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. Thesis

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INTRODUCTION

Nitric oxide (NO) is one of the most important mediators in the regulation of peripheral resistance 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 and 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 N^{ω}-nitro-L-arginine (L-NNA), NG-monomethyl- L-arginine (L-NMMA) or N^{ω}-nitro-L-arginine-methyl-ester (L-NAME) have been shown to inhibit NOS with the consequent elimination of NO-mediated dilations of vessels. In several human diseases, such as hyperhomocysteinemia (HHcy), diabetes mellitus, hypertension and praeeclampsia there is an increased serum level of methylated L-arginines, such as asymmetric dymethylarginine (ADMA). Results from animal experiments suggest that ADMA represents not only a risk marker but also a risk factor for cardiovascular events. Up to now, ADMA was thought to be an endogenous inhibitor of NOS, thereby affecting arteriolar tone. However, recent studies have shown that exogenous ADMA may have other effects, such as eliciting production of ROS.

It has been established that angiotensin II (Ang II) plays an important role in the activation of the vascular NAD(P)H oxidases and, thus superoxide production, whereas recent studies have also shown that exogenous ADMA elicits superoxide generation. Also, Agn 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.

Biochemically, homocysteine and ADMA are linked via several ways. It was found that higher ADMA, but not homocysteine, levels were associated with cardiovascular disease. 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.

On the basis of the aforementioned 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.

SPECIFIC AIMS OF THE STUDY

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

MATERIALS AND METHODS

Experiments were conducted on isolated gracilis muscle arterioles (inside active diameter: 153±4 μm and passive diameter 235±3 μm, at 80 mmHg) of Male Wistar rats (weight 300-350 g). The inner diameter of arterioles was measured by videomicroscopy equipped with a microangiometer and recorded on a chart recorder, or using analogue-digital converter Powerlab.

To test the function of arterioles the endothelium-dependent dilator agent acetylcholine and the endothelium-independent dilator agent sodium nitroprusside were used. To exclude the potential contribution of prostaglandins, experiments were performed in the presence of indomethacin, inhibitor of cyclooxygenases. 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 NAD(P)H oxidase inhibitor apocynin, xanthine oxidase inhibitor oxypurinol, ACE inhibitor quinapril or in the absence of the endothelium. In the presence of ADMA L-arginine, or the free-radical scavengers superoxide dismutas and catalase (SOD/CAT), or apocynin or oxypurinol or quinapril or the angiotensin 1 receptor blocker (ARB) losartan was administered and flow-induced responses were obtained. In another series of experiments in the presence of ADMA vasomotor responses were assessed to increasing concentrations of acetylcholine and the NO donor sodium nitroprusside in the presence or absence of SOD/CAT.

Vascular $O_2^{\cdot-}$ production was assessed in isolated femoral artery samples by the lucigenin chemiluminescence (CL) method, whereas hydroethidine fluorescense was used to localize $O_2^{\cdot-}$ production.

RESULTS

Basal arteriolar tone and agonist induced responses vs. ADMA

ADMA elicited a significant decrease in the basal diameter of isolated gracilis muscle arterioles. Compared to control, the basal arteriolar diameters were significantly reduced in the presence of ADMA. The presence of apocynin, quinapril or endothelium removal abolished the constrictor effect of ADMA on the basal diameter. Previous incubation with oxypurinol did not eliminate the constrictor effect of ADMA on basal diameter. Dilations to increasing concentrations of acetylcholine were not affected by 10^{-4} M ADMA, whereas ADMA significantly reduced dilations to the NO donor sodium nitroprusside, which were improved by administration of SOD/CAT.

Flow-induced responses vs. ADMA

In control conditions step increases in intraluminal flow (5, 10, 15, and 20 μL/min) elicited substantial dilations of arterioles (maximum 28 ± 2%). After returning flow to zero, the diameter of arteriole returned to the control level. However, in the presence of ADMA step increases in flow did not elicit dilations (maximum from 31 ± 2% to 3 ± 1%). Flow-induced dilations were restored to the control level by the presence of SOD/CAT, whereas presence of L-arginine and ADMA did not have significant effect. Presence of apocynin restored dilations to increases in flow in ADMA-treated arterioles (maximum from 4 ± 1% to 25 ± 3%). We have also found that the presence of oxypurinol did not restore flow-induced dilations in the presence of ADMA. In contrast, presence of quinapril or losartan also restored flow-induced dilations to the control level (maximum 32 ± 2% and 23±2%, respectively).

Assessment of Vascular Superoxide Production in the presence of ADMA

Measurements of EB fluorescence in control and ADMA- incubated arterial sections indicated an increased EB fluorescence in ADMA- incubated vessels as compared to the control. Simultaneous incubation of ADMA with apocynin, quinapril, or losartan decreased the EB fluorescence in the arterial wall, close to the control levels.

To further assess and localize the presence of superoxide anion, lucigenin enhanced chemiluminescence (CL) of femoral arterial section was studied. ADMA elicited an enhanced lucigenin chemiluminescence in the arterial wall, which was significantly inhibited by pre-incubation of the vessels with the NO donor, SNP or an angiotensin type 1 receptor blocker.

DISCUSSION

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

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

2) ADMA inhibited flow-induced dilations, which were not restored by L-arginine or xanthine oxidase inhibitor, oxypurinol, but were restored by scavengers 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) ADMA elicited vascular oxidative stress, 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.

By now, it seems to be well established that elevated plasma levels of ADMA is associated with cardiovascular diseases in humans and experimental animals. In several human diseases, such as hypertension, diabetes and hyperhomocysteinaemia, the serum levels of methylated L-arginines, such as ADMA are increased. On the basis of previous studies (primarily biochemical, in the absence of cells, other enzymes or proteins) two mechanisms of action of ADMA have been proposed: 1) ADMA inhibits eNOS thus release of NO and 2) in the presence of ADMA eNOS became "uncoupled" due to the absence of appropriate level of the eNOS substrate L-arginine resulting in production of superoxide rather than NO.

Interestingly, however, in living tissue experiments, ADMA seems to have effects that are unrelated to the activity of eNOS. For example, ADMA induced coronary microvascular lesions in wild-type and eNOS-KO mice, which obviously could not be related to NO or uncoupled eNOS especially, because they were not antagonized by administration of L-arginine. In addition, ADMA increased superoxide production in monocytes, epithelial, endothelial and even in cardiac cells.

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, diabetes, or hyperhomocysteinaemia. Interestingly, in these human diseases, the serum levels of methylated L-arginines, such as ADMA are increased. 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 likely due to activation of NAD(P)H oxidase. Because angiotensin II is a known activator of

NADP(H) oxidase the potential role of renin-angiotensin system (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.

First, we confirmed that ADMA elicits significant constriction of arterioles.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. We have found that, in the presence of ADMA, a NAD(P)H oxidase inhibitor apocynin restored the basal diameter of arterioles as well. 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 oxypurinol. Collectively these findings suggest that the decrease in diameter of arterioles was due to increased levels of superoxide interfering with NO and that ROS is produced by NAD(P)H oxidase, rather than xanthin oxidase. Previous studies have provided evidence that L-arginine is the precursor of the formation of NO and supplementation of L-arginine optimizes the formation of NO and that in case of eNOS “uncoupling” the excess formation of superoxide by NOS can be prevented by L-arginine. However, in the present experiments L-arginine did not restore flow-induced dilations in the ADMA-treated arterioles.

To further test the hypothesis that ADMA act via superoxide we have used the NO donor, SNP to elicit dilations of isolated gracilis arterioles. We have found that ADMA significantly reduced the NO donor, SNP-induced arteriolar dilations, which were restored by SOD/CAT, suggesting that increased level of ROS in the presence of ADMA interfered with the NO released from SNP is responsible for the reduced dilations.

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. Also, previous studies proposed a potential interaction between ADMA and the RAS. Thus, we hypothesized that the arteriolar RAS is involved in the ADMA-induced oxidative stress. Indeed, we have found that the ACE inhibitor quinapril and AT_1R blocker losartan restored flow-induced dilations in arterioles in the presence of ADMA and quinapril also inhibited a reduction of diameter by ADMA. In addition, we have also found that ADMA increased DHE fluorescence, 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 which was inhibited by the prior incubation with the NO donor, SNP or the AT_1R blocker, telmisartan. Collectively, we suggest that ADMA, via as yet unknown mechanism(s), activates the

microvascular RAS, which leads to an increased level of angiotensin II in the microvascular wall, and AT_1 receptors are involved in the ADMA-angiotensin II pathway producing reactive oxygen species.

In the present study 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.

Previous studies showed important links between ADMA and homocystein metabolism and vascular actions. Like many other cardiovascular risk factors, hyperhomocysteinemia produces endothelial dysfunction due to impaired bioavailability of NO. The molecular mechanisms responsible for decreased NO bioavailability in hyperhomocysteinemia 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. Indeed, 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 addition to revealing several pathophysiological mechanisms related to the elevated levels of ADMA, our findings provide a theoretical base for the clinical use of antioxidants, inhibitors of renin-angiotensin-aldosterone system, possibly novel, more specific NAD(P)H oxidase inhibitors in the presence of elevated levels of ADMA present in various disease conditions.

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