Improvement of Endothelial Function of the Corpus Cavernosum in Apolipoprotein E Knockout Mice Treated with Irbesartan

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ABSTRACT

Angiotensin receptor blockers enhance endothelial function and are suggested to improve erectile function. The effects and underlying mechanisms of treatment with the angiotensin receptor blocker irbesartan on penile endothelial function in apolipoprotein E (ApoE)−/− mice were determined. Wild-type (C57/B6) and ApoE−/− mice were fed with a high-fat, cholesterol-rich diet for 7 weeks and treated with irbesartan (50 mg/kg · day) or hydralazine (250 mg/l). Vital parameters were measured with the tail-cuff method. Endothelial (aortic rings) and erectile function (corpora cavernosa) were assessed by pharmacological stimulation in an organ bath chamber. Oxidative stress and angiotensin receptor expression were determined. Blood pressure was significantly decreased in irbesartan- and hydralazine-treated ApoE−/− mice (p < 0.05) compared with controls and wild-type mice. Endothelial function of the aorta and corpus cavernosum was significantly impaired in ApoE−/− mice (p < 0.05) and could be restored by treatment with irbesartan (p < 0.05). Consistently, nitric oxide production of corpora cavernosa was impaired in ApoE−/− mice (p < 0.01), with a restoration in irbesartan- but not hydralazine-treated mice. Dihydroethidium-stained sections and lipid peroxidase assay revealed a reduction of superoxide production in irbesartan (p < 0.05) compared with hydralazine-treated and control ApoE−/− mice. In summary, irbesartan improves penile endothelial function in ApoE−/− mice by reduction of vascular and cavernosal oxidative stress. This result emphasizes the beneficial effect of inhibition of the renin-angiotensin system even in terms of erectile function.

Erectile dysfunction (ED) is defined as the inability to attain or maintain penile erections sufficient for satisfactory sexual performance (NIH Consensus Development Panel on Impotence, 1993). In the Western industrialized countries, prevalence of ED in the general population is approximately 20 to 30%, with an economical and significant psychological impact (Feldman et al., 1994; Braun et al., 2000). In cardiovascular high-risk patients, prevalence of ED rises up to 75%, indicating the strong association of ED with the known cardiovascular risk factors and cardiovascular diseases (Baumhäkel and Böhm, 2007; Böhm et al., 2007). Impairment of endothelial function by cardiovascular risk factors such as hypertension seems to be the pathophysiological link, because nitric oxide synthesis plays a crucial role in physiology of penile erection (Yavuzgil et al., 2005).

Inhibition of the renin-angiotensin system is known to reduce blood pressure effectively. Moreover, recent studies suggested a beneficial effect of antihypertensive treatment with angiotensin-converting enzyme inhibitors or angiotensin receptor antagonists on erectile function (Fogari et al., 1998, 2001; Dusing, 2003). The beneficial effect of angiotensin receptor blockade with irbesartan on erectile function was also observed in patients with metabolic syndrome and hypertension. The local synthesis of angiotensin II in the corpus cavernosum initiating the detumescence phase in humans could be responsible (Becker et al., 2001). Moreover, angiotensin II is known to stimulate reactive oxygen species in the endothelial monolayer, with consecutive oxidative stress and development of endothelial dysfunction and likely erectile dysfunction (Griendling et al., 1994; Rajagopalan et al., 1996). Thus, treatment with angiotensin receptor blockers is suggested to improve endothelial function of the corpus cavernosum and consecutive erectile function due to a reduction of oxidative stress. Although plausible, studies on these
underlying mechanisms in an atherosclerotic disease model are presently lacking.

The objective of this study was to determine the effect and possible mechanisms of treatment with the angiotensin receptor antagonist irbesartan on endothelial function of the corpus cavernosum as a surrogate for erectile function in cholesterol-fed ApoE−/− mice with atherosclerosis.

Materials and Methods

Animals and Procedures. Animal procedures were performed in accordance with institutional guidelines, the German animal protection law, and the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication 85-23, revised 1996). Male C57BL/6J mice (wild type) and ApoE−/− mice (C57BL/6J genetic background; Charles River, Sulzfeld, Germany) were used for this study and were demonstrated previously to be an appropriate model investigating erectile function in atherosclerotic mice (Behr-Roussel et al., 2006; Xie et al., 2007). The animals were maintained at 22°C on a 12-h light/dark cycle. All mice were fed with a high-fat, cholesterol-rich diet (21% fat, 19.5% casein, and 1.25% cholesterol; Sniff, Soest, Germany) for 7 weeks, starting at the age of 10 weeks. Additional groups of male ApoE−/− mice were either treated with irbesartan, an angiotensin receptor antagonist (orally via chow; 50 mg/kg·day), or with the vasodilator hyalurazine-HCl as a control (orally via drinking water; 250 mg/kg). Systolic blood pressure and heart rate were measured noninvasively with the tail-cuff method in conscious mice as described previously (BP-2000; Visitech-Systems, Apex, NC) (Wassmann et al., 2004). Plasma lipid concentrations were measured, with LDL-cholesterol calculated by using the Friedewald formula. After the treatment period, mice were killed, and tissue and blood samples were collected immediately. Chemicals were obtained from Sigma-Aldrich (Taufkirchen, Germany). All chemicals were dissolved in distilled water.

Aortic Ring Preparation and Tension Recording. After excision, the descending thoracic aorta was immersed in Tyrode’s solution containing 118.0 mM NaCl, 2.5 mM CaCl2, 4.73 mM KCl, 1.2 mM MgCl2, 1.2 mM KH2PO4, 25.0 mM NaHCO3, 0.026 mM Na-EDTA, and 5.5 mM D(+)-glucose, pH 7.4. Adventitial tissue was carefully removed. Three-millimeter rings were mounted in organ bath chambers filled with the Tyrode’s solution described above (37°C; aerated with 95% O2 and 5% CO2) and attached to a force transducer recording isometric tension. Aortic rings (AoR) were stretched to a resting tension of 10 mN, which was maintained throughout the experiment. Pharmacologically induced contraction of aortic rings was performed with an α-agonist, ([R]-)-phenylephrine-HCl (10 μM). Drugs were added in increasing concentrations to obtain cumulative concentration-response curves for carbachol (carbamylcholine chloride; 1 nM–1 μM) and glyceryl-trinitrate (100 nM–100 μM). Drugs were washed out before adding the next substance. The relaxing effect of carbachol was abolished by adding N-nitro-L-arginine methyl ester (1 μM). Relaxation of corpora cavernosa mimics erectile function, and decreased relaxation to a stimulus indicates erectile dysfunction (Buyukasik and Un, 2003). CCS without any response to relaxation with carbachol (<10% relaxation) were excluded from statistical analysis due to the possible absence of the endothelium.

Staining Procedures. Aortic sinus, ascending aorta, and corpora cavernosa were snap-frozen at −80°C and sectioned on a cryostat (10 μm in thickness; Leica, Wetzlar, Germany). At least five consecutive sections per animal per staining were used for analysis. For detection of atherosclerotic lesions in the aortic sinus and ascending aorta, oil red O staining was performed as described previously (Laufs et al., 2005). Morphometric analysis was performed with Lucia Measurement Software 4.6 (Nikon UK Ltd., Surrey, UK), measuring lipid-staining plaque area related to vessel diameter. To assess vascular superoxide production in situ, dihydroethidium (DHE) fluorescence of aortic tissue and corpora cavernosa sections was used. Aortas were prepared as described above, and 4-mm segments were embedded in Tissue Tek OCT embedding medium (Bayer Corp., Emeryville, CA). Aortic rings and corpora cavernosa strips were snap-frozen and stored at −80°C. Sections were sectioned on a cryostat (10 μm in thickness; Leica) and placed on glass slides. Krebs-HEPES buffer containing 2 μM DHE was topically applied to each tissue section, and sections were incubated in a dark humidified chamber at 37°C for 30 min. In situ production of superoxide was visualized by fluorescence microscopy. Aortas and CCS from each treatment group were processed in parallel, and images were acquired with identical acquisition parameters and were stored digitally. To assess penile collagen content, Sirius Red staining of corpora cavernosa sections was performed. Corpora cavernosa strips were snap-frozen and stored at −80°C. Sections were sectioned on a cryostat (10 μm in thickness; Leica) and placed on glass slides, incubated with the Sirius Red agent. Collagen content was quantified using fluorescence microscopy. CCS from each treatment group was processed in parallel, and images were acquired with identical acquisition parameters and were stored digitally.

Nitric oxide production was quantified with diaminofluorescein (DAF) staining (4,5-diaminofluorescein; Sigma-Aldrich, Schnelldorf, Germany). Corpora cavernosa sections were obtained as described above. Immediately before staining, sections were air-dried and bordered with water-repellent bromopropene/dipentene-pen. Slides were then incubated in phosphate-buffered saline for 15 min to rinse them from freezing medium. DAF was diluted in Krebs-Henseleit phosphate buffer to a concentration of 10 μM instantly before use, and 75 μl was applied to each section. Slides were incubated at 37°C for 4 h. Digital images of the sections were produced immediately afterward (fluorescence microscopy; fluorescein isothiocyanate-fluorescence filter; 100-fold magnification). Exposition of DAF to artificial light or daylight was avoided at any time. Images were subsequently converted to gray-scale, and ImageJ software (National Institutes of Health, Bethesda, MD) was used to assess the fluorescence intensity by outlining cavernosal tissue and measuring brightness in pixel values (black = 0; white = 255).

Measurement of Lipid Peroxidation. Ascorbic tissue/corpus cavernosum was homogenized in phosphate-buffered saline, pH 7.4, containing butylated hydroxytoluene (4 mM). Lipid hydroperoxides were determined using the Lipid Peroxidation Assay Kit II (Calbiochem, Darmstadt, Germany) and are expressed as micromoles per milligram of protein (Laufs et al., 2005).

Angiotensin Receptor Expression. Immunoblotting for AT1 receptor (AT1 [N-10], rabbit polyclonal IgG; Santa Cruz Biotechnology, Heidelberg, Germany) and glyceraldehyde-3-phosphate dehydrogenase protein expression to control for equal protein loading using the enhanced chemiluminescence kit (GE Healthcare, Munich,
Germany) were performed as described previously (Nickenig et al., 2000).

Statistical Analysis. All data are expressed as mean ± S.E.M. Statistical significance was assumed at a p level of <0.05. Intergroup differences were assessed with analysis of variance, with the Newman-Keuls post hoc analysis (GraphPad Prism 4.03; GraphPad Software Inc., San Diego, CA).

Results

Vital Parameters and Lipid Levels. Systolic blood pressure and heart rate of all animals after treatment with a high-cholesterol diet for 7 weeks are shown in Table 1. There were no significant differences in heart rate or systolic blood pressure in WT and ApoE−/− mice. Treatment with hydralazine or irbesartan decreased systolic blood pressure significantly compared with untreated mice, without significant differences within these treatment groups. All cholesterol levels measured (total cholesterol, LDL, and high-density lipoprotein), but not triglycerides, were significantly increased in ApoE−/− mice irrespective of additional medical treatment (Table 1).

Atherosclerotic Lesion Formation. After 7 weeks of a cholesterol-rich diet, atherosclerotic lesion formation was quantified in the aortic sinus and the ascending aorta in all groups by histological analysis of Oil Red O stainings. WT mice showed no signs of atherosclerotic changes. In ApoE−/− mice, atherosclerosis was severely displayed, with a significant reduction in hyaluronic- and irbesartan-treated ApoE−/− mice (Fig. 1).

Endothelial Function of the Aorta and the Corpus Cavernosum. Erectile function was determined as endothelial relaxation of CCS (two CCS/animal; 9–12 animals/treatment group), endothelial function was determined by relaxation of AoR (four AoR/animal; 9–12 animals/treatment group) in all groups. Response to muscarinic stimulation with carbachol was significantly impaired in aortic rings of ApoE−/− mice compared with WT mice (Fig. 2A). Parallel to endothelial function in the aorta, endothelium-dependent relaxation of corpora cavernosal strips was significantly decreased in ApoE−/− mice (Fig. 2B). Both AoR and CCS revealed an impaired efficacy of muscarinic stimulation in ApoE−/− mice. In addition, the log EC50 value calculated from the concentration-response curves to carbachol was shifted to the right in CCS of ApoE−/− mice (log EC50 of WT, −6.74 ± 0.06; ApoE−/−, −6.22 ± 0.19; p < 0.01). Relaxation of AoR and CCS related to carbachol was significantly improved in irbesartan-treated mice, with a restoration of endothelial function of the aorta and the corpus cavernosum comparable with WT mice (Fig. 2, A and B). In CCS, both efficacy and potency of muciainergic stimulation with carbachol were significantly increased in irbesartan-treated mice (log EC50 of −6.87 ± 0.30, p < 0.001 versus ApoE−/−). Efficacy of muscarinic stimulation of AoR and CCS was not improved in hydralazine-treated mice (Fig. 2, A and B). In contrast, potency of carbachol stimulation was improved in CCS treated with hydralazine (log EC50, −6.80 ± 0.07, p < 0.01 versus ApoE−/−). Endothelium-independent relaxation induced by nitroglycerin of aortic rings and corpora cavernosa was not significantly different between all groups (N.S.).

Vascular Oxidative Stress. As a global parameter of oxidative stress, lipid peroxidation of the aortic wall (n = 5) was measured. Lipid hydroperoxides were significantly increased in ApoE−/− mice compared with WT mice, with a restoration in irbesartan-treated mice (Fig. 3A). Treatment with the vasodilator hydralazine had no effect on lipid per-
oxidation. In situ detection of superoxide anion production by DHE fluorescence microscopy in aortic sections revealed a similar result (Fig. 4A). ROS release was significantly increased in ApoE<sup>−/−</sup> mice. Irbesartan treatment, but not hydralazine treatment, reduced ROS release to an extent comparable with WT mice.

**Penile Oxidative Stress.** Lipid peroxidation of seven corpora cavernosa per group was measured. Comparable with the vascular oxidative stress of the aorta, treatment with irbesartan, but not hydralazine, restored increased lipid peroxidation in ApoE<sup>−/−</sup> mice (Fig. 3B). Additionally, DHE staining revealed a significant reduction of ROS in the corpora cavernosa in irbesartan-treated mice (Fig. 4B). Treatment with hydralazine had no effect on ROS release in the penile tissue.

**Angiotensin Receptor Expression.** Western blot analysis of corpora cavernosa revealed a significant increase of AT1 receptor expression in ApoE<sup>−/−</sup> mice compared with WT. Protein expression was significantly reduced in irbesartan-treated but not hydralazine-treated mice (Fig. 5).

**Collagen Content.** Collagen content, as a parameter of fibrotic changes in the aortic root and the corpus cavernosum, was calculated as described above. Content of collagen fibers was significantly enhanced in ApoE<sup>−/−</sup> mice, with a restoration in irbesartan and in part in hydralazine-treated ApoE<sup>−/−</sup> mice (Fig. 6).

**Nitric Oxide Production.** Production of nitric oxide in corpora cavernosa strips was determined with DAF staining and subsequent quantification by fluorescence microscopy (Fig. 7). Nitric oxide production was significantly decreased in ApoE<sup>−/−</sup> mice with a restoration in irbesartan-treated but not hydralazine-treated mice.

**Discussion**

Erectile function is dependent on nitric oxide synthesis of the endothelial monolayer of the penile arteries and the corpus cavernosum and has been shown to represent an early symptom of generalized atherosclerosis (Feldman et al., 1994; Yavuzgil et al., 2005). In our study, impairment of both endothelial function of the aorta and the corpus cavernosum in atherosclerotic ApoE<sup>−/−</sup> mice compared with WT mice was similar (Fig. 3). Nitric oxide production was significantly decreased in ApoE<sup>−/−</sup> mice with a restoration in irbesartan-treated but not hydralazine-treated mice.
tors, is known to be related to erectile dysfunction (Müller et al., 1991). Despite few reports indicating a decreasing erectile function after initiation of antihypertensive treatment, several trials demonstrated favorable effects of blood pressure reduction on erectile function (Modebe, 1990; Llisterri et al., 2001; Hale et al., 2002). Recent trials indicated an advantage of improvement of erectile function for substances inhibiting the renin-angiotensin system. This was demonstrated in hypertensive men as well as in patients with a metabolic syndrome (Fogari et al., 1998, 2001). Even switching antihypertensive therapy from carvedilol to AT1 antagonists was shown to be advantageous for sexual performance (Dusing, 2003). These results might suggest a favorable effect of inhibition of the renin-angiotensin system beyond blood pressure reduction. In multivariate analysis, predictors of change of erectile function after 6 months of treatment with the AT1 antagonist irbesartan indicated that beneficial effects were at least in part independent of blood pressure reduction. However, direct evidence in controlled experimental conditions was lacking until now. In this study, treatment with the AT1 antagonist irbesartan and the vasodilator hy-

![Fig. 4. Oxidative stress. Superoxide production in aortic segments (A) and corpora cavernosa (B) was evaluated by DHE staining and fluorescence microscopy. Mean ± S.E.M., n = 5 per group. *, p < 0.05 versus ApoE/−; and **, p < 0.05 versus ApoE/−-hydralazine. Representative DHE-stained sections of corpora cavernosa are shown in B.](image)

![Fig. 5. Angiotensin receptor expression. Protein expression of the AT1-receptor was quantified densitometrically after Western blot analysis. Mean ± S.E.M., n = 4 to 5. *, p < 0.05 versus WT/ApoE/−-irbesartan (AT1-R, AT1 receptor; glyceraldehyde-3-phosphate dehydrogenase [GAPDH], housekeeping protein). A representative Western blot is shown.](image)

![Fig. 6. Collagen content. Collagen content was quantified using Sirius Red staining following fluorescence microscopy in aortic (A; n = 5) and corpora cavernosa tissues (B; n = 5; representative sections were shown). Mean ± S.E.M. *, p < 0.001 versus WT/ApoE/−-irbesartan; **, p < 0.05 versus ApoE/−; and ***, p < 0.05 versus WT/ApoE/−-irbesartan.](image)
Induced oxidative stress, endothelial dysfunction, and atherosclerosis (Harrison, 1997). Thus, cholesterol-ionization, which impairs endothelial cell function in the early stages of atherosclerosis (Rajagopalan et al., 1996; Nickenig et al., 1997; Warnholtz et al., 1999). Oxidative stress is an important mechanism in endothelial and erectile function, with enhanced ROS demonstrated to increase AT1 receptor gene expression and oxidative stress, restoring endothelial function and nitric oxide production, and improved atherosclerotic lesion formation as well as collagen content significantly, whereas vasodilation with hydralazine had only minor effects.

Angiotensin II was demonstrated to be synthesized in endothelial cells lining blood vessels and smooth muscle bundles within the corpus cavernosum, inducing the phase of detumescence (Kifor et al., 1997; Becker et al., 2001). Moreover, injection of angiotensin II terminates erection in dogs, with the angiotensin receptor antagonist losartan increasing intracavernosal pressure and erectile function (Kifor et al., 1997). Consistent with the pharmacological observations, recent clinical trials suggest beneficial effects of inhibition of the renin-angiotensin system regarding erectile function. In our study, treatment with irbesartan increased endothelial function and thereby erectile function in the corpus cavernosum. Comparable with the vascular effects, irbesartan but not hydralazine, reduced radical load and oxidative stress in the corpus cavernosum. These results further support the correlation of endothelial and erectile function and are in favor of the notion that protection by angiotensin receptor antagonism against oxidative stress with consecutive endothelial and erectile dysfunction is directly mediated by angiotensin receptor blockade and is independent of blood pressure reduction. Consistently, nitric oxide production of the corpora cavernosa tissue, known to be a key player in physiology of erectile function, was restored in irbesartan-treated but not hydralazine-treated mice. The data provide underlying mechanisms for advantageous effects of AT1 antagonists in patients with hypertension as described previously.

Hypercholesterolemia was recently demonstrated to increase expression of the AT1 receptor, with activation of NADPH oxidase and consecutive enhanced ROS production leading to endothelial dysfunction (Nickenig et al., 1997). In corpora cavernosa of hypercholesterolemic ApoE−/− mice, protein expression of the AT1 receptor was significantly increased, with restoration to WT level in irbesartan-treated mice only. Considering improvement of endothelial function and nitric oxide production by a reduction of oxidative stress in irbesartan-treated mice, down-regulation of the AT1 receptor might be one possible pathophysiological link explaining the results of the presented study. Moreover, irbesartan decreased the collagen content of the penile tissue to a greater extent than did hydralazine. These effects were comparable with vascular effects of irbesartan in ApoE−/− mice already demonstrated in comparison with the calcium channel blocker amlodipine (Candido et al., 2004). Thus, a decrease of fibrotic changes is also likely to improve endothelial function of the aorta and the corpus cavernosum and is suggested to be related to a decrease of oxidative stress.

In conclusion, treatment with the angiotensin receptor antagonist irbesartan restores impairment of endothelial function of the corpus cavernosum, indicating improvement of
erectile function in ApoE−/− mice. These beneficial effects are largely blood pressure independent and are associated with a reduction of vascular and cavernosal oxidative stress related to a normalization of AT1 receptor expression with consecutive improvement of endothelial function in the corpus cavernosum. The results of this study emphasize the important role of inhibition of the renin-angiotensin system with regard to endothelial and especially erectile function. The clinical significance of these results is currently evaluated in the prospective, randomized ONTARGET/TRANSCEND erectile dysfunction substudy. Moreover, this trial will provide information whether inhibition of the angiotensin-converting enzyme, antagonism of the angiotensin receptor, or the combination thereof is advantageous for cardiovascular high-risk patients with erectile dysfunction.

References


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