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RESEARCH PAPER

Telmisartan, ramipril and their combination improve endothelial function in different tissues in a murine model of cholesterol-induced atherosclerosis

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BACKGROUND AND PURPOSE

Erectile dysfunction correlates with cardiovascular disease and its common risk factors due to the development of endothelial dysfunction. Positive effects on endothelial and erectile function have been described for substances inhibiting the renin-angiotensin-system. Here, we investigated in an atherosclerosis model, whether telmisartan (angiotensin receptor blocker) and ramipril (angiotensin converting enzyme inhibitor) are equivalent or the combination of both is superior in improving endothelial function in the aorta and the corpus cavernosum and in reducing atherosclerosis.

EXPERIMENTAL APPROACH

Wild-type (WT, C57/B6) and apolipoprotein-E-deficient ($ApoE^{-/-}$) mice were treated with a cholesterol-rich diet for 8 weeks. $ApoE^{-/-}$ mice were supplemented with either telmisartan (20 $mg \cdot kg^{-1} \cdot day^{-1}$), ramipril (2.5 $mg \cdot kg^{-1} \cdot day^{-1}$) or the combination thereof.

KEY RESULTS

Systolic blood pressure significantly decreased in treatment groups ($P < 0.001$), with significantly smaller reduction under ramipril monotherapy ($P < 0.05$). Endothelial function (assessed by pharmacological stimulation of aortic rings and corpus cavernosum in organ bath chambers) was impaired in $ApoE^{-/-}$ mice compared to WT animals, which was improved by all three treatments to a comparable extent ($P < 0.05$). Atherosclerotic lesion size in the ascending aorta and aortic sinus ($P < 0.001$), the amount of lipid peroxides in cavernosal and aortic tissue ($P < 0.05$) and free radical load (dihydroethidium-stain) ($P < 0.05$) were enhanced in untreated $ApoE^{-/-}$ mice in comparison to WT animals and were significantly reduced by either treatment. In penile tissue, expression of eNOS could be restored by renin-angiotensin-aldosterone system blockade.

CONCLUSIONS AND IMPLICATIONS

Telmisartan and ramipril significantly improved endothelial function of aortic and cavernosal tissues in $ApoE^{-/-}$ via reduction of oxidative stress. Combination of both agents does not enhance beneficial effects significantly.

Abbreviations

ACE, angiotensin converting enzyme; Ang II, angiotensin II; CCS, corpora cavernosal strips; DHE, dihydroethidium; ED, erectile dysfunction; eNOS, endothelial nitric oxide synthase; L-NAME, N_{ω}-nitro-l-arginine methyl ester; RAAS, renin-angiotensin-aldosterone system; WT, wild-type

Introduction

Erectile dysfunction (ED), defined as the inability to attain and/or maintain an erection sufficient for satisfactory sexual intercourse for a period of at least 6 months, does not only represent a highly prevalent health problem of considerable socioeconomic impact, but is considered an early end-organ manifestation of atherosclerosis as well (NIH, 1993; Feldman *et al.*, 2000; Kirby *et al.*, 2001). Therefore, diagnosis of ED, especially in younger patients gains greater importance, as it should prompt investigation of cardiovascular risk factors (Thompson *et al.*, 2005). Hypertension, dyslipidemic conditions, diabetes and further cardiovascular risk factors correlate strongly with presence and extent of ED (Esposito *et al.*, 2004). These conditions are associated with development of endothelial dysfunction, that is, impaired release of nitric oxide from endothelial cells leading to decreased vasomotor responses (Harrison, 1997). Because of the small diameter of the helical arteries and a relatively high content of endothelium and smooth muscle within the corpora cavernosa, erectile tissue is prone to early endothelial dysfunction caused by oxidative stress and reduced bioavailability of nitric oxide, which occurs early in the process of atherosclerosis (Yavuzgil *et al.*, 2005; Billups *et al.*, 2008).

Angiotensin II (Ang II) and expression, as well as activity of the angiotensin $(AT)_1$ receptor (receptor nomenclature follows Alexander *et al.*, 2009) mediates vascular oxidative stress and inflammatory processes and finally endothelial dysfunction, while genetic disruption of the AT_1 receptor leads to inhibition of vascular oxidative stress, endothelial dysfunction and formation of atherosclerotic lesions (Griendling *et al.*, 1994; Rajagopalan *et al.*, 1996; Wassmann *et al.*, 2004).

Moreover, besides being subject to systemic changes in the activity of the renin-angiotensin-aldosterone system (RAAS), the local angiotensin homeostasis of erectile tissue has to be taken into account, as the corpus cavernosum produces and secretes physiologically relevant amounts of Ang II which contribute to the development of detumescence (Kifor *et al.*, 1997). Previously, we observed reduction of vascular oxidative stress and endothelial dysfunction with subsequent improvement of endothelial function of penile tissue in apolipoprotein-E-deficient ($ApoE^{-/-}$) treated with the AT_1 receptor antagonist irbesartan, which is in line with clinical findings of advantageous effects of AT_1 receptor blockade in hypertensive patients (Fogari *et al.*, 2001; Dusing, 2003; Baumhakel *et al.*, 2008). For reduction of Ang II formation via angiotensin converting enzyme (ACE) inhibitors, comparable effects have been described (Dorrance *et al.*, 2002; Speel *et al.*, 2005).

However, local Ang II formation in the vasculature, as well as in erectile tissue is supposed to play a major role in promoting oxidative stress and inflammatory processes, independent of ACE functionality (Hirono *et al.*, 2007). On the other hand, blockade of AT_1 receptors without ACE-inhibition leads to accumulation of Ang II, stimulating the AT_2 receptor, while degradation of bradykinin through ACE reduces bradykinin B_2 receptor-mediated stimulation of endothelial nitric oxide synthesis in erectile tissue (Becker *et al.*, 2001). Thus, the combination of both modes of action, ACE inhibition and AT_1 receptor blockade, could lead to synergistic effects, greater than the results achievable with monotherapy using either class of drug.

The aim of this study was to determine the effect of the AT_1 receptor antagonist telmisartan, the ACE inhibitor ramipril and the combination of both substances on vascular and cavernosal endothelial function as well as on oxidative stress and lipid peroxidation in aortic tissue and corpora cavernosa of cholesterol fed $ApoE^{-/-}$ mice.

Methods

Animals and procedures

All animal care and experimental procedures were in accordance with institutional guidelines, the German animal protection law and the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication no. 85–23, revised 1996). Male C57BL/6J mice (wild-type, WT) and $ApoE^{-/-}$ mice (C57BL/6J, genetic background, Charles River, Sulzfeld, Germany), previously demonstrated to be an appropriate model for the assessment of cavernosal endothelial function in atherosclerotic mice, were used in this study (Xie *et al.*, 2007). The animals were maintained at 22°C with a 12 h light/dark cycle. All mice were fed a high-fat, cholesterol-rich diet (21% fat, 19.5% casein and 1.25% cholesterol, Ssniff, Soest, Germany).

Twenty $ApoE^{-/-}$ mice were used as a positive, and 20 WT mice as a negative control respectively. These animals received the atherogenic diet throughout 8 weeks, while three groups, each consisting of 20 $ApoE^{-/-}$ mice were fed the same diet, supplemented orally with either telmisartan in drinking water (20 $mg \cdot kg^{-1} \cdot day^{-1}$), ramipril in chow-pellets (2.5 $mg \cdot kg^{-1} \cdot day^{-1}$) or the combination thereof at the same doses throughout the treatment period.

Systolic blood pressure and heart rate were measured non-invasively via the tail-cuff method in conscious mice (BP-2000, Visitech-Systems, Apex, NC, USA). After the treatment period, mice were killed and tissue and blood samples were collected immediately. Plasma lipid concentrations were measured with low density lipoprotein (LDL)-cholesterol being calculated with the Friedewald formula. Chemicals were obtained from Sigma-Aldrich, Taufkirchen, Germany. All chemicals were dissolved in distilled water.

Preparation of corpus cavernosum strips and aortic rings and tension recording

Penises were removed and immersed in chilled Tyrode-buffer containing in mM: NaCl 118.0, $CaCl_2$ 2.5, KCl 4.73, $MgCl_2$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0, EDTA 0.026, D(+)glucose 5.5 (pH 7.4). The glans penis and urethra were excised and adherent tissue was removed, keeping the tunica albuginea intact. Strips of corpora cavernosal strips (CCS) were separated, cleaned and suspended in organ baths chambers filled with the Tyrode solution described above (37°C, aerated with 95% O_2 and 5% CO_2). CCS were attached to a force transducer recording isometric tension and subsequently equilibrated at a resting tension of 3 mN, which was maintained throughout the experiment. Following equilibration, CCS were precontracted with the α-adrenoceptor agonist (R)-(-)-phenylephrine-HCl (5 μM). After a steady state of contraction

had stabilized, drugs were added in increasing concentrations to obtain cumulative concentration-response curves for carbachol (representing an endothelium-dependent relaxing agent, 1 nM–1 μM) and glyceryl trinitrate (as an nitric oxide-donor, 100 nM–100 μM). Drugs were washed out before adding the next substance. The relaxing effect of carbachol was abolished by adding N^{ω}-nitro-L-arginine methyl ester (L-NAME, 1 μM). CCS without any response to carbachol (relaxation < 10%) were excluded from statistical analysis due to a presumable damage of the endothelium. Relaxation of cavernous smooth muscle as a key step in haemodynamics of erection is strongly dependent on nitric-oxide production by the epithelial lining of the cavernosal sinusoids, subsequent to nervous and endocrine stimuli. A deterioration of endothelium-dependent relaxation to carbachol indicates dysfunction of the cavernosal endothelium (Buyukafsar and Un, 2003).

After excision, the descending thoracic aorta was immersed in Tyrode solution (as described above) immediately and adventitial tissue was carefully removed. Three-millimetre rings were mounted in organ bath chambers and attached to a force transducer as described above. Aortic rings were equilibrated at a resting tension of 10 mN, which was maintained throughout the experiment. Contraction of aortic rings was induced by adding (R)-(-)-phenylephrine-HCl (10 μM). Cumulative concentration-response curves for carbachol (1 nM–100 μM) and glyceryl trinitrate (100 nM–100 μM) were obtained as described above, abolishing the relaxing effect of carbachol by adding L-NAME (1 μM).

Staining procedures

Corpora cavernosa and hearts including the ascending aorta were snap-frozen at –80°C and sectioned on a Leica cryostat, obtaining transverse sections of 10 μm thickness. At least five consecutive sections per animal and per staining were used for analysis.

For assessment of atherosclerotic lesion size, sections of the aortic sinus as well as the ascending aorta were stained with Oil-Red O, as described before (Laufs *et al.*, 2005). Morphometric analysis of microscopic images was performed by using Image-J software 1.37v (National Institutes of Health, USA) to determine the relation of lipid-stained plaque area to vessel diameter.

Fluorescence produced by the oxidation of dihydroethidium (DHE) by superoxide anions, was used to quantify superoxide production *in situ* for both tissues. Krebs-HEPES buffer containing DHE (2 μM) was topically applied to each tissue section and subsequently incubated in a dark humidified chamber at 37°C for 30 min. Aortic tissue and CCS from each treatment group were processed in parallel and images were immediately acquired and digitally stored, using fluorescent microscopy with acquisition parameters kept identical at all times. Intensity of fluorescence was subsequently quantified using Image-J software 1.37v. Prior to measurement, images were converted to greyscale and the epithelial regions of vessels and cavernae of erectile tissue were outlined. Each pixel within the outlined area was digitally allocated a numeric value (0 = black, 255 = white) according to its brightness which were subsequently averaged.

Measurement of lipid peroxidation

Corpus cavernosal as well as abdominal aortic tissue was homogenized in distilled water. Oxidative degradation of cell membrane lipids, dependent on the equilibrium of cellular oxidative load and antioxidant potential was assessed via redox reactions with ferric ions performed in deoxygenated chloroform-methanol, as in the instructions of the Lipid Peroxidation Assay Kit II (Calbiochem, Darmstadt, Germany). Subsequent to protein assessment, hydroperoxide concentrations were expressed as $nmol \cdot mg^{-1}$ protein (Laufs *et al.*, 2005).

Immuno-staining for endothelial nitric oxide synthase (eNOS)

Corpora cavernosa segments were sectioned as described above. Immediately prior to staining, sections were air-dried and bordered with water-repellent bromopropane/dipentene-pen. Slides were then incubated in 4%-paraformaldehyde for 2 min and subsequently rinsed in phosphate-buffered saline (PBS) for 5 min. Following which, slides were treated twice with a 0.5% solution of *tert*-octylphenoxypolyethanol (Igepal, Sigma Aldrich, Deisenhofen, Germany) in PBS for 5 min before being incubated in a 0.5% solution of goat serum (Sigma Aldrich, Deisenhofen, Germany) in PBS. The primary antibody (eNOS/NOS type III rabbit-anti-mouse, isotype IgG, Becton Dickinson, Heidelberg, Germany) was diluted 1:100 in 0.5% goat serum in PBS and 100 μL applied to each section. Slides were incubated for 90 min in a humid chamber and were then rinsed in PBS three times before being incubated with the secondary antibody using the same amount (TRITC-conjugated goat-anti-rabbit, affinity-isolated and adsorbed with human IgG, Sigma Aldrich, Deisenhofen, Germany, dilution 1:100 in 0.5% goat serum in PBS) for 45 min in a humid chamber. Slides were subsequently rinsed in PBS three times for 10 min and embedded in mounting medium for fluorescence microscopy (Dako Deutschland GmbH, Hamburg, Germany). Digital images of the sections were acquired immediately afterwards (fluorescence microscopy, G-2A-fluorescence filter, excitation 510–560 nm, 100-fold magnification). Exposure of the TRITC-conjugated secondary antibody to artificial light or daylight was avoided at all times. Images were subsequently converted to grey-scale and Image-J software was used to assess the fluorescence intensity by outlining cavernosal tissue and measuring brightness in pixel values (black = 0, white = 255).

Statistical analysis

All data are expressed as mean ± SEM. Statistical significance was assumed when $P < 0.05$. In organ bath chamber experiments, the arithmetic mean of the response of all aortic rings and CCS was calculated for each animal. Those mean values were averaged for the treatment group. Intergroup differences were assessed with the ANOVA test followed by Newman–Keuls *post hoc* analysis (GraphPad Prism 4.03, GraphPad, San Diego, CA, USA). Quantification and analysis of all assays was performed without knowledge of the treatments.

Results

Vital parameters

Systolic blood pressure significantly decreased in all treatment groups in comparison to untreated ApoE$^{-/-}$ and WT animals (Table 1). Treatment with telmisartan alone or in combination with ramipril decreased systolic blood pressure to a greater extent than ramipril monotherapy. Heart rate was significantly lower in untreated ApoE$^{-/-}$ mice than in all other groups. Irrespective of intervention, total cholesterol and LDL cholesterol levels of all ApoE$^{-/-}$ groups were higher than those measured in WT animals (Table 1).

Atherosclerotic lesion size

Ascending aortae, as well as the aortic sinus of untreated ApoE$^{-/-}$ mice exhibited a greater extent of atherosclerotic lesion formation, as shown by Oil Red-O staining than WT-animals. All interventions reduced plaque formation to a similar extent, compared with ApoE$^{-/-}$ mice without treatment (Figure 1).

Endothelial function in CCS

Contractile responses to phenylephrine of samples of both tissues, corpora cavernosa and aorta, were not different between all treatment groups (Table 2). CCS from untreated ApoE$^{-/-}$ animals showed a reduced relaxation to carbachol in comparison to WT animals which was significant at all concentrations above 0.03 μM carbachol. All treatment groups showed an improvement of endothelium-dependent relaxations to carbachol with no differences between treatment groups (Figure 2). Additionally, the potency of carbachol to induce endothelium dependent relaxation (as pD_2) was impaired in ApoE$^{-/-}$ animals, with improvement in all treatment groups (Table 3). Neither the maximum effect nor the potency to induce endothelium-independent relaxation to glyceryl trinitrate differed at any concentration, between groups (Table 3).

Vascular endothelial function

Endothelium-dependent relaxation of aortic tissue to carbachol was impaired in untreated ApoE$^{-/-}$ animals in comparison to WT mice at all concentrations. All three treatments improved this endothelial function equally, that is, there were no differences between the treatment groups (Figure 3). The pD_2 of carbachol also improved significantly in all treatment groups, whereas endothelium-independent relaxations to glyceryl trinitrate did not differ significantly at any concentration between groups (Table 3).

Dihydroethidium fluorescence

Intensity of the fluorescence signals measured subsequent to staining with DHE was higher in ascending aorta and CCS of untreated ApoE$^{-/-}$ mice in contrast to that in tissues from WT animals. ApoE$^{-/-}$ animals treated with telmisartan, ramipril, or both showed a significant reduction in DHE fluorescence signals in comparison to untreated ApoE$^{-/-}$ mice while there were no differences in this variable between treatment groups (Figure 4).

Lipid peroxidation

The content of hydroperoxides in aortic and cavernosal tissue of ApoE$^{-/-}$ mice which had not received any treatment exceeded that measured in WT animals. Intervention with telmisartan, ramipril or their combination reduced the formation of lipid hydroperoxides in both tissues, without differences between the treatment groups (Figure 5).

Endothelial nitric oxide synthase

The eNOS was quantified by immunohistochemistry-staining of corpora cavernosa sections. In ApoE$^{-/-}$ mice, eNOS was

Table 1

Cardiovascular parameters and lipid values of experimental groups

	Systolic blood pressure (mmHg)	Heart rate (bpm)	Total cholesterol (mg·L^{-1})	LDL cholesterol (mg·L^{-1})	HDL cholesterol (mg·L^{-1})	Triglycerides (mg·L^{-1})
WT	111.7 ± 2.5	640.8 ± 9.9^{+}	21.2 ± 1.9	5.3 ± 1.7	14.6 ± 2.7	7.9 ± 0.8
ApoE$^{-/-}$	105.6 ± 2.3	593.4 ± 10.9	117.8 ± 17.1**	90.6 ± 14.1**	26.0 ± 4.2	10.8 ± 3.1
ApoE$^{-/-}$ + ramipril (2.5 mg·kg^{-1}·day^{-1})	91.1 ± 2.5**,$^{++}$	635.8 ± 6.2^{+}	114.6 ± 18.3**	85.2 ± 14.8**	32.6 ± 4.7	13.2 ± 3.3
ApoE$^{-/-}$ + telmisartan (20 mg·kg^{-1}·day^{-1})	79.8 ± 3.3**,$^{++,\#}$	621.2 ± 4.3^{+}	121.3 ± 14.6**	90.7 ± 12.4**	28.5 ± 5.0	11.6 ± 2.5
ApoE$^{-/-}$ + telmisartan/ramipril (20 mg·kg^{-1}·day^{-1}; 2.5 mg·kg^{-1}·day^{-1})	79.7 ± 2.7**,$^{++,\#}$	639.1 ± 10.2^{+}	80.3 ± 16.2*	68.8 ± 11.3*	21.5 ± 9.4	12.2 ± 3.2

Systolic blood pressure and heart rate were determined in all animals. Lipid levels were measured in five to nine mice per group. Data shown are means ± SEM.

*P < 0.05 versus WT; **P < 0.001 versus WT; ^{++}P < 0.001 versus ApoE$^{-/-}$; ^{+}P < 0.05 versus ApoE$^{-/-}$; $^{\#}P$ < 0.05 versus ApoE$^{-/-}$ + ramipril.

ApoE$^{-/-}$, apolipoprotein-E-deficient; HDL, high density lipoprotein; LDL, low density lipoprotein; WT, wild-type.

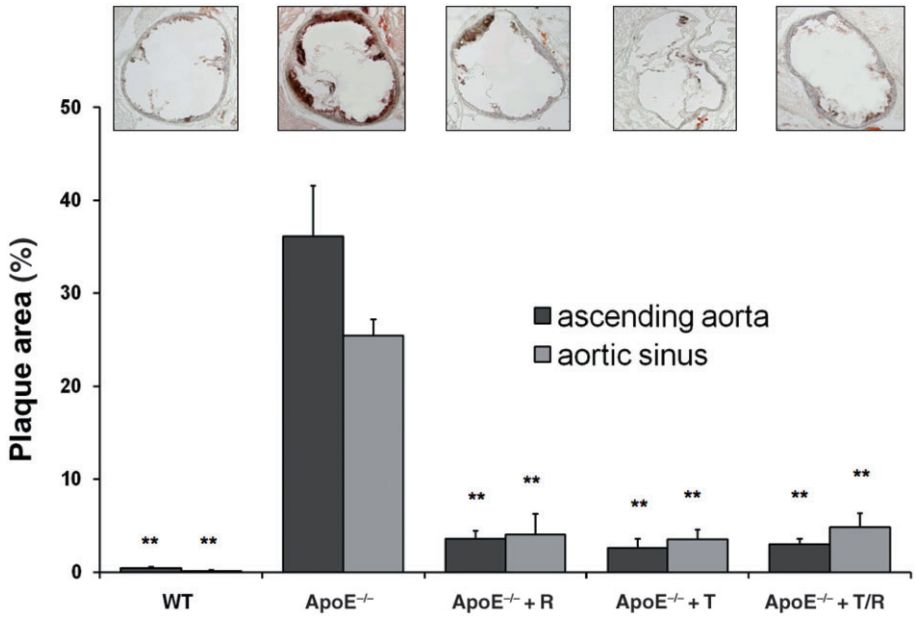

Figure 1

Atherosclerotic lesion size. Extent of atherosclerotic lesions was measured in the aortic sinus (representative images shown) and the ascending aorta. Mice were treated with ramipril (R), or telmisartan (T) or in combination (T/R) for 8 weeks. Data shown are means ± SEM from five animals per group. $^{**}P < 0.001$, different from values for ApoE$^{-/-}$ mice. ApoE$^{-/-}$, apolipoprotein-E-deficient; WT, wild-type.

Table 2

Phenylephrine-induced contraction of corpora cavernosal strips and aortic rings

% tension at baseline	Wild-type	ApoE$^{-/-}$	ApoE$^{-/-}$ + ramipril	ApoE$^{-/-}$ + telmisartan	ApoE$^{-/-}$ + telmisartan/ramipril
Aortic tissue					
Carbachol	19.03 ± 0.97	22.63 ± 2.10	20.93 ± 3.40	24.47 ± 2.14	22.20 ± 0.92
GTN	20.41 ± 2.64	20.00 ± 1.88	20.80 ± 2.28	27.02 ± 3.21	27.01 ± 1.78
Cavernosal tissue					
Carbachol	7.69 ± 0.55	9.68 ± 0.78	10.41 ± 0.89	10.23 ± 1.68	8.78 ± 1.32
GTN	9.44 ± 0.81	11.22 ± 0.89	11.14 ± 1.57	10.58 ± 1.57	9.83 ± 1.50

Contractile responses (% of baseline tension) of aortic and cavernosal tissue to phenylephrine before relaxation with carbachol or GTN. No significant difference within all treatment groups (n.s.). Five to ten animals per group. Mean ± SEM.
ApoE$^{-/-}$, apolipoprotein-E-deficient; GTN, glyceryl trinitrate.

significantly decreased and could partly restored by treatment with telmisartan, ramipril, or the combination (Figure 6).

Discussion

The present data show that the AT_1 receptor antagonist telmisartan and the ACE inhibitor ramipril were equally effective in restoring aortic and cavernosal endothelial function and in reducing atherosclerosis in a murine model of cardiovascular disease, supposedly via reduction of excessive amounts of reactive oxygen species (ROS) in both tissues with restoration of eNOS as the pathophysiological link. Combination of both drugs did, however, not provide any further amelioration of vascular damage.

The predictive value of end-organ damage for cardiovascular events and, vice versa, their regression by treatment for cardiovascular protection is well accepted. The effects of differentially blocking the RAAS on end-organ damage in individuals bearing cardiovascular risk factors such as hypertension and dyslipidaemia have been widely discussed in terms of left ventricular function, renal function, cerebrovascular function and congestive heart failure (Friedrich *et al.*, 2006; Werner *et al.*, 2008). Little attention has been paid to functional changes in erectile tissue as a very sensitive target-organ in cardiovascular disease. The corpora cavernosa

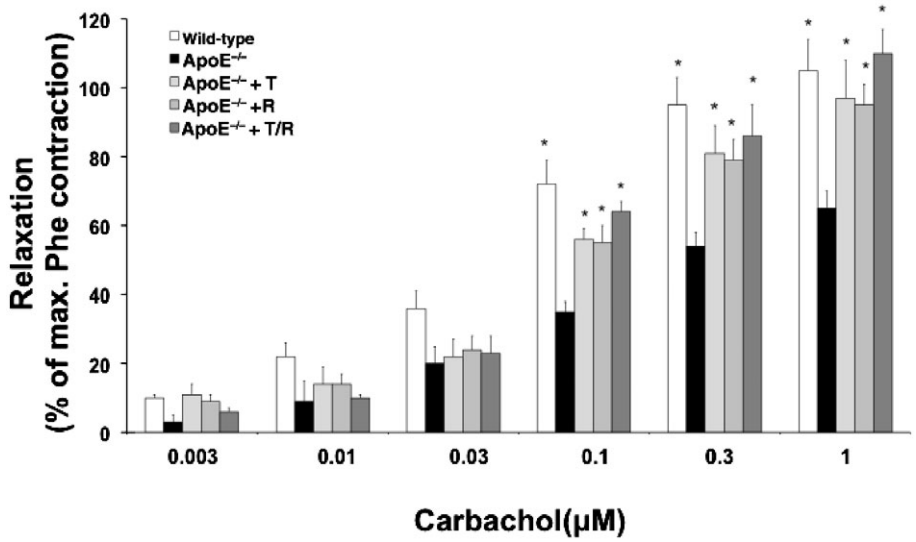

Figure 2

Corpus cavernosum isometric tension recording. Relaxation to carbachol, as % of phenylephrine (Phe)-induced contraction, was assessed in corpora cavernosal strips. Apolipoprotein-E-deficient ($ApoE^{-/-}$) mice were treated with ramipril (R), or telmisartan (T) or in combination (T/R). Data shown are means ± SEM from six to nine animals per group. *$P < 0.05$, different from values for $ApoE^{-/-}$ mice.

Table 3

Parameters (pD_2, maximum effect) of endothelium-dependent or -independent relaxations in aortic rings and corpora cavernosal strips

	Wild-type	$ApoE^{-/-}$	$ApoE^{-/-}$ + ramipril	$ApoE^{-/-}$ + telmisartan	$ApoE^{-/-}$ + telmisartan/ramipril
Aortic tissue, response to carbachol					
pD_2 (-log)	–5.85 ± 0.22*	–4.71 ± 0.20	–6.27 ± 0.34*	–5.47 ± 0.18*	–5.70 ± 0.31*
Maximum relaxation (%)	76.4 ± 4.9*	49.3 ± 5.4	81.2 ± 7.7*	75.7 ± 7.7*	69.5 ± 4.1*
Cavernosal tissue, response to carbachol					
pD_2 (-log)	–7.45 ± 0.07*	–7.16 ± 0.18	–7.54 ± 0.11*	–7.58 ± 0.08*	–7.66 ± 0.08*
Maximum relaxation (%)	105.3 ± 9.5*	64.5 ± 4.5	95.3 ± 5.6*	97.2 ± 11.6*	109.2 ± 13.3*
Aortic tissue, response to glyceryl trinitrate					
pD_2 (-log)	–6.22 ± 0.08	–6.36 ± 0.12	–6.40 ± 0.20	–6.69 ± 0.09	–6.72 ± 0.15
Maximum relaxation (%)	124.3 ± 11.8	141.9 ± 9.1	142.9 ± 11.4	108.2 ± 6.1	113.5 ± 7.1
Cavernosal tissue, response to glyceryl trinitrate					
pD_2 (-log)	–5.56 ± 0.14	–5.69 ± 0.13	–5.54 ± 0.20	–5.57 ± 0.13	–5.73 ± 0.19
Maximum relaxation (%)	46.4 ± 6.5	57.3 ± 6.9	43.5 ± 3.3	46.6 ± 5.9	53.6 ± 7.4

Endothelium-dependent relaxations were induced by carbachol and endothelium-independent relaxations by glyceryl trinitrate. Data shown are means ± SEM from six to ten mice per group.
*$P < 0.05$ versus $ApoE^{-/-}$ mice.
$ApoE^{-/-}$, apolipoprotein-E-deficient.

of the penis represent the most abundantly endothelialized tissue in the human body as physiology of erection is strongly dependent on the release of nitric oxide from the cavernous endothelium, making ED an indicator of endothelial dysfunction appearing 3 to 12 years prior to other cardiovascular manifestations of end-organ damage (Bookstein *et al.*, 1990; Harrison, 1997; Speel *et al.*, 2003; Baumhakel and Bohm, 2007).

Despite evidence for the improvement of ED via monotherapy with inhibitors of the RAAS, the ONgoing Telmisartan Alone and in Combination with Ramipril Global Endpoint Trial (ONTARGET) and the Telmisartan Randomized AssessmeNT Study in ACE-INtolerant Subjects with Cardiovascular Disease (TRANSCEND), were the first long-term prospective trials on cardioprotective actions in high-risk patients to address the effects of dual RAAS blockade on

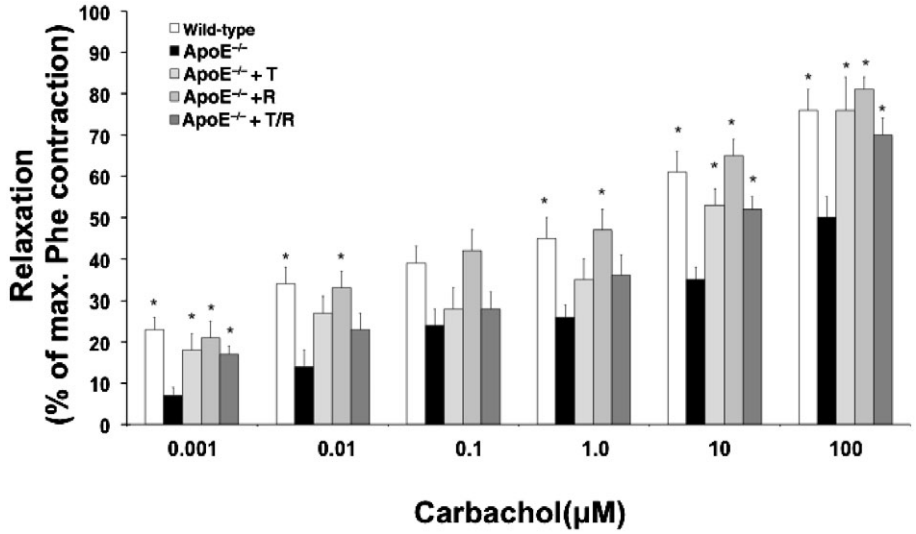

Figure 3

Aortic ring isometric tension recording. Relaxation to carbachol in % of phenylephrine (Phe)-induced contraction was assessed in aortic rings. Apolipoprotein-E-deficient ($ApoE^{-/-}$) mice were treated with ramipril (R), or telmisartan (T) or in combination (T/R). Data shown are means ± SEM from seven to ten animals per group. *$P < 0.05$, different from values for $ApoE^{-/-}$ mice.

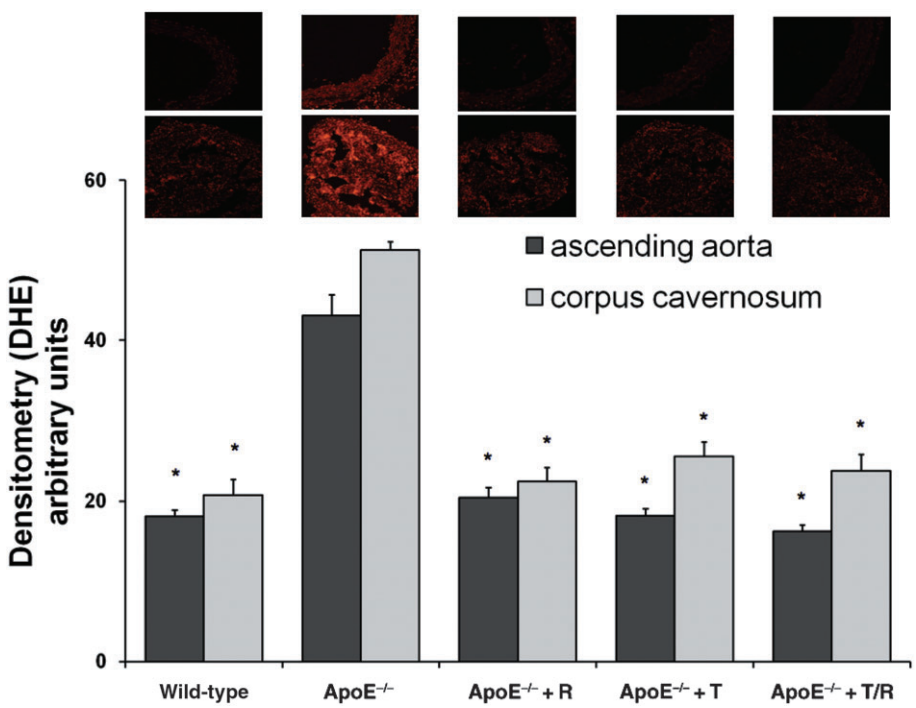

Figure 4

Oxidative stress [dihydroethidium (DHE) fluorescence]. Ascending aortae (representative images above) and corpora cavernosa (representative images below) were stained with DHE (five per group). Apolipoprotein-E-deficient ($ApoE^{-/-}$) mice were treated with ramipril (R), or telmisartan (T) or in combination (T/R).Data shown are means ± SEM. *$P < 0.05$, different from values for $ApoE^{-/-}$ mice.

erectile function (Bohm *et al.*, 2007; Yusuf *et al.*, 2008a,b). Effects due to reduction in tissue Ang II effects through telmisartan on the one hand and reduced formation of Ang II and degradation of bradykinin via ramipril, on the other, could have exerted synergistic effects on erectile tissue, given its sensitivity to changes in erectile function.

The present study evaluated functional and molecular effects of dual RAAS blockade on endothelial tissues in

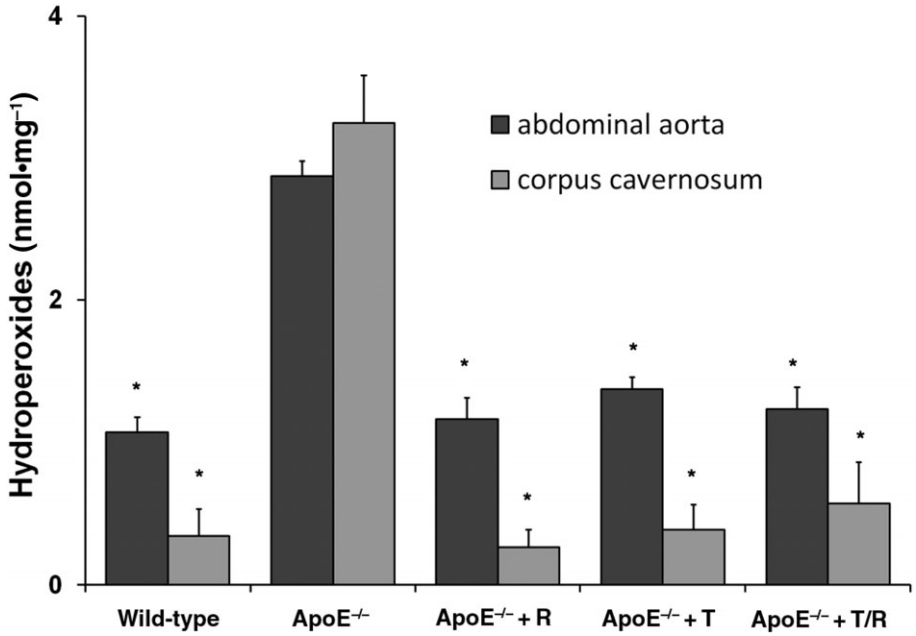

Figure 5

Oxidative stress (lipid peroxidation). Levels of hydroperoxides were assessed in abdominal aortic segments and corpora cavernosal tissue and expressed in nmol (mg protein)$^{-1}$. Apolipoprotein-E-deficient (ApoE$^{-/-}$) mice were treated with ramipril (R), or telmisartan (T) or in combination (T/R). Data shown are means ± SEM from five animals per group. *P < 0.05, different from values for ApoE$^{-/-}$ mice.

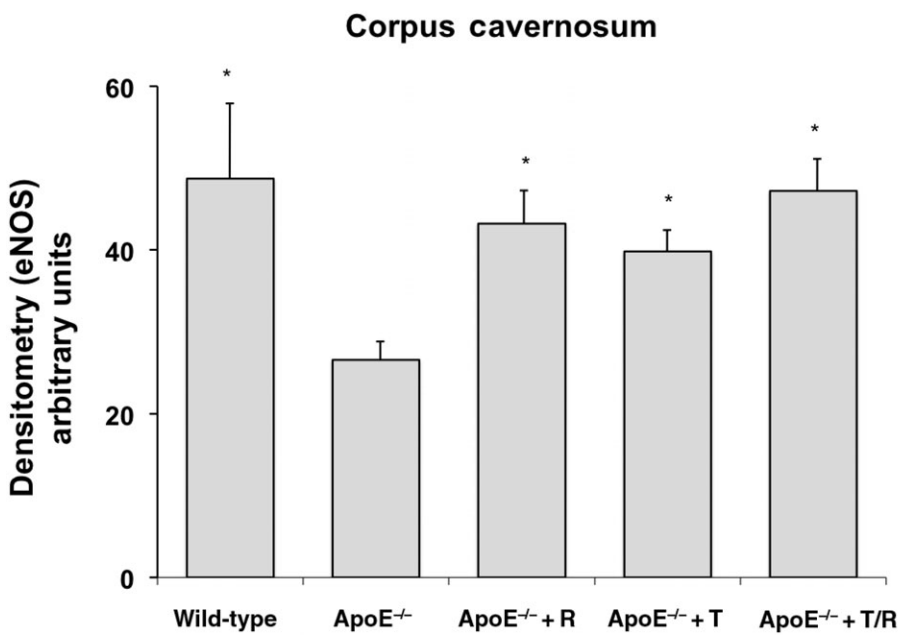

Figure 6

Immunohistochemical staining for eNOS. The amounts of eNOS were quantified by immunohistochemical staining of corpora cavernosa sections following fluorescence microscopy. Apolipoprotein-E-deficient (ApoE$^{-/-}$) mice were treated with ramipril (R), or telmisartan (T) or in combination (T/R). Data shown are means ± SEM from five mice per group. *P < 0.05, different from values for ApoE$^{-/-}$ mice.

ApoE$^{-/-}$ mice, an established murine model of atherosclerosis and ED (Behr-Roussel *et al.*, 2006; Kolovou *et al.*, 2008). All ApoE$^{-/-}$ mice developed equal hypercholesterolaemia, as a pre-condition of the severity of onset in metabolic changes initiating endothelial dysfunction and haemodynamic changes (Kolovou *et al.*, 2008). However, there was a not significant reduction in serum levels of total cholesterol as well as LDL- and high density lipoprotein-cholesterol in the

combination treatment group. This trend might be explained by complete inhibition of detrimental effects of Ang II on inflammation parameters and insulin signalling with consequent increase of cholesterol levels (Munoz *et al.*, 2009). Nevertheless, these effects are not likely to influence results on endothelial function and atherosclerosis.

Blood pressure was reduced by either means of RAAS blockade, in comparison to untreated animals. Lowering of systolic blood pressure in the telmisartan and combination therapy groups was slightly and not significantly greater than that achieved by ramipril treatment alone. RAAS blockade by any intervention significantly improved endothelium-dependent relaxation, equally for all treatments. Similarly, RAAS blockade attenuated atheromatous plaque formation in ApoE$^{-/-}$ mice, with no differences between treatments.

Reduction in blood pressure below a certain level in patients treated with telmisartan, ramipril or both has recently been shown not to any increased benefit in myocardial infarction, while cardiovascular mortality was unchanged or increased in a large population of cardiovascular high-risk patients from the ONTARGET-trial (Sleight *et al.*, 2009). Furthermore, it has been suggested that cardioprotective effects attributed to blockade of the RAAS are only in part due to a reduction in blood pressure (Baumhakel *et al.*, 2008; Jankowski *et al.*, 2009). The AT_1 receptor promotes pro-inflammatory, pro-fibrotic, pro-thrombotic and apoptotic effects, which lead to endothelial dysfunction and are largely independent of pressure-mediated endothelial damage in experimental settings (Jankowski *et al.*, 2009). Activation of interleukins, cytokines, adhesion molecules and growth factors is dependent on AT_1 receptor stimulation by Ang II (Skultetyova *et al.*, 2007). Activation of AT_1 receptors also leads to imbalance of the oxidative homeostasis of endothelial cells through excessive production of ROS, mainly via AT_1 receptor-responsive enzymes such as nicotinamide adenine dinucleotide phosphate-oxidase (Taniyama and Griendling, 2003). Excessive production of ROS leads to dysfunction of the endothelium by scavenging nitric oxide and subsequently reducing its protective effects on the endothelium (Li and Forstermann, 2000; Landmesser and Harrison, 2001). Consistent with these earlier reports, oxidative stress was increased in aortic and penile tissues in this study.

In the corpus cavernosum, Ang II leads to trabecular contraction, thereby initiating detumescence, by activating cGMP-dependent protein kinases which reduce endothelial nitric oxide production via eNOS, thereby reducing trabecular smooth muscle cell relaxation via guanylyl cyclase while impeding the nitric oxide-mediated inhibition of calcium influx into trabecular smooth muscle cell (Blatter and Wier, 1994; Kifor *et al.*, 1997; Park *et al.*, 1997). This interaction of Ang II and nitric oxide in erectile tissue provides the mechanistic rationale for RAAS blockade to improve erectile function. The more than twofold increase of ROS in ApoE$^{-/-}$ tissues might be involved in the development of endothelial dysfunction in aortic and cavernosal tissues in the present study. Consistent with this possibility, RAAS blockade led to significant reduction in ROS formation in both tissues along with the observed improvement in endothelial function. Moreover, semiquantitative measurement of eNOS by immunohistological methods, revealed a recovery of eNOS in animals with RAAS blockade. The combination of RAAS blockers was no better than each blocker alone in improving endothelial dysfunction and atherogenesis and also in reducing ROS load.

The fact that telmisartan and ramipril monotherapies improved vascular and cavernosal endothelial function and oxidative stress to values, almost equal to those in WT animals, shows that appropriate doses of either an AT_1 receptor antagonist or an ACE inhibitor will provide adequate vascular protection, leaving little room for improvement by dual RAAS blockade. In clinical trials on heart failure patients, additional therapy with a AT_1 receptor antagonist had favourable effects when ACE inhibitor doses below the median were used (Krum *et al.*, 2004). Furthermore, the theoretical assumption that the reduced bradykinin inactivation under dual RAAS blockade, compared to monotherapy with a AT_1 receptor antagonist might yield favourable effects seems to be less applicable to states of advanced vascular injury. Bradykinin effects in rabbits fed atherogenic diet were less pronounced when compared to animals on a standard Western diet (Weckler *et al.*, 2003). However, the role of bradykinin remains unclear in the results presented. One indication of additional effects of bradykinin on endothelial function might be that, in ramipril-treated animals, the improvement of endothelial function was comparable to that in telmisartan treated animals, in spite of a more pronounced blood pressure reduction in the latter animals.

Finally, it is possible that potentially protective AT_2 receptor-mediated pathways might be diminished in presence of ACE inhibitors, due to decreased Ang II levels. Regardless of the contribution of these possibilities to the physiologically observed equivalence of dual RAAS blockade in this study, our results show that oxidative stress in aortic and cavernosal tissue and subsequent peroxidation of membrane lipids can be effectively reduced by any type of RAAS blockade at appropriate doses.

Limitations of this study

The ApoE$^{-/-}$ mouse used for the experiments is a transgenic model of hypercholesterol-induced atherosclerosis. Thus, a significant decrease of blood pressure in the treatment groups in these normotensive animals might be an expression of overdosage and translation of the results into humans remains to be demonstrated. Nevertheless, the lack of intergroup difference might be the same in humans.

Functional organ bath chamber experiments assessed endothelial function of the corpus cavernosum, which plays a major role in physiology of penile erection. However, despite evidence for impaired erectile function in ApoE$^{-/-}$ mice, we did not measure improvement of erectile function itself, by treatment with RAAS blockade, but used a suggested surrogate for this function (Behr-Roussel *et al.*, 2006).

In conclusion, telmisartan and ramipril proved to be equally effective in restoring impairment of endothelial function of the aorta and corpus cavernosum, the latter as a possible surrogate of improvement of erectile function, in ApoE$^{-/-}$ mice. Furthermore atheromatous plaque load occurring subsequent to endothelial dysfunction was reduced to a similar extent. These beneficial effects are largely blood pressure-independent and are associated with a reduction of vascular and cavernosal oxidative stress as well as expression

of eNOS, as one pathophysiological link. Dual RAAS blockade did not lead to further improvement of these parameters at the respective doses.

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Conflict of interest

M Böhm was a member of the steering committee of the ONTARGET/TRANSCEND trials.

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