of humans and animals also supported a role of oxidative stress in the development of vascular dysfunction in HHcy, because oral administration of the antioxidant ascorbic acid prevented HHcy-induced endothelial dysfunction in both conduit and resistance vessels.[270, 271] Interestingly, homocysteine can elicit generation of oxygen free radicals either via autooxidation of the sulfhydryl group[272] or by decreasing the antioxidant mechanisms, such as glutathion-peroxidase or SOD.[273] On the basis of these findings and the above mentioned studies we suggest that oxidative stress plays an important role in the altered flow-induced responses in HHcy.

Correspondingly, we have found that in saphenous veins isolated from HHcy rats there was an increased oxidative stress as indicated by the elevated number of EB staining in the wall of vessels compaired to controls (**Figure 20**) and scavengers of ROS (SOD/CAT) abolished flow-induced constriction in HHcy venules (**Figure 20**).

Previous studies have shown that ROS, such as superoxide anion and $H_2O_2$ elicit constrictions of isolated venules[274] primarily by activating $TXA_2$ receptors. Thus, it is possible that in venules isolated from HHcy rats – in addition to $TxA_2$ - ROS also contribute to the development of flow-induced constrictions, either directly via facilitation of constrictor prostanoid production or via decreasing the bioavailability of NO (**Figure 21**). The potential sources of ROS can be the upregulation of renin angiotensin system[275, 276] or arachidonic acid metabolism via cyclooxygenase, lipoxygenase[277] and $PGH_2$ synthase.[278]

The pathophysiological and clinical importance of our findings is that, in addition to changes in coagulation and anticoagulation systems,[279, 280] shown previously, HHcy alters the function of arteriolar and venular endothelium. Intact function of endothelium is important both in the regulation of vasomotor tone and rheological properties of blood. In HHcy, the increased release of $TxA_2$ and ROS may significantly alter the regulation of the resistance of small veins and venules. It is known that during physical activity, such as exercise venular blood flow increases substantially, which in normal conditions would increase the diameter of venular vessels[281] allowing the increase in venular and venous blood flow. However, in HHcy

the increased release of $TxA_2$ and ROS to flow could increase the resistance of venular circulation, hence reduce venous return and may promote platelet aggregations as well.[282]

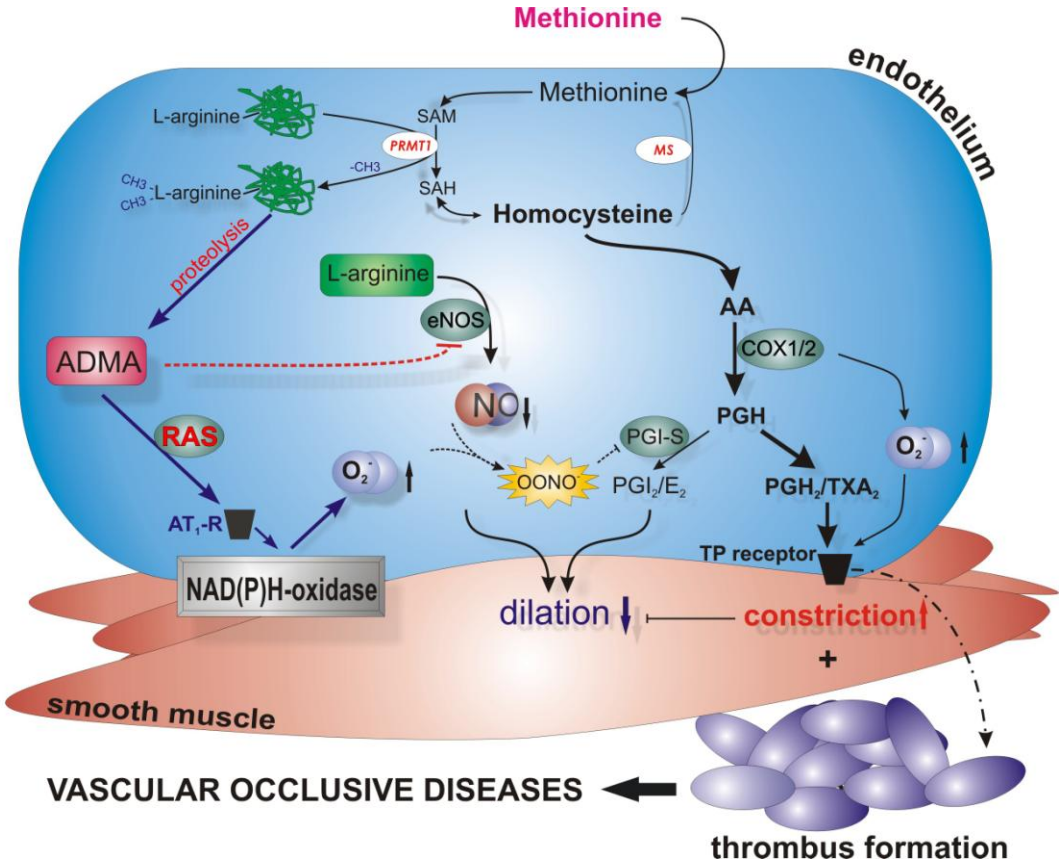


**Figure 21.** Proposed intracellular mechanisms responsible for flow/shear stress-induced vasomotor responses in venules isolated from rats with hyperhomocysteinemia (HHcy) and elevated level of ADMA. In control venules, dilator nitric oxide, prostaglandin $E_2/I_2$ ($PGI_2$ and $PGE_2$) and constrictor prostaglandins ($PGH_2/TxA_2$) are produced, eliciting dilation. In HHcy production of $PGH_2/TxA_2$, overcoming the dilator effects of prostaglandin $I_2/E_2$ and nitric oxide, resulting in constrictions. Also, an elevated production of reactive oxygen species likely due to the upregulation of arachidonic acid metabolism and ADMA-angiotensin II-NAD(P)H oxidase- superoxide pathways may contribute to flow-induced constrictions via activating the $PGH_2/TXA_2$ pathway and TP receptors and via reducing the bioavailability of NO by producing peroxynitrite ($ONOO^-$). Peroxynitrite has also been shown to inhibit $PGI_2$ synthase further facilitating the production of $TXA_2$. Increased levels of $TxA_2$ and reactive oxygen species increase the resistance of venular circulation and favor the development of platelet aggregation and thrombus formation, all of which could result in the development of occlusive vascular diseases in hyperhomocysteinemia.

## 6. CONCLUSIONS

Our findings demonstrated that elevated levels of ADMA reduced NO donor- and flow-induced dilations and basal tone of arterioles, all of which were restored by scavengers of reactive oxygen species, inhibitors of the microvascular renin angiotensin system, or NAD(P)H oxidase (but not by L-arginine). These findings suggest that ADMA activates the renin-angiotensin system in the arteriolar wall, leading to increased production of angiotensin II, which then elicits oxidative stress by activating the NAD(P)H oxidase resulting in reduced bioavailability of NO. Elevated levels of reactive oxygen species and microvascular dysfunction are key factors in the development of cardiovascular diseases, such as hypertension, atherothrombosis, peripheral vascular diseases and hyperhomocysteinaemia.[283] Thus, understanding the mechanisms responsible for the reduced availability of NO and increased production of superoxide in the presence of elevated levels of ADMA, shown to be present many of these diseases has the potential to identify novel therapeutic targets and modalities aiming to improve the regulation of endothelial function by local mechanisms. We have found that endothelial regulation of venular tone is substantially altered in HHcy (known to be associated with elevated levels of ADMA), which is due to the increased production of thromboxane $A_2$ and elevated levels of reactive oxygen species. These factors can increase the resistance of venular blood circulation and at the same time could contribute to increased platelet aggregation and thrombus formation, all of which favoring the development of occlusive vascular diseases.

In future studies decreasing the level of methylated L-arginines could be therapeutically targeted by enzymes regulating ADMA levels, such as protein arginine methyltransferase inhibitors or dimethylarginine dimethylaminohydrolase gene transfer.[89] and by use of more specific NAD(P)H oxidase inhibitor. Whereas at present, our findings provide a mechanistic base for the clinical use of inhibitors of the tissue renin-angiotensin system at various levels, which may prevent or delay the development of microvascular diseases due to increased cellular production of ADMA present in pathophysiological conditions.

## 7. SUMMARY

Nitric oxide (NO) is an important mediator in the regulation of peripheral resistance in microvessels and thus tissue blood flow. Because NO is a very labile molecule it can react with reactive oxygen species (ROS) that are also act as signaling molecules modulating vascular tone, development and progression of cardiovascular diseases. NO is synthesized from L-arginine by a family of NO synthases (NOS). NOS can be stimulated by administration of its substrate L-arginine resulting in arteriolar dilation. Methylated forms of L-arginine, such as G-monomethyl- L-arginine (L-NMMA) or $N^{\omega}$-nitro-L-arginine-methyl-ester (L-NAME) has been shown to inhibit NO synthase with the consequent elimination of NO mediated dilations of vessels. In several human diseases, such as hyperhomocysteinemia (HHcy), hypertension or preeclampsia, there is an increased production of methylated L-arginines, such as asymmetric dymethylarginine (ADMA). Up to now, ADMA was thought to be an endogenous inhibitor of NOS, thereby affecting arteriolar tone. However, recent studies have shown that ADMA may have other effects, such as eliciting production of ROS.

Thus, we hypothesized, that ADMA by activating the vascular renin angiotensin system, especially angiotensine type 1 receptors ($AT_1R$), upregulates the function of NAD(P)H oxidase, which then leads to increased superoxide generation interfering with NO mediation of arteriolar dilations.

In order to test this hypothesis we have investigated the effects of exogenously administered ADMA on the vasomotor function of isolated rat skeletal muscle arterioles.

We have found that (in the presence of indomethacin), exogenous ADMA elicited significant constrictions of arterioles from rat gracilis muscle and eliminated the dilations to increases in intraluminal flow. In the presence of ADMA, superoxide dismutase (SOD) plus catalase (CAT) restored dilations to flow. Incubation of arterioles with the NAD(P)H oxidase inhibitor apocynin or the angiotensin-converting enzyme (ACE) inhibitor quinapril inhibited ADMA-induced constrictions. In addition, apocynin, quinapril or the $AT_1R$ blocker losartan restored flow-induced dilations reduced by ADMA, but the xanthin oxidase inhibitor oxypurinol or L-

arginine did not improve the dilations to increases in intraluminal flow. ADMA-induced increased production of superoxide - assessed by dihydroethidium fluorescence - was inhibited by apocynin, quinapril or losartan. We have also found that ADMA significantly reduced dilations to the NO donor, sodium nitroprusside (SNP), which was partially restored by superoxide-dismutase. Furthermore, the ADMA-enhanced lucigenin chemiluminescence was normalized by SNP and $AT_1R$ blocker.

Collectively, these findings suggests that elevated level of ADMA activates the renin-angiotensin system in the arteriolar wall, and via angiotensin II activates NAD(P)H oxidase, which then increases ROS production. Elevated level of ROS interferes with the bioavailability of NO resulting in diminished NO mediated dilations. These alterations may also contribute to increased tone in diseases associated with elevated levels of ADMA, such as during altered homocysteine metabolism, activating arachidonic cascade and prostaglandin synthesis as well, leading together to the development of microvascular vasomotor dysfunction.

## 8. ÖSSZEFOGLALÁS

A szöveti vérkeringés és a mikroerek perifériás rezisztenciájának egyik kulcsfontosságú mediátora a nitrogén-monoxid (NO). A reaktív oxigén gyökök (ROS) a NO-dal reakcióba lépve csökkentik annak biológiai hozzáférhetőségét, ezáltal részt vesznek az erek tónusának szabályozásában, valamint a kardiovaszkuláris betegségek kialakulásában és progressziójában is lényeges tényezők. Az NO szintézisét L-arginin aminosavból az ún. nitrogén-monoxid szintáz (NOS) enzim végzi. Az L-arginin szintetikusan előállított metilszármazékai, mint az NG-monometil-L-arginin (L-NMMA) vagy az Nω-nitro-L-arginin-metil-észter (L-NAME) gátolják a működését, ezáltal befolyásolják a NO-mediált vazodilatációt. Ismert, hogy az emberi szervezetben is keletkeznek metilált L-argininek, pl. az aszimmetrikus dimetil-L-arginin (ADMA), amelynek a szintje kóros körülmények között, mint például hiperhomociszteinémiában (HHcy), hipertóniában vagy praeeclampsiában megemelkedik. Az ADMA vélhetően NO-szintáz gátlásán keresztül szabályozza az arteriolák tónusát. Legutóbbi tanulmányok viszont azt mutatták ki, hogy az ADMA nem csak az NO szintézisét csökkenti, hanem szuperoxid termelést is indukál.

Feltételezésünk az volt, hogy az ADMA aktiválja a szöveti renin-angiotenzin rendszert, főként az angiotenzin 1 receptorokat ($AT_1R$), amely a NAD(P)H oxidáz funkcióját fokozza, ezáltal oxidatív stresszt okoz, amely rontja az NO-függő dilatációt, melynek eredményeképpen vázizom arteriolákon vazomotor diszfunkció jön létre. Indomethacin jelenlétében patkány gracilis izomból izolált arteriolákat ADMA-val ($10^{-4}$ mol/L) inkubáltuk, ami szignifikáns vazokonstrikciót váltott ki és megszüntette a megnövelt intraluminális áramlás okozta dilatációt. ADMA jelenlétében a szuperoxid-dizmutáz (SOD) + kataláz visszaállította az áramlás-indukálta dilatációt. Endotél denudáció illetve az arteriolák inkubációja NAD(P)H oxidáz inhibitor apocyninben vagy az angiotenzin-konvertáló enzim gátló quinaprilban megszüntette az ADMA-kiváltotta konstrikciót, a xantin oxidáz gátló oxypurinol viszont hatástalan volt. Ezek mellett az apocynin, a quinapril vagy az angiotenzin 1 receptor blokkoló losartan visszaállította az áramlás-indukálta dilatációt, amit az ADMA csökkentett, míg az oxypurinol és L-arginin adása nem javított azon. Az ADMA-indukálta megnövekedett szuperoxid termelés, ami

dihydroethidium fluoreszcenciával követhető apocyninnel, quinaprillal illetve losartannal gátolható. ADMA jelenlétében szignifikánsan csökkent dilatációt találtunk az NO donor nátrium nitroprusszid (SNP) hatására, melyet a SOD/CAT jelentősen javított. ADMA fokozta az erek chemilumineszcenciáját, amit SNP és angiotenzin 1 receptor blokkoló meggátolt.

Ezek az adatok arra utalnak, hogy az ADMA aktiválja a helyi renin-angiotenzin-rendszert és az angiotenzin II aktiválja a NAD(P)H oxidázt, az általa temelődő szuperoxidok gátolják az NO biológiai hozzáférhetőségét ezáltal csökkentve az NO-függő dilatációt. Ezek a mechanizmusok hozzájárulhatnak a megnövekedett tónus kialakulásához az emelkedett ADMA-szintekhez társuló betegségekben; így például homocisztein anyagcsere-betegségben az aktivált arachidonsav kaszkáddal és prosztaglandin szintézis fokozódásával együtt szerepet játszhat a mikrovaszkuláris vazomotor diszfunkció létrejöttében, ezáltal elősegítve az okklúziós vaszkuláris betegségek kifejlődését.

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**ABSTRACTS**

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