Angiotensin II-Derived Hydrogen Peroxide Impairs Endothelial Function in a Murine Model

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Excessive production of reactive oxygen species (ROS) leads to inactivation of nitric oxide (NO) and subsequent endothelial dysfunction. All cardiovascular risk factors (hypertension, dyslipidemia, diabetes, etc.) have been reported to elicit vascular oxidative stress. Angiotensin II (Ang II) is one of the most important mediators of vascular dysfunction in the presence of oxidative stress. Most of the actions of Ang II in endothelial cells are known to be associated with increased production of superoxide anion (O_2^-) which is the major responsible for endothelial NO synthase uncoupling. The aim of the present study was to assess the contribution of hydrogen peroxide (H_2O_2) production in murine vessels to endothelial dysfunction after Ang II treatment. Rat aortic segments were incubated in the presence vs. absence of Ang II or H_2O_2. Subsequently, the tissue was either studied in organ bath system for the vasomotor function or used for measurements of H_2O_2 production by the FOX (Ferric iron Xylenol Orange) assay. The results showed an attenuation of vascular relaxation response after Ang II application; this effect was at least partially mediated via the the increased H_2O_2 generation and involved the impairment of NO production.

Keywords: experimental endothelial dysfunction, angiotensin II, hydrogen peroxide

Several vasomotor mediators are released by the endothelial layer: i) vasodilators – prostaglandins, nitric oxide (NO) and endothelium-derived-hyperpolarizing factor, and ii) vasoconstrictors – tromboxane and endothelin [1]. NO plays a key role in vascular homeostasis via the control of vascular tone, inhibition of platelet aggregation, regulation of local cell growth, thus maintaining the physiological endothelial function [2, 3].

The vast majority of cardiovascular risk factors (hypertension, dyslipidemia, diabetes, etc.) have been reported to elicit vascular oxidative stress as demonstrated *in vitro* by the hyperproduction of reactive oxygen species (ROS) [4]. Small amounts of ROS are critical for the vascular tone regulation being involved in several signaling pathways [5]. Conversely, excessive ROS production leads to a disruption of normal ROS signaling and endothelial dysfunction through different mechanisms, including NO inactivation. The term ROS collectively refers to a couple of noxious compounds such as: superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH), hypochlorous acid (HOCl) and peroxynitrite ($ONOO^-$) [6] generated within the vascular system by a variety of cell, e.g. the vascular smooth muscle cells, endothelial cells and monocytes [7].

Activation of the renin-angiotensin system is strongly associated with cardiovascular pathologies [8] with angiotensin II (Ang II) being the major culprit for the deleterious effects. Most of the actions of Ang II in endothelial cells are known to be associated with the overproduction of superoxide anion (O_2^-), which leads to decreased levels of NO via endothelial NO synthase (eNOS) dysfunction/uncoupling [9, 10].

The aim of the present study was to assess the effect of Ang II treatment on H_2O_2 production in isolated murine vessels.

Experimental part

Materials and methods

Study design and animal procedures

Experiments were performed in vascular preparations (thoracic aortas) isolated from male Sprague-Dawley rats weighing 250-350 g (n=6/group). The arterial segments were carefully cleared of connective tissue so that to damage not the intimal surface and cut into 2-3 mm wide rings.

Animals were fed ad libitum and housed under standard conditions (constant temperature and humidity of 22.5 ± 2° C and 55 + 5%, 12-h light/dark cycle). Twenty-four hours prior the experiment solid food was withdrawn with no limitation in water supply.

All experimental procedures used in this study were conducted in accordance with the Directive 2010/63/EU on the protection of animals used for scientific purposes. The experimental protocol was approved by the Committee for Ethics Research of "Victor Babes" University for Medicine and Pharmacy of Timişoara, Romania.

Most reagents were from Sigma Aldrich, Germany.

Organ Culture

Rat aortic 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 Ang II (100 nmol/L) or hydrogen peroxide (100 microM).

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Organ Bath Experiments

Organ bath experiments were performed in aortic rings in the presence of diclofenac (10 μmol/L). The concentration of phenylephrine (Phe) used for pre-constriction, was adjusted to obtain an identical preconstriction level of 80% of the contraction elicited by KCl (80 mmol/L). Endothelium-dependent relaxation to increasing concentrations of acetylcholine (ACh) was registered. It was tested also the vascular contractility to L-NAME (Nω-Nitro-L-arginine methyl ester hydrochloride, 10 μM).

Reactive Oxygen Species Measurement

Hydrogen peroxide production was measured in rat aortic segments by means of FOX (Ferric iron Xylenol Orange) oxidation method using PeroxyDetect Kit (Sigma Aldrich). 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 colored adduct with xylenol orange (XO, 3,32 -bis[N,N-bis(carboxymethyl)aminomethyl]-o-cresolsulfonephthalein, sodium salt), which is observed at 560 nm.

Statistics

All values are mean±SEM. Relaxations were calculated from individual dose-response curves. Statistical analysis was carried out by ANOVA or ANOVA for repeated measurements followed by Fisher LSD posthoc test. Values of $p<0.05$ were considered statistically significant.

Results and discussions

In vitro Exposure to Ang II Results in Vascular Function Impairment

In rat aortic segments incubated for 24 h with Ang II we measured the vascular contractility and relaxation responses in the organ bath setup (Schuler organ bath). The vascular contractility after Ang II stimulation was significantly increased in response to cumulative doses of phenylephrine ($p<0.05$ vs. control, fig.1A). Also, endothelium-dependent relaxation, after Ang II pretreatment was significantly attenuated ($p<0.05$ vs. control, fig.1B). In Ang II-treated segment the contractility to L-NAME (Nω-Nitro-L-arginine methyl ester hydrochloride, 10μM) was significantly increased (fig.1C), observation suggestive for the important role of NO in vascular vasomotor function. Indeed, by inhibiting eNOS, less NO with vasodilator effect will be generated by the endothelial cells with a subsequent increase in contractility. DELTA was calculated as the difference between the maximum contractility to L-NAME and the value that represents 10% from the maximal KCl contraction in response to Phe.

Pretreatment with High Concentration of H_2O_2 Leads to Vascular Dysfunction

The most investigated radical species in relation with endothelial dysfunction is O_2^- produced by several enzymatic pathways. In this study we aimed to investigate the effect of H_2O_2 on the vasomotor function. In this regard we incubated overnight rat aortic segments with H_2O_2 and evaluated the endothelium-dependent relaxation in organ bath. We obtained a significant reduction of the vasodilator response, suggestive for the deleterious effects of H_2O_2 in high concentration (fig. 2).

Exposure to Ang II increase H_2O_2 production

Since exogenous H_2O_2 administration elicited endothelial dysfunction, we further investigated whether treatment with AII could be responsible for the increased endogenous production of H_2O_2. The results of FOX assay showed a significant increase in H_2O_2 production after Ang II stimulation.

In the present study we assessed the vasomotor function and H_2O_2 production of rat aortic segments after Ang II stimulation. We reported an attenuation of relaxation in response to Ang II pretreatment, an effect that was, at least partially, mediated by the increased H_2O_2 generation that in turn induced an impairment of NO production. This effect is supported by an excessive augmentation of contractility to L-NAME as demonstrated by the study on vasomotor function.

It is widely accepted that the main free radical with unequivocal role in the progression of endothelial dysfunction is the superoxide anion (O_2^-). Indeed, O_2^- is considered the only responsible for scavenging NO with the generation of peroxynitrite that further triggers eNOS uncoupling [11, 12].

In physiological concentrations H_2O_2 has been reported to exert beneficial effects on vascular function. In particular, hydrogen peroxide increased the endothelial level of calcium with subsequent activation of eNOS, NO production and vascular relaxation [13, 14]. However, since hydrogen peroxide is freely diffusible through cell membranes, in high concentrations it can further activate inflammation and promote endothelial dysfunction [15].

Treatment with Ang II is a classic method to induce experimental endothelial dysfunction [16] via activating several enzymatic systems that generate either O_2^- or H_2O_2.

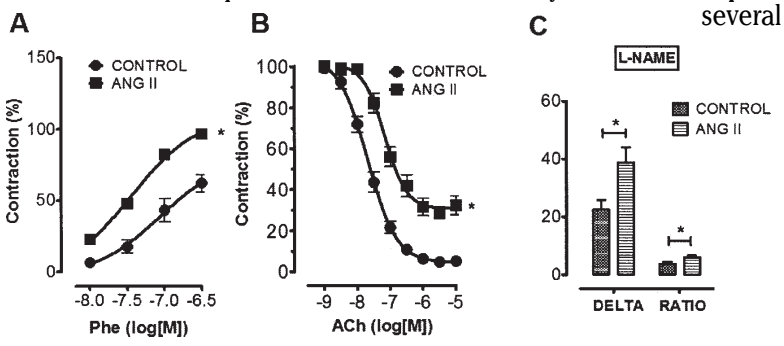

Fig. 1. The effects of Ang II on vasomotor vascular function. Aortic segments from rats were treated in vitro 24 h with Ang II (organ culture, 100 nM) and evaluated in organ bath system. A. Phenylephrine-induced contractions, n=6, *p<0.05 with and without Ang II treatment. B. Acetylcholine-induced endothelium-dependent relaxation. n=6, *p<0.05 with and without Ang II treatment. C. Contraction to L-NAME (Nω-Nitro-L-arginine methyl ester hydrochloride), DELTA= Max. contr. – 10% Phe contr., RATIO= Max. contr. / 10% Phe contr. (Max.contr.= maximal contraction to KCl 80 M, 10% Phe.contr.= 10% from maximal contraction to KCl induced by Phe)

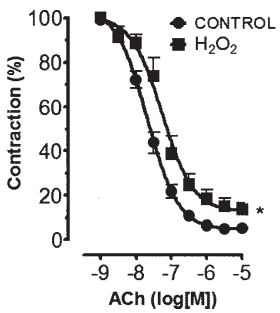

Fig. 2. The effects of H_2O_2 on vasomotor function. Aortic segments from rats were treated in vitro for 24 h with H_2O_2 - organ culture, 100 μM and evaluated in organ bath system. Acetylcholine-induced endothelium-dependent relaxation. (n=6, *p<0.05 vs. non- treatment)

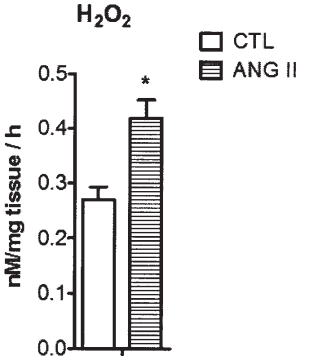

Fig. 3. Vascular H_2O_2 production after Ang II stimulation. Aortic segments from rats were treated in vitro 24 h with H_2O_2 (organ culture, 100 μM). H_2O_2 formation by FOX Assay, n=6, *p<0.05 with and without Ang II treatment

The major ROS sources are NADPH oxidases [17] and mitochondrial respiratory chain [18]. Superoxide anion is produced by: isoform 2 of NADPH (nicotinamide-adenine dinucleotide phosphate) oxidase [19, 20], uncoupled endothelial NO synthase (eNOS), cyclooxygenase, defective mitochondrial electron transport [21], whereas isoform 4 of NADPH oxidase [19, 20], xanthine oxidase [22] and, more recently, p66 [23] and monoamine oxidases [24] are responsible for hydrogen peroxide generation. All these enzymatic systems contribute to the aggravation of endothelial dysfunction and the progression of atherosclerotic vascular disease.

Despite the fact that we confirmed the contribution of AngII-derived H_2O_2 to the development of the *in vitro* endothelial dysfunction, no data are provided about the intracellular sources of H_2O_2 which is a limit of our study.

Conclusions

The present study performed in murine vascular rings exposed to angiotensin II demonstrated that increased generation of hydrogen peroxide elicited an impairment of the nitric oxide-dependent relaxation. Whether this observation can be recapitulated in vascular rings harvested from diabetic vs. non-diabetic patients with endothelial dysfunction as well as the signal transduction pathways is a topic of our current research.

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References

1. HIGASHI, Y., NOMA, K., YOSHIZUMI, M., KIHARA, Y., Endothelial function and oxidative stress in cardiovascular diseases, Circ. J., **73**, nr. 3, 2009, p. 411-418.
2.VANHOUTTE, P.M., SHIMOKAWA, H., TANG, E.H.C., FELETOU, M., Endothelial dysfunction and vascular disease, Acta Physiologica, **196**, 2009, p.193-222.
3.FORSTERMANN, U., LI, H., Therapeutic effect of enhancing endothelial nitric oxide synthase (eNOS) expression and preventing eNOS uncoupling, Br. J. Pharmacol., **164**, 2011, p. 213-223.
4.VICTOR, V.M., APOSTOLOVA, N., HERANCE, R., HERNANDEZ-MIJARES, A., ROCHA, M., Oxidative stress and mitochondrial dysfunction in atherosclerosis: mitochondria-targeted antioxidants as potential therapy, Curr. Med. Chemistry, **16**, nr. 35, 2009, p. 4654-4667.
5.ZHANG, D.X., GUTTERMAN, D.D., Mitochondrial reactive oxygen species-mediated signaling in endothelial cells, Am. J. Physiol. Heart Circ. Physiol., 292, 2006, p. H2023- H2031.
6.FÖRSTERMANN, U., Oxidative stress in vascular disease: causes, defense mechanisms and potential therapies, Nat. Clin. Pract. Cardiovasc. Med., **5**, nr. 6, 2008, p. 338-349.
7.TANIYAMA, Y., GRIENDLING, K.K., Reactive oxygen species in the vasculature: molecular and cellular mechanisms, Hypertension, **42**, nr. 6, 2003, p. 1075-81.
8.PATEL, B.M., MEHTA, A.A., Aldosterone and angiotensin: Role in diabetes and cardiovascular diseases, Eur. J. Pharmacol., **697**, nr. 1-3, 2012, p. 1-12.
9.SHATANAWI, A., ROMERO, M.J., IDDINGS, J.A., CHANDRA, S., UMAPATHY, N.S., VERIN, A.D., CALDWELL, R.B., CALDWELL, R.W., Angiotensin II-induced vascular endothelial dysfunction through RhoA/Rho kinase/p38 mitogen-activated protein kinase/arginase pathway, Am. J. Physiol. Cell Physiol., **300**, nr. 5, 2011, p. C1181-C1192.
10.RABELO, L.A., ALENINA, N., BADER, M., ACE2-angiotensin-(1-7)-Mas axis and oxidative stress in cardiovascular disease, Hypertens. Res., **34**, nr. 2, 2011, p. 154-60.
11.JUNG, O., MARKLUND, S.L., GEIGER, H., PEDRAZZINI, T., BUSSE, R., BRANDES, R.P., Extracellular superoxide dismutase is a major determinant of nitric oxide bioavailability: in vivo and ex vivo evidence from ecSOD-deficient mice, Circ. Res., **93**, nr. 7, 2003, p. 622-629.
12.WIDDER, J., BEHR, T., FRACCAROLLO, D., HU, K., GALUPPO, P., TAS, P., ANGERMANN, C.E., ERTL, G., BAUERSACHS, J., Vascular endothelial dysfunction and superoxide anion production in heart failure are p38 MAP kinase-dependent, Cardiovasc Res., **63**, nr. 1, p. 161-167.
13.FÖRSTERMANN, U., MÜNZEL, T., Endothelial nitric oxide synthase in vascular disease: from marvel to menace, Circulation, **113**, nr. 13, 2006, p. 1708-1714.
14.CAI, H., Hydrogen peroxide regulation of endothelial function: origins, mechanisms, and consequences, Cardiovasc. Res., **68**, 2005, p. 26-36.
15.WITTMANN, C., CHOCKLEY, P., SINGH, S.K., PASE, L., LIESCHKE, G.J., GRABHER, C., Hydrogen Peroxide in Inflammation: Messenger, Guide, and Assassin, Advances in Hematology, 2012; Article ID **541471**, doi:10.1155/2012/541471
16.BRANDES, R.P., MILLER, F.J., BEER, S., HAENDELER, J., HOFFMANN, J., HA, T., HOLLAND, S.M., GÖRLACH, A., BUSSE, R., The vascular NADPH oxidase subunit p47phox is involved in redox-mediated gene expression, Free Radic. Biol. Med., **32**, nr. 11, 2002, p. 1116-22.
17.OELZE, M., DAIBER, A., BRANDES, R.P., HORTMANN, M., WENZEL, P., HINK, U., SCHULZ, E., MOLLNAU, H., VON SANDERSLEBEN, A., KLESCHYOV, A.L., MÜLSCH, A., LI, H., FÖRSTERMANN, U., MÜNZEL, T., Nebivolol inhibits superoxide formation by NADPH oxidase and endothelial dysfunction in angiotensin II-treated rats, Hypertension, **48**, nr. 4, 2006, p. 677-684.
18.KRÖLLER-SCHÖN, S., JANSEN, T., SCHÜLER, A., OELZE, M., WENZEL, P., HAUSDING, M., KERAHRODI, J.G., BEISELE, M., LACKNER, K.J., DAIBER, A., MÜNZEL, T., SCHULZ, E., Peroxisome proliferator-activated receptor ă, coactivator 1á deletion induces angiotensin II-associated vascular dysfunction by increasing mitochondrial oxidative stress and vascular inflammation, Arterioscler. Thromb. Vasc. Biol., **33**, nr. 8, 2013, p. 1928-1935.
19.SCHRÖDER, K., ZHANG, M., BENKHOFF, S., MIETH, A., PLIQUETT, R., KOSOWSKI, J., KRUSE, C., LUEDIKE, P., MICHAELIS, U.R., WEISSMANN, N., DIMMELER, S., SHAH, A.M., BRANDES, R.P., Nox4 is a protective reactive oxygen species generating vascular NADPH oxidase, Circ. Res., **110**, nr. 9, 2012, p. 1217-25.
20.BRANDES, R.P., TAKAC, I., SCHRÖDER, K., No superoxide—no stress?: Nox4, the good NADPH oxidase!, Arterioscler. Thromb. Vasc. Biol., **31**, nr. 6, 2011, p. 1255-1257.
21.ERUSALIMSKY, J.D., MONCADA, S., Nitric oxide and mitochondrial signaling: from physiology to pathophysiology, Arterioscler. Thromb. Vasc. Biol., **27**, nr. 12, 2007, p. 2524-2531.
22.PARK, J.H., JIN, Y.M., HWANG, S., CHO, D.H., KANG, D.H., JO, I., Uric acid attenuates nitric oxide production by decreasing the interaction between endothelial nitric oxide synthase and calmodulin in human umbilical vein endothelial cells: a mechanism for uric acid-induced cardiovascular disease development, Nitric Oxide, **32**, 2013, p. 36-42.
23.LI, M., CHIOU, K.R., KASS, D.A., Shear stress inhibition of H(2)O(2) induced p66(Shc) phosphorylation by ASK1-JNK inactivation in endothelium, Heart Vessels, **22**, nr. 6, 2007, p. 423-427.
24.STURZA, A., LEISEGANG, M.S., BABELOVA, A., SCHRÖDER, K., BENKHOFF, S., LOOT, A.E., FLEMING, I., SCHULZ, R., MUNTEAN, D.M., BRANDES, R.P., Monoamine oxidases are mediators of endothelial dysfunction in the mouse aorta, Hypertension, **62**, nr. 1, 2013, p. 140-146.

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