ELSEVIER

European Journal of Pharmacology 362 (1998) 77–81

ejp

Short communication

Hydralazine prevents endothelial dysfunction, but not the increase in superoxide production in nitric oxide-deficient hypertension

Johann Bauersachs [a,*], Anne Bouloumié [b], Daniela Fraccarollo [a], Kai Hu [a], Rudi Busse [b], eorg Ertl [a]

[a] *II. Medizinische Klinik, Universitätsklinikum Mannheim, Fakultät für Klinische Medizin Mannheim der Universität Heidelberg, Mannheim, Germany*
[b] *Institut für Kardiovaskuläre Physiologie, Zentrum der Physiologie, Klinikum der J.W. Goethe-Universität, Frankfurt am Main, Germany*

Received 14 October 1998; accepted 20 October 1998

Abstract

Dilator responses, superoxide anion-production, endothelial nitric oxide (NO) synthase and soluble guanylyl cyclase expression were determined in aortic rings from Wistar rats treated for 5 weeks either with the NO synthase inhibitor N^G-nitro-L-arginine-methylester (L-NAME), L-NAME plus hydralazine or placebo. In the L-NAME-treated group, acetylcholine-induced relaxation was significantly attenuated whereas it was nearly normal in the L-NAME/hydralazine group. This difference was even more pronounced following inhibition of the endogenous superoxide dismutase using diethyldithiocarbamate. Aortic superoxide production was significantly elevated in both L-NAME-treated groups and hydralazine had no acute effect on superoxide formation. Expression of endothelial NO synthase was similar in all three groups whereas the attenuated soluble guanylyl cyclase expression in rats treated with L-NAME was nearly normalised by concomitant hydralazine treatment. These results demonstrate that in NO-deficient hypertension hydralazine treatment improves vasodilator responses but not the increased superoxide production.

Keywords: Hypertension; Endothelium; Superoxide anion; Nitric oxide (NO) synthase; N^G-nitro-L-arginine-methylester; Hydralazine; Guanylyl cyclase, soluble

1. Introduction

Endothelial dysfunction is a common feature of essential hypertension in patients (Panza et al., 1990) as well as several experimental models of hypertension and has been attributed to the generation of an endothelium-derived constrictor prostanoid and/or an apparent decrease in the production of bioactive nitric oxide (NO) (Lüscher and Vanhoutte, 1986; Lüscher et al., 1988). More recently, endothelial dysfunction in genetic as well as angiotensin II-induced hypertension has been ascribed to the increased vascular production of superoxide anions (Grunfeld et al., 1995; Rajagopalan et al., 1996), which scavenge NO. Indeed, despite an superoxide-mediated reduction in bioactive NO, the actual generation of NO may even be increased (Bouloumié et al., 1997).

An enhanced vascular formation of superoxide has been implicated in the development of nitrate tolerance, and could be prevented by treatment with the vasodilator hydralazine, which significantly attenuated vascular superoxide production in the rabbit aorta (Münzel et al., 1995, 1996).

In the present study, we investigated whether an enhanced superoxide formation or a reduced expression of the endothelial NO synthase contributes to the endothelial dysfunction induced by chronic NO-deficient hypertension, and whether hydralazine, which restored blood pressure to normal values, improves endothelial function.

2. Materials and methods

Female Wistar rats (250–300 g) were treated for 5 weeks either with the NO synthase inhibitor N^G-nitro-L-arginine-methylester (L-NAME, 67 mg/100 ml of drinking water), with the NO synthase inhibitor plus hydralazine (8 mg/100 ml of drinking water, L-NAME/hydralazine) or with placebo. In order to exclude acute effects of the study drugs, treatment was stopped one day before hemodynamic and organ bath studies. Hemodynamic studies were performed as described (Fraccarollo et al., 1997) under light ether anaesthesia and spontaneous respiration.

* Corresponding author. Tel.: +49-621-383-2503; Telefax: +49-621-383-3821; E-mail: johann.bauersachs@med2.ma.uni-heidelberg.de

0014-2999/98/$ - see front matter
PII: S0014-2999(98)00763-8

2.1. Vascular reactivity studies

The descending thoracic aorta was dissected following removal of the heart, cleaned of connective tissue and cut into three sections as described (Bauersachs et al., 1998). The upper section (15 mm) was immediately frozen in liquid nitrogen for Western blot analysis. The lower section (15 mm) was used for measurement of superoxide anion production, while the remainder was cut into rings (3 mm in length) which were mounted in an organ bath (Föhr Medical Instruments, Seeheim, Germany) for isometric force measurement. The rings were equilibrated for 30 min under a resting tension of 2 g in oxygenated (95% O_2; 5% CO_2) Krebs–Henseleit solution (pH 7.4, 37°C) of the following composition: NaCl, 118 mM; KCl, 4.7 mM; $MgSO_4$, 1.2 mM; $CaCl_2$, 1.6 mM; K_2HPO_4, 1.2 mM; $NaHCO_3$, 25 mM; glucose, 12 mM) and the cyclooxygenase inhibitor diclofenac (1 μM). Rings were repeatedly contracted by KCl (50 mM) until reproducible responses were obtained. Thereafter, the rings were preconstricted with phenylephrine (0.3–3 μM) to comparable constriction levels (2.1 ± 0.3 g, 2.3 ± 0.3 g and 2.6 ± 0.4 g for the placebo group, the L-NAME-treated group and the L-NAME/hydralazine-treated group, respectively) and the relaxant responses to cumulative doses of acetylcholine and sodium nitroprusside were assessed with and without inhibition the endogenous superoxide dismutase using diethyldithiocarbamate (DETC, 1 mM, 40 min, and repeated washout).

2.2. Western blot analysis

Crude protein extracts were subjected to polyacrylamide gel electrophoresis and transferred to nitro-cellulose membranes (Bio-Rad) as previously described (Fleming et al., 1995). Prestained molecular weight marker proteins (Bio-Rad) were used as standards. A Ponceau staining was performed to verify the quality of the transfer and the equal amount of protein in each lane. Proteins were detected using their respective antibodies and were visualised by enhanced chemiluminescence using a commercially available kit (Amersham, Germany). The autoradiographs were analysed by scanning densitometry.

2.3. Measurement of superoxide anion formation

The superoxide generation of the rings was assessed by lucigenin-enhanced chemiluminescence as described previously (Bauersachs et al., 1998). Briefly, aortic segments (5 mm) were transferred into tubes containing 0.5 ml HEPES buffer, maintained at 37°C for at least 30 min, before lucigenin (250 μM) was added. The luminometer (LKB Wallac 1251, Freiburg, Germany) was set to report arbitrary units of light emitted and integrated over a 30-s interval, repeated measurements were made over 3 min and averaged. The specific chemiluminescence signal was calculated after subtraction of background activity and expressed as counts per mg dry weight of samples [cpm/mg].

2.4. Materials

All biochemicals were obtained in the highest purity available from Sigma (Deisenhofen, Germany). The mono-

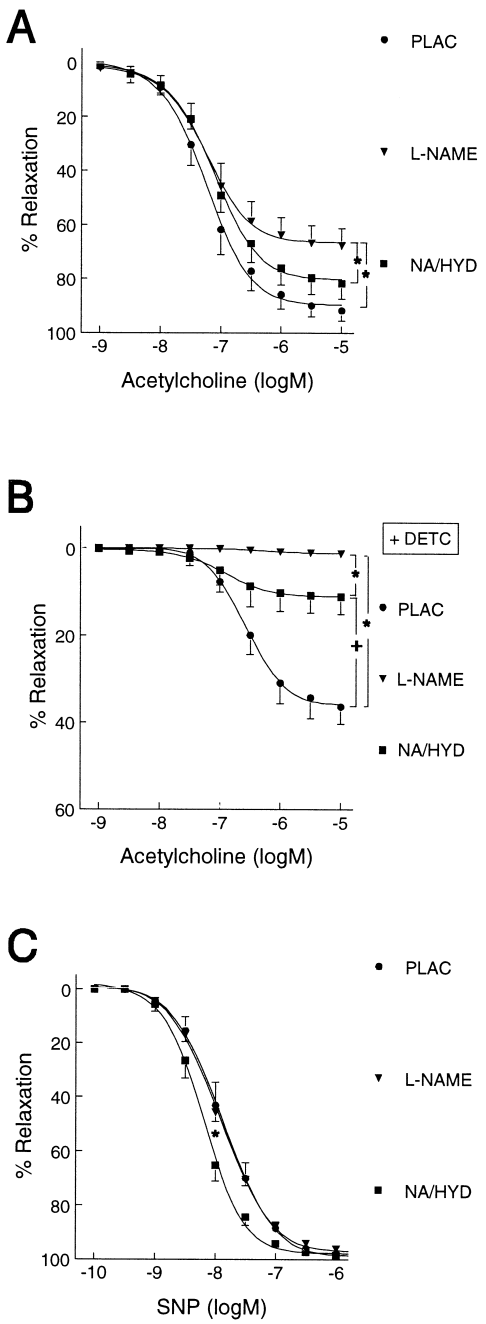

Fig. 1. Acetylcholine- (A, B) and sodium nitroprusside (SNP, C)-induced relaxations of phenylephrine-preconstricted aortic rings from Wistar rats treated for 5 weeks either with the NO synthase inhibitor N^G-nitro-L-arginine-methylester (L-NAME, ▼), the NO synthase inhibitor plus hydralazine (NA/HYD, ■) or placebo (●). Experiments in (B) were performed following inhibition the endogenous superoxide dismutase using diethyldithiocarbamate (DETC, 1 mM, 40 min). Results are expressed as the mean ± S.E.M. from 8–10 separate experiments. *, $P < 0.05$ vs. L-NAME; +, $P < 0.05$ placebo vs. NA/HYD.

clonal endothelial NO synthase antibody was purchased from Transduction Laboratories (Affinity, Exeter, England) and the antibody against the β_1-subunit of the soluble guanylyl cyclase kindly provided by Dr. Peter Yuen, Memphis, TN, USA.

2.5. Statistics

Dilator responses were given as percentage dilatation relative to the preconstriction level. All data in the figures and in the text are expressed as mean ± S.E.M. of *n* experiments with segments from different arteries. Statistical analysis was performed by One-way analysis of variance (ANOVA) followed by a Bonferroni *t*-test or by the two-tailed Student's *t*-test for unpaired data, where appropriate, with *P*-values < 0.05 considered statistically significant.

3. Results

3.1. Global parameters

Mean arterial pressure was significantly elevated in L-NAME-treated rats (142 ± 4 mm Hg) whereas no difference was observed between the placebo group (112 ± 5 mmHg) and the L-NAME/hydralazine group (113 ± 4 mmHg). Similarly, left ventricular systolic pressure was significantly higher in L-NAME-treated rats (173 ± 6 mmHg) as compared to placebo (147 ± 6 mm Hg) and the L-NAME/hydralazine groups (148 ± 4 mmHg). Heart rate was similar in all animals (placebo: 414 ± 17 min^{-1}, L-NAME: 411 ± 15 min^{-1}, L-NAME/hydralazine: 404 ± 8 min^{-1}).

3.2. Vasodilator responses in aortic rings

In phenylephrine-preconstricted aortic rings, acetylcholine induced a concentration-dependent relaxation which was blunted in aortae from L-NAME-treated rats (Fig. 1A). In contrast, in aortae from the L-NAME/hydralazine-treated group, endothelium-dependent relaxation was not different from placebo. Acetylcholine-induced relaxations were mediated by NO since they were abolished following incubation with the NO synthase inhibitor N^G-nitro-L-arginine (0.3 mM, 30 min). Maximum relaxation in response to acetylcholine (10 μmol/l) under this conditions were 4 ± 1%, 6 ± 1% and 5 ± 1% for the placebo group, the L-NAME-treated group and the L-NAME/hydralazine-treated group, respectively.

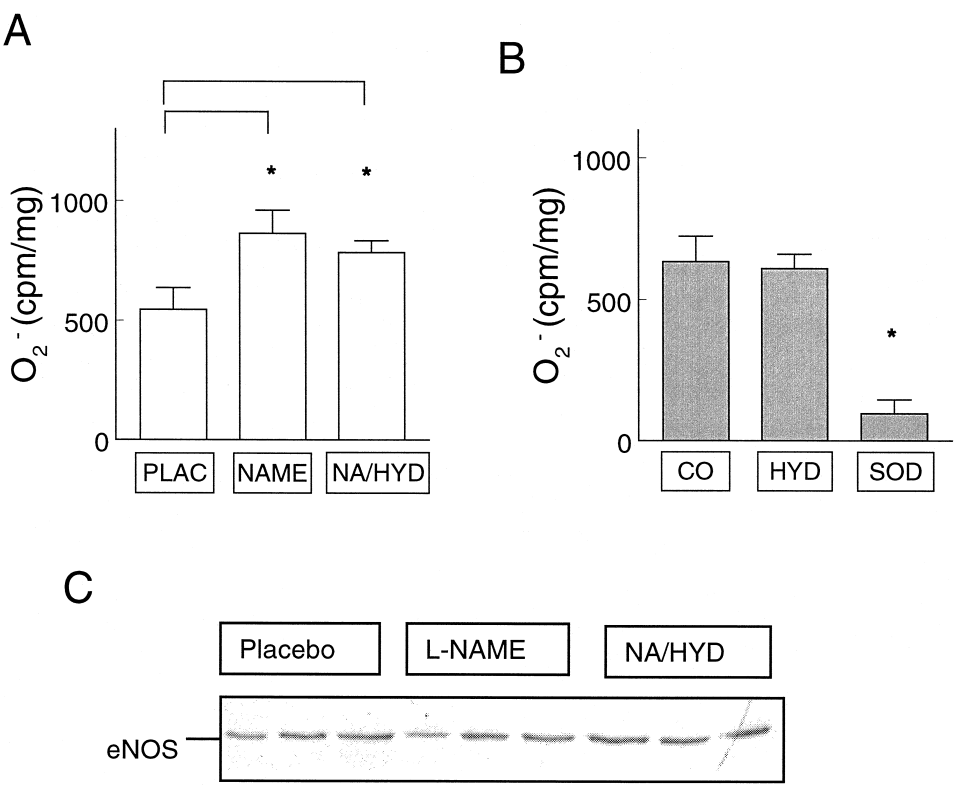

Fig. 2. (A) Superoxide anion production in aortic rings from Wistar rats treated for 5 weeks either with the NO synthase inhibitor N^G-nitro-L-arginine-methylester (L-NAME), the NO synthase inhibitor plus hydralazine (NA/HYD) or placebo (PLAC). *, $P < 0.05$ vs. PLAC. (B) Superoxide anion production in aortic rings from Wistar rats in the absence (CO) or presence of either hydralazine (HYD, 10 μM, 30 min) or superoxide dismutase (SOD, 600 U/ml, 20 min). *, $P < 0.05$ vs. CO. Results are expressed as the mean ± S.E.M. from 8–10 separate experiments. (C) Western blots showing endothelial NO synthase protein levels in the aorta from Wistar rats treated for 5 weeks either with the NO synthase inhibitor N^G-nitro-L-arginine-methylester (L-NAME), the NO synthase inhibitor plus hydralazine (NA/HYD) or placebo. Proteins were separated by polyacrylamide gel electrophoresis and endothelial NO synthase protein was detected using a specific anti-endothelial NO synthase antibody as described in Section 2.

To determine whether an enhanced production of superoxide accounts for the attenuated vasodilator responses, the acetylcholine-induced relaxation in aortic rings was studied following inhibition of the endogenous superoxide dismutase using diethyldithiocarbamate (1 mM, 40 min). Thereafter, the inhibition of acetylcholine-induced relaxation was significantly more pronounced in NO-deficient animals, and the relaxation was almost abolished in the L-NAME-treated group (Fig. 1B).

The concentration–response curve to the endothelium-independent vasodilator sodium nitroprusside was not different between the placebo and L-NAME-treated group, whereas it was slightly, but significantly shifted to the left in aortae from the L-NAME/hydralazine-treated animals. Maximum relaxant responses were identical in all three groups (Fig. 1C).

3.3. Production of superoxide anions

Superoxide generated by aortic rings was assessed by lucigenin-enhanced chemiluminescence. Superoxide release was greater in aortae from NO-deficient animals (Fig. 2A), but was unaffected by hydralazine treatment. Removal of the endothelium slightly, but not significantly reduced radical production in all three groups (endothelium-intact: placebo 547 ± 90 cpm/mg, L-NAME 865 ± 97, L-NAME/hydralazine 786 ± 49; endothelium-denuded: placebo 513 ± 68, L-NAME 748 ± 130, L-NAME/hydralazine 732 ± 132).

Acute incubation of aortic rings with hydralazine (1 and 10 μM, 30 min) did not affect superoxide formation, whereas superoxide dismutase (600 U/ml, 20 min) practically abolished superoxide production (Fig. 2B).

3.4. Endothelial NO synthase and soluble guanylyl cyclase expression in the aorta

Protein levels of the endothelial NO synthase were determined by Western blot analysis in aortic segments from the three groups of rats, however, no difference in expression could be detected (Fig. 2C). In contrast, soluble guanylyl cyclase expression was attenuated in rats treated with L-NAME, and concomitant treatment with hydralazine nearly normalised the expression of soluble guanylyl cyclase (densitometrical analysis: placebo 4.6 arbitrary units, L-NAME 1.15, L-NAME/hydralazine 3.2, $n = 3$).

4. Discussion

The results of the present study demonstrate that the endothelial dysfunction observed in chronic NO-deficient hypertension was associated with an increase in vascular superoxide formation, whereas endothelial NO synthase expression was unchanged. Hydralazine treatment normalised endothelium-dependent relaxation but did not prevent the increase in superoxide production.

An endothelial dysfunction in L-NAME-induced hypertension has been described (Küng et al., 1995), however, the underlying mechanisms have not been clarified. An enhanced formation of endothelium-derived vasoconstrictor prostanoids has been implicated, but can not account for the dysfunction observed in our study, since in all experiments the cyclooxygenase inhibitor diclofenac was present. Our data demonstrate that in chronic NO deficiency, vascular superoxide production is significantly elevated and may lead to an enhanced inactivation of endothelium-derived NO and thus contribute to endothelial dysfunction. The source of superoxide formation appears to be the vascular smooth muscle layer, since removal of the endothelium did not significantly attenuate radical production. Cultured and native vascular smooth muscle cells are able to generate superoxide in response to the vasoconstrictor peptide angiotensin II, which stimulates the expression of an NAD(P)H-dependent oxidase (Griendling et al., 1994; Rajagopalan et al., 1996). Tissue angiotensin converting enzyme activity in the heart as well as in the aorta is markedly elevated after 4 weeks of L-NAME-induced hypertension (Takemoto et al., 1997). Therefore, an enhanced local formation of angiotensin II may lead to an enhanced vascular superoxide formation through the expression of an NAD(P)H dependent oxidase in aortic smooth muscle cells (Griendling et al., 1994; Rajagopalan et al., 1996).

An imbalance between NO and superoxide production has been associated with endothelial dysfunction in several models of hypertension such as spontaneously hypertensive rats (Grunfeld et al., 1995; Bauersachs et al., 1998) and aortic coarctation (Bouloumié et al., 1997) and appears also to be a common feature of many cardiovascular diseases such as atherosclerosis, hypercholesterolemia and diabetes. In addition, under these pathophysiological circumstances, NO and superoxide react to the powerful oxidant peroxynitrite ($OONO^-$), which can form hydroxyl radicals and nitrate protein tyrosine residues (Bouloumié et al., 1997).

Anti-hypertensive therapy has been associated with beneficial effects on endothelial function in NO-deficient hypertension, e.g., angiotensin converting enzyme inhibitors as well as calcium antagonists restored acetylcholine-induced relaxation in hypertension induced by 6-week treatment with L-NAME (Küng et al., 1995; Takemoto et al., 1997). An important mechanism of angiotensin converting enzyme inhibitor treatment appears to be a correction of the NO/superoxide imbalance since ramipril increased the production of NO through an enhanced expression of endothelial NO synthase and decreased superoxide accumulation in spontaneously hypertensive rats (Wiemer et al., 1997). In contrast to the angiotensin converting enzyme inhibitor cilazapril (Clozel et al., 1990), hydralazine treatment did not improve endothelium-dependent relaxation in spontaneously hypertensive rats. However, the dose of hydralazine used in that study was relatively low

(2.5 mg/kg vs. 8 mg/kg in our study), and in contrast to cilazapril did not restore blood pressure to normal levels.

The lack of effect of in vivo as well as in vitro treatment with hydralazine on vascular superoxide formation in our study was surprising given a recent report of hydralazine-mediated inhibition of an NADH-dependent oxidase (Münzel et al., 1996) and may be explained by species differences in the type of the oxidase(s) involved in superoxide formation. Since neither endothelial NO synthase expression nor superoxide formation were affected by hydralazine treatment the improvement of acetylcholine-induced relaxation in rat aorta does not appear to involve a modulation of the NO/superoxide balance. Hydralazine slightly potentiated the smooth muscle relaxant response to the NO donor SNP, an observation which has already been made for the angiotensin converting enzyme inhibitor trandolapril (Küng et al., 1995), pointing to an enhanced cGMP accumulation in vascular smooth muscle cells following stimulation with SNP. Although a reduced rate of cGMP degradation by phosphodiesterases cannot be excluded, the enhanced soluble guanylyl cyclase expression in rats treated with both L-NAME and hydralazine may be an important mechanism for the normalisation of the dilator response in these animals.

In conclusion, our data indicate that an increased vascular superoxide generation contributes to endothelial dysfunction in NO-deficient hypertension. Although Hydralazine treatment did not affect aortic superoxide formation and endothelial NO synthase expression, vascular relaxant responses were improved through an increase in soluble guanylyl cyclase expression.

Acknowledgements

This work was supported in part by the Deutsche Forschungsgemeinschaft (Ba 1742/1-1) and the Forschungsfonds der Fakultät für Klinische Medizin Mannheim (0067/1997). The authors wish to thank Claudia Liebetrau and Elke Burmeister for expert technical assistance.

References

Bauersachs, J., Bouloumié, A., Mülsch, A., Wiemer, G., Fleming, I., Busse, R., 1998. Vasodilator dysfunction in aged spontaneously hypertensive rats: changes in NO synthase III and soluble guanylyl cyclase expression, and in superoxide anion production. Cardiovasc. Res. 37, 772–779.

Bouloumié, A., Bauersachs, J., Linz, W., Schölkens, B.A., Wiemer, G., Fleming, I., Busse, R., 1997. Endothelial dysfunction coincides with an enhanced NO synthase expression and superoxide anion production. Hypertension 30, 934–941.

Clozel, M., Kuhn, H., Hefti, F., 1990. Effects of angiotensin-converting enzyme inhibitors and of hydralazine on endothelial function in rats. Hypertension 16, 532–540.

Fleming, I., Fisslthaler, B., Busse, R., 1995. Calcium-signalling in endothelial cells involves activation of tyrosine kinases and leads to the activation of mitogen-activated protein kinases. Circ. Res. 76, 522–529.

Fraccarollo, D., Hu, K., Galuppo, P., Gaudron, P., Ertl, G., 1997. Chronic endothelin receptor blockade attenuates progressive ventricular dilatation and improves cardiac function in rats with myocardial infarction. Possible involvement of myocardial endothelin system in ventricular remodeling. Circulation 96, 3963–3973.

Griendling, K.K., Minieri, C.A., Ollerenshaw, J.D., Alexander, R.W., 1994. Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. Circ. Res. 74, 1141–1148.

Grunfeld, S., Hamilton, C.A., Mesaros, S., McClain, S.W., Dominiczak, A.F., Bohr, D.F., Malinski, T., 1995. Role of superoxide in the depressed nitric oxide production by the endothelium of genetically hypertensive rats. Hypertension 26, 854–857, [part 1].

Küng, C.F., Moreau, P., Takase, H., Lüscher, T.F., 1995. L-NAME hypertension alters endothelial and smooth muscle function in rat aorta: prevention by trandolapril and verapamil. Hypertension 26, 744–751.

Lüscher, T.F., Vanhoutte, P.M., 1986. Endothelium-dependent contractions to acetylcholine in the aorta of the spontaneously hypertensive rat. Hypertension 8, 344–348.

Lüscher, T.F., Diederich, D., Weber, E., Vanhoutte, P.M., Bühler, F.R., 1988. Endothelium-dependent responses in carotid and renal arteries of normotensive and hypertensive rats. Hypertension 11, 573–578.

Münzel, T., Sayegh, H., Freeman, B.A., Tarpey, M., Harrison, D.G., 1995. Evidence for enhanced vascular superoxide anion production in nitrate tolerance. J. Clin. Invest. 95, 187–194.

Münzel, T., Kurz, S., Rajagopalan, S., Thoenes, M., Berrington, W.R., Thompson, J.A., Freeman, B.A., Harrison, D.G., 1996. Hydralazine prevents nitroglycerin tolerance by inhibiting activation of a membrane-bound NADH oxidase: a new action for an old drug. J. Clin. Invest. 98, 1465–1470.

Panza, J.A., Quyyumi, A.A., Brush, J.E., Epstein, S.E., 1990. Abnormal endothelium-dependent vascular relaxation in patients with essential hypertension. N. Engl. J. Med. 323, 22–27.

Rajagopalan, S., Kurz, S., Münzel, T., Tarpey, M., Freeman, B.A., Griendling, K.K., Harrison, D.G., 1996. Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. J. Clin. Invest. 97, 1916–1923.

Takemoto, M., Egashira, K., Usui, M., Numaguchi, K., Tomita, H., Tsutsui, H., Shimokowa, H., Sueshi, K., Takeshita, A., 1997. Important role of tissue angiotensin-converting enzyme activity in the pathogenesis of coronary vascular and myocardial structural changes induced by long-term blockade of nitric oxide synthesis in rats. J. Clin. Invest. 99, 278–287.

Wiemer, G., Linz, W., Hatrik, S., Schölkens, B.A., Malinski, T., 1997. Angiotensin-converting enzyme inhibition alters nitric oxide and superoxide release in normotensive and hypertensive rats. Hypertension 30, 1183–1190.