Reversible Inhibition Monoamine Oxidase - A Improves Vascular Dysfunction in Canine Carotid Arteries Exposed to Angiotensin II

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Monoamine oxidase (MAO) is a flavinic enzyme, with two isoforms (A and B), located at the outer mitochondrial membrane that constantly generate hydrogen peroxide (H_2O_2) as by-product of their catalytic cycle. We investigated the role of monoamine oxidase (MAO) in development of endothelial dysfunction in isolated dog carotid arteries after in vitro exposure to angiotensin 2 (Ang II). Canine carotid artery segments were incubated with Ang II in the presence or absence of moclobemide (a reversible MAO-A inhibitor) or catalase. Subsequently, the fragments were used for organ bath studies of vascular reactivity and for the hydrogen peroxide measurements. Pretreatment of vascular rings revealed that incubation with Ang elicited endothelial dysfunction via H_2O_2 production whereas co-treatment with moclobemide reduced the H_2O_2 levels and improved the vascular function in Ang II-treated vessels; in presence of L-NAME the effect of moclobemide was absent. In conclusion, MAO is an important source of ROS production, activated in response to Ang II and treatment with MAO inhibitors represents a possibility to reverse this negative effect.

Keywords: Monoamine oxidase, angiotensin 2, endothelial dysfunction, oxidative stress, moclobemide

The endothelium is the main target of cardiovascular risk factors, being mostly involved in the progression of vascular inflammation and atherosclerosis. Although low levels of reactive oxygen species (ROS) play a key role as signaling molecules, elevated levels of ROS are major contributors to the pathophysiology of cardiovascular dysfunction. The enzymatic systems currently considered responsible for the generation of reactive oxygen species (ROS) in the development of endothelial dysfunction via limiting the bioavailability of nitric oxide (NO) are: NADPH oxidases, xanthine oxidase, mitochondrial respiratory chain, cyclooxygenase, lipooxygenase and uncoupled endothelial NO synthase (eNOS) [1-6]. In the past decade novel mitochondrial enzymes have been characterized as unequivocal sources of oxidative stress. Monoamine oxidases (MAOs) are flavoenzymes, with two isoforms, MAO-A and MAO-B, located at the outer mitochondrial membrane that constantly generate H_2O_2 as by-product of their catalytic cycle [7-9]. The primary physiological role of MAO is the degradation of endogenous monoamine neurotransmitters and exogenous amines. In peripheral tissues, MAO is involved in oxidative catabolism of amines and prevention of the ingress of dietary amines (such as tyramine from fermented cheese) in circulation [10].

MAOs have been extensively studied for their involvement in pathogenesis of neurodegenerative and psychiatric diseases [7, 11]. In this regard, a wide range of MAO inhibitors (MAOIs) are available and proven to have therapeutic value in several pathologies, including affective disorders, neurodegenerative diseases, stroke and senescence [7]. MAOIs are classified into 3 categories: i) non-selective irreversible inhibitors such as phenelzine and tranylcypromine; ii) irreversible and selective inhibitors such as selegiline for MAO-B and clorgyline for MAO-A; iii) reversible and selective inhibitors such as moclobemide for MAO-A and lazabemide for MAO-B. Despite the fact that MAO inhibitors are not the most widely prescribed antidepressants, they still represent an important therapeutic option in psychiatric and neurological diseases [10] being also recognized in recent years for their neuroprotective properties [12]. In this regard, moclobemide (a reversible inhibitor of MAO-A) was found to have anti-parkinsonian activity and neuroprotective effects in a model of cerebral ischemia via MAO-A inhibition and also independent of this effect [7, 10].

In the past decade, MAO has been reported to contribute to the ischemia-reperfusion injuries in kidney [13] and heart [14], and in maladaptive cardiomyocyte hypertrophy and its evolution to heart failure [15]. However, less information is available in the literature with regard to the effect of MAO in vasculature. Accordingly, one study reported a significant reduction in contractility of the basilar arteries harvested from the hypertensive by the treatment with MAO-A inhibitors [16]. In another study the treatment with angiotensin II converting enzyme inhibitors reduced MAO activity, suggesting that hyperactivation of renin-angiotensin-aldosterone system will potentiate the enzyme activity [17]. We previously reported an increased expression of both MAO isoforms in two murine models associated with vascular inflammation and hypertension responsible for subsequent endothelial dysfunction that was partially reversed by MAO inhibitors [18].

In the present study we aimed at assessing the effect of angiotensin II exposure on MAO in canine carotid vascular segments. We hypothesized that *in vitro* treatment with Ang II will impair vascular relaxation in a MAO-dependent

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manner and that MAO inhibition will improve the vascular outcome, respectively.

Experimental part

Materials and methods

Canine vascular segments were obtained from adult mongrel dogs of either sex weighing 8-16 kg from the Department of Pharmacology and Pharmacotherapy, University of Szeged, Hungary within the framework of a collaborative research mobility. All the experimental procedures were conducted in accordance with the Directive 2010/63/EU regarding the protection of animals used for scientific purposes. The protocols were approved by the review board of the Department of Animal Health and Food Control of the Ministry of Agriculture and Rural Development, Hungary (XII./01031/000/2008 and XIII./1211/2012).

Organ Culture

Carotid artery segments were dissected under sterile conditions, cleaned, and incubated for the times indicated at 37°C in EBM culture medium containing 0.1% BSA, in the presence or absence of angiotensin II (100 nmol/L) with or without moclobemide (10 microM, Sigma-Aldrich) or PEG-Catalase (100 U/mL, Sigma-Aldrich). Subsequently, the tissue was used for organ bath experiments and for ROS measurement, respectively.

Studies of Vascular Function

Vascular function of carotid artery rings was studied in the presence of diclofenac (10 μmol/L). The concentration of phenylephrine, used for preconstriction, was adjusted to obtain an identical preconstriction level of 80% of the contraction elicited by KCl (80 mmol/L). Endothelium-dependent relaxation to cumulatively increasing concentrations of acetylcholine was recorded.

Measurements of Reactive Oxygen Species

Hydrogen peroxide production was measured in carotid segments by the Ferric iron xylenol orange oxidation method (FOX Assay, Sigma Aldrich) as previously reported [18]. The principle of the assay is that peroxides oxidize Fe^{2+} to Fe^{3+} ions at acidic *p*H. The Fe^{3+} ion will form a coloured adduct with xylenol orange (XO, 3,33' -carboxy-methyl)aminomethyl]-o-cresolsulfonephthalein, sodium salt), which is observed at (XO, 3,33' -bis[N,Nbis (carboxy-methyl)aminomethyl]-o-cresolsulfonephthalein, sodium salt), which is observed at 560 nm.

Statistics

All values are expressed as mean ± SEM and were analysed using one-way ANOVA and Tukey's post hoc analysis. Relaxations were calculated from individual dose-response curves. Data analysis of the dose-effect response curves was performed using the ANOVA F-test (comparisons of bottom and top values, EC50 and the Hill slope). Values of $p<0.05$ were considered statistically significant.

Results and discussions

In vitro treatment of carotid arteries with Ang II elicited H_2O_2–mediated endothelial dysfunction

Incubation of vascular rings with Ang II is a classical method to induce *in vitro* endothelial dysfunction. There is unanimous consensus in the literature regarding the major role of superoxide anion ($O_2^{\cdot-}$) in mediating endothelial dysfunction. In the present study we questioned the contribution of H_2O_2 production after Ang II stimulation to the vascular dysfunction of canine carotid rings. To this aim we incubated the vascular preparations for 24 h with Ang II (100 nmol/L) in the presence vs. the absence of the H_2O_2 scavenger catalase (100 U/ml). The vascular contractility in response to phenylephrine after Ang II stimulation was significantly increased as compared to the non-treated rings and this effect was reduced by the presence of catalase (fig. 1A). Also, after Ang II treatment the endothelium-dependent relaxation was significantly impaired. This effect was partially attenuated by co-incubation with catalase (fig. 1B). Finally, we evaluated by means of the FOX assay, the H_2O_2 production in vessels treated with Ang II *vs.* control in the presence or absence of catalase. The results showed a significant increase in H_2O_2 production, effect that was partially attenuated in the presence of catalase (fig.1C). Thus we confirmed the contribution of H_2O_2 to the Ang II-induced endothelial dysfunction in isolated carotid rings.

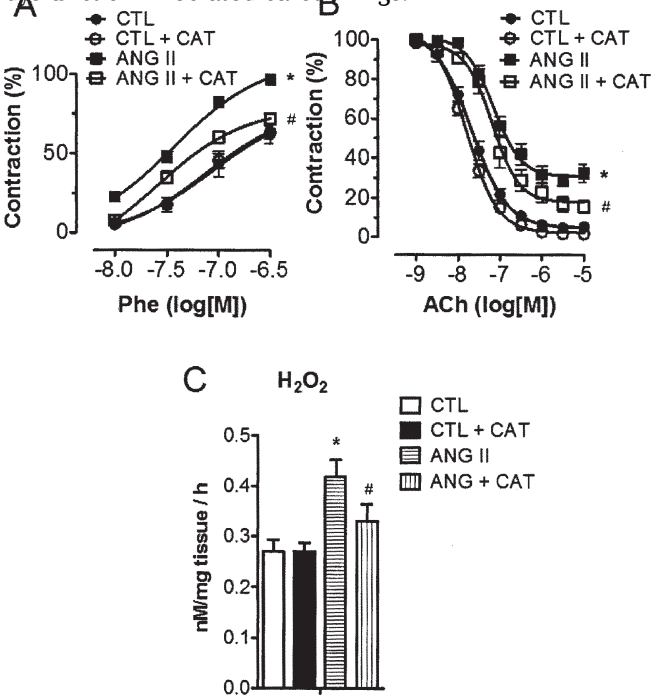

Fig. 1. Role of H_2O_2 in functional vascular impairment induced by *in vitro* treatment with Ang II. A) Phenylephrine-induced contractions, n=6, *p<0.05 with and without Ang II treatment, # p<0.05 with and without catalase (PEG-Catalase, 100 U/mL). B) Acetylcholine-induced endothelium-dependent relaxation. n=6, *p<0.05 with and without Ang II treatment, #p<0.05 with and without Catalase. C) H_2O_2 formation by FOX assay in the presence vs. absence of PEG-catalase (100 U/mL), n=6, *p<0.05 with and without Ang II treatment, #p<0.05 with and without catalase

Co-treatment with MAO inhibitor reduced the H_2O_2 production and improved vascular function in Ang II-treated preparations

MAOs are constant sources of mitochondrial H_2O_2 production. Whether MAO-derived H_2O_2 contributes to the endothelial dysfunction induced by Ang II in canine has not been previously investigated. We treated carotid arteries with Ang II in the presence *vs.* the absence of moclobemide, the reversible MAO-A inhibitor. Ang II induced a strong attenuation of the endothelium-dependent relaxation in response to acetylcholine. Co-incubation with MAO-A inhibitor restored partially the relaxation response (fig. 2A) and the level of H_2O_2 (fig. 2B).

Inhibition of eNOS by L-NAME prevented the beneficial effects of MAO inhibition

In order to investigate the mechanism underlying the MAO-induced endothelial dysfunction after Ang II

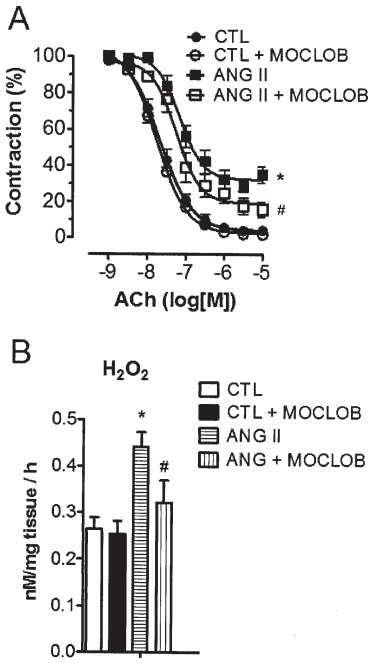

Fig. 2. Role of MAO-derived H_2O_2 in Ang II-induced endothelial dysfunction and the effect of MAO-A inhibition. A) Acetylcholine-induced endothelium-dependent relaxation. n=6, *p<0.05 with and without Ang II treatment, #p<0.05 with and without moclobemide (10 μM). B) H_2O_2 formation by FOX assay in the presence of absence of the moclobemide (10 μM), n=6, *p<0.05 with and without Ang II treatment, #p<0.05 with and without moclobemide

stimulation, we assessed the effect of endothelial NO synthase (eNOS) inhibition with L-NAME in vessels treated with Ang II with and without moclobemide. Interestingly, in Ang II-treated carotid arteries, after eNOS inhibition, moclobemide had no further beneficial effects on the endothelial-dependent relaxation (fig. 3). These results suggest that one of the pathways affected by MAO derived H_2O_2 includes eNOS and NO signalling. If eNOS is suppressed MAOi fail to improve the vascular response.

Results and discussions

In this study we were focused on the role of MAO in development of endothelial dysfunction in experimental setting using Ang II as promotor of the vascular impairment.

We have previously demonstrated in the murine model that Ang II elicited a significant increase in the amount of H_2O_2 generated in isolated vascular preparations [19]. We confirm that this is also the case in the canine model of in vitro induced endothelial dysfunction as determined by the FOX assay. We also report that at least partially H_2O_2 is generated via the MAO-A isoform. In fact, Ang II elicited a 2 times-increase in the H_2O_2 production in aortic rings whereas incubation (30 min) with moclobemide, the MAO-A inhibitor, reduced by 50% the amount of this ROS.

The level of H_2O_2 generated *via* this mitochondrial enzyme induced a serious alteration of the vascular relaxation in Ang II-treated vascular segments. This effect was partial, but significantly improved in the presence of moclobemide.

Another important finding of this study is that, in control vessels, MAO inhibition did not interfere with H_2O_2 production and vascular reactivity, suggesting that in basal conditions MAO activity is reduced and the amount of ROS produced is low and thus unable to induce endothelial dysfunction.

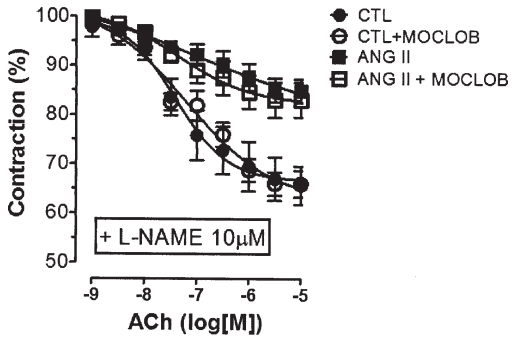

Fig. 3. Effect of eNOS inhibition on Moclobemide (MAO-A inhibitor) effect in Ang II-stimulated dog carotid arteries. Carotid segments from dogs were treated in vitro 24 h with Ang II (organ culture, 100 nM) in the presence *vs.* absence of Moclobemide (reversible MAO-A inhibitor) +/- eNOS inhibitor (L-NAME, 10 μM). Acetylcholine-induced endothelium-dependent relaxation, n=6.

Increased production of reactive oxygen species is associated to different type of pathologies such as hypertension, atherosclerosis, diabetes [20, 21]. ROS-induced endothelial dysfunction is a well established pathomechanism but currently this phenomenon was attributed to the increased formation of the superoxide anion - responsible for scavenging NO with the generation of peroxynitrite and subsequent eNOS uncoupling [22], and not to the generation of hydrogen peroxide. However, *since hydrogen peroxide* is freely diffusible through *cell* membranes, it can further activate inflammation, induce apoptosis and promote endothelial dysfunction [24]. We hereby demonstrated that H_2O_2 contributes to acute experimental endothelial dysfunction dogs as it did in rats.

The role of MAOs in terminating the action of neurotransmitters in the central and peripheral nervous system and in the oxidation of dietary amines in extraneuronal tissues have been extensively studied [7]. Thus, it has been shown that *in vitro* or *in vivo* application of L-deprenyl, an irreversible MAO-B inhibitor, produced vasodilatation via an increase in the amount of NO in brain tissue and cerebral blood vessels [24]. NO inhibits mitochondrial monoamine oxidase activity and decreases outer mitochondria membrane fluidity, yet, the mechanism by which NO inhibits MAO remains unknown at present [25, 26].

MAO inhibitors are currently used in psychiatry and neurology for the treatment of anxiety and depressive disorders, Parkinson's and Alzheimer's diseases, respectively [7]. Therefore, they can be easily tested in the settings of cardiovascular diseases, especially since there is a growing consensus that they are increasingly associated with depression. MAO inhibition was already used with good results in the late 60s – early 70s in the treatment of angina pectoris [27, 28]. Therefore, further studies addressing the potential role of MAO inhibitors in reversing an impaired vascular dysfunction are clearly warranted.

Conclusions

We have here demonstrated that monoamine oxidase A is a relevant source of ROS production, activated in response to Ang II and that MAO-derived H_2O_2 impairs endothelial function most probably via an NO mediated mechanism. Treatment with a MAO-A reversible inhibitor which is in current use for other pathologies partially reversed this negative effect.

Further studies are clearly required to elucidate the pathogenesis of the MAO-mediated endothelial dysfunction for the proper use of available therapeutic resources.

Acknowledgements: This work was supported by a grant of the Ministry of National Education, CNCS – UEFISCDI, project number PN-II-ID-PCE-2012-4-0512. Adrian Sturza and Danina Muntean are MC members of the COST Action BM 1005 „European Network on Gasotransmitters". We kindly thank Dr. András Varró and Dr. János Pataricza (Department of Pharmacology and Pharmacotherapy, Faculty of Medicine, University of Szeged) for providing the vascular samples and for teaching useful practical skills.

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Manuscript received: 22.12.2014