Angiotensin II Induces Endothelial Xanthine Oxidase Activation
Role for Endothelial Dysfunction in Patients With Coronary Disease

Ulf Landmesser, Stephan Spiekermann, Christoph Preuss, Sajoscha Sorrentino, Dieter Fischer, Constantina Manes, Maja Mueller, Helmut Drexler

Objective—Xanthine oxidase (XO), a major source of superoxide, has been implicated in endothelial dysfunction in atherosclerosis. Mechanisms, however, leading to endothelial XO activation remain poorly defined. We tested the effect of angiotensin II (Ang II) on endothelial XO and its relevance for endothelial dysfunction in patients with coronary disease.

Methods and Results—XO protein levels and XO-dependent superoxide production were determined in cultured endothelial cells in response to Ang II. In patients with coronary disease, endothelium-bound XO activity as determined by ESR spectroscopy and endothelium-dependent vasodilation were analyzed before and after 4 weeks of treatment with the AT1-receptor blocker losartan, the XO inhibitor allopurinol, or placebo. Ang II substantially increased endothelial XO protein levels and XO-dependent superoxide production in cultured endothelial cells, which was prevented by NAD(P)H-oxidase inhibition. In vivo, endothelium-bound XO activity was reduced by losartan and allopurinol, but not placebo therapy in patients with coronary disease. XO inhibition with oxypurinol improved endothelium-dependent vasodilation before, but not after losartan or allopurinol therapy.

Conclusions—These findings suggest a novel mechanism whereby Ang II promotes endothelial oxidant stress, ie, by redox-sensitive XO activation. In patients with coronary disease, losartan therapy reduces endothelium-bound XO activity likely contributing to improved endothelial function. (Arterioscler Thromb Vasc Biol. 2007;27:000-000.)

Key Words: endothelium ■ coronary disease ■ free radicals ■ xanthine oxidase ■ angiotensin II

Increased vascular production of reactive oxygen species (ROS) is thought to contribute importantly to endothelial dysfunction in experimental atherosclerosis and in patients with coronary disease, in part because of rapid inactivation of endothelial nitric oxide (NO) by superoxide.3–6 Furthermore, accumulating evidence suggests that endothelial dysfunction as determined by impaired endothelium-dependent vasodilation is closely associated with cardiovascular events.7–10 Therefore, there is a major interest to further understand mechanisms underlying increased vascular production of reactive oxygen species in atherosclerosis.

Xanthine oxidase (XO) has been identified as a major endothelial source of superoxide1,2,11,12 that is activated in experimental atherosclerosis.1,2 Moreover, we and others have recently demonstrated that endothelial XO activity and protein levels are substantially increased in patients with coronary disease or carotid stenosis,11,13,14 and inversely related to endothelium-dependent vasodilation.11 In addition, serum levels of uric acid, the product of xanthine oxidase, have been suggested as a predictor of cardiovascular disease mortality.15,16 The mechanisms, however, leading to increased endothelial XO activation in atherosclerosis remain to be determined.

Angiotensin II (Ang II) represents a major stimulus of endothelial superoxide production in experimental atherosclerosis and likely in patients with coronary disease.17–19 We therefore hypothesized that Ang II may impact on endothelial xanthine-oxidase protein levels and XO-mediated superoxide production and determined the effect of Ang II on endothelial XO protein levels and XO-mediated superoxide production as assessed by electron spin resonance (ESR) spectroscopy in vitro. Of note, xanthine oxidoreductase is synthesized as xanthine dehydrogenase and needs to be converted to XO to become a source of superoxide.9,20 Recent studies have suggested that reactive oxygen species may trigger XO-mediated superoxide production in endothelial cells, and endothelial cells from mice deficient in the NAD(P)H oxidase subunit p47phox had reduced XO activity.20,21 We therefore tested the question of whether another source of reactive oxygen species may contribute to endothelial XO activation by Ang II and examined in particular the role of the NAD(P)H oxidase in this process, because Ang II is known to stimulate endothelial NAD(P)H oxidase activity.22,23

Furthermore, we hypothesized that Ang II may play an important role for increased endothelial XO activation in

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patients with coronary disease. To address this question we determined the effect of chronic AT1 receptor blocker therapy as well chronic XO inhibition by allopurinol on endothelial-bound xanthine-oxidase activity in vivo measured by electron spin resonance spectroscopy in patients with coronary disease. Moreover, we evaluated the effect of acute XO inhibition by oxypurinol on endothelium-dependent vasodilation before and after chronic treatment with losartan or allopurinol in patients with coronary disease.

Methods

An expanded methods section is available online (please see http://atvb.ahajournals.org).

Endothelial Cell Culture

Bovine aortic endothelial cells (BAECs; Clonetics, East Rutherford, NJ) were used between passages 3 and 6 for experiments. BAECs were stimulated with 10^-5 mol/L of Ang II for 12 hours, a time point that was selected according to the time course of XO protein levels in response to Ang II (Figure 1A).

NAD(P)H oxidase activation was inhibited by pretreatment (12 hours) with apocynin (500 μmol/L) or siRNA transfection for p47phox as described in detail in the online supplement. To exclude that the effect of Ang II on endothelial XO-mediated superoxide production was species specific we performed additional experiments in human aortic endothelial cells (HAECs; PromoCell, Heidelberg, Germany) that were used between passages 3 and 5.

In Vivo Protocol in Patients With CAD

15 patients with angiographically documented stable CAD (left ventricular ejection fraction >45%) were randomized to 4 weeks of treatment with the XO inhibitor allopurinol (300 mg bid), and endothelium-bound XO and endothelium-dependent vasodilatation were determined before and after chronic oral XO inhibition.

Patients with an acute coronary syndrome; diabetes mellitus; uncontrolled hypertension; hematologic, renal, or hepatic dysfunction; heparin therapy within the last 48 hours; or patients taking antioxidant vitamin supplements were excluded. Vasoactive medications were withheld, and alcohol and caffeine were prohibited for at least 12 hours before the study. Written informed consent was obtained for all subjects, and the protocol was approved by the local Ethics Committee.

Statistical Analysis

All data are expressed as mean±SEM. Comparisons of >2 measurements were done by one-way ANOVA. For the clinical study, both treatment group and time in addition to oxypurinol treatment were considered in the statistical analysis by performing a mixed model analysis using the PROC MIXED statistical procedure (SAS statistical software version 9.1) with "patients" as the random factor and "treatment group", "time", "oxypurinol", and the resulting interactions thereof as fixed effects. This analysis yielded a probability value for the effect "treatment group" × "time" of \(P=0.0097\). One-sided paired \(t\) tests or \(t\) tests for independent groups were used for further analysis, resulting in probability values as depicted in Figures 4 and 5. The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results

Effect of Ang II on Endothelial XO Protein Levels and XO-Mediated Superoxide Production In Vitro

Ang II administration (10^-7 mol/L) caused a marked increase in XO protein levels in cultured BAECs (Figure 1A). Endothelial superoxide production as determined by electron spin resonance (ESR) spectroscopy (spin trap: CP-H) was increased with a similar time course as compared with endothelial XO protein levels after exposure to Ang II (Figure 1B). Importantly, Ang II-induced endothelial superoxide production was markedly reduced by two structurally different XO inhibitors, ie, oxypurinol and tungsten, suggesting that XO

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contributes critically to Ang II-induced endothelial superoxide formation (Figure 1C and 1D). In preliminary experiments neither higher (10^{-6} mol/L) nor lower (0.5 \times 10^{-7}) concentrations of Ang II resulted in a more pronounced stimulation of endothelial XO protein levels (data not shown). There was no effect of Ang II on endothelial xanthine dehydrogenase protein levels (data not shown).

Of note, the Ang II-induced increase of both endothelial XO protein levels and superoxide production was blocked by cotreatment with the AT1 receptor antagonist losartan, but not by the AT2-receptor antagonist PD123319 (supplemental Figure I), suggesting that endothelial XO activation is mediated by the AT1-receptor.

Role of the NAD(P)H-Oxidase for Ang II-Induced Endothelial XO Activation

Importantly, both treatment with the NAD(P)H-oxidase inhibitor apocynin or transfection with small interference RNA (siRNA) specific for the NAD(P)H oxidase subunit p47phox prevented Ang II–induced increase of endothelial XO protein levels (Figure 2). Moreover, in endothelial cells treated with the NAD(P)H oxidase inhibitor apocynin or transfected with p47phox specific siRNA there was no increase of superoxide production after stimulation by Ang II (Figure 2).

To further characterize mechanisms leading to Ang II–induced increases in endothelial XO protein levels we pretreated endothelial cells with N-acetyl-cysteine, the hydrogen peroxide scavenger catalase, with catalase and superoxide dismutase and with the NO synthase inhibitor L-NAME (supplemental Figure II). Catalase and NO-synthase inhibition with L-NAME reduced the increase of endothelial XO protein levels in response to Ang II (supplemental Figure II). These experiments suggest that both hydrogen peroxide and peroxynitrite are involved in Ang II–induced increases of endothelial XO protein levels.

To exclude that the effect of Ang II on endothelial XO-mediated superoxide production was species specific we performed experiments in HAECs, which have been previously shown to contain XO activity.24 In these experiments we observed a similar inhibition of Ang II–stimulated superoxide production by the XO inhibitors oxypurinol and tungsten as observed in BAECs (data not shown). To further determine the relevance of Ang II–induced endothelial XO activation in patients with coronary disease we performed a clinical study:

Effect of AT1-Receptor Antagonism by Losartan and Chronic XO Inhibition by Allopurinol on Endothelium-Bound Xanthine-Oxidase Activity in Patients With Coronary Disease In Vivo

As shown in Figure 3, there was a significant release of XO activity from the endothelium into plasma after heparin bolus injection in patients with coronary disease as detected by ESR spectroscopy. The left panel shows increase of plasma XO activity at 3, 5, 7, and 10 minutes after heparin bolus injection before and after 4 weeks of treatment with placebo (A), losartan (B), or allopurinol (C). The right panel shows endothelium-bound XO activity (area under the curve of XO activity released by heparin bolus injection).
The major findings of the present study are: (1) Ang II markedly increases endothelial XO protein levels and XO-dependent endothelial superoxide production, suggesting that XO is a major source of superoxide activated by Ang II. (2) Ang II–induced increases of endothelial XO protein levels and superoxide production from XO are prevented by NAD(P)H-oxidase inhibition, suggesting that Ang II activates endothelial XO by a redox-sensitive pathway involving the NADPH oxidase. (3) In vivo, in patients with coronary disease, endothelium-bound XO activity is substantially reduced after 4 weeks of AT1-receptor blocker therapy with losartan, but not after placebo treatment as determined by electron spin resonance spectroscopy, suggesting that Ang II is important for endothelial XO activation in human coronary disease. (5) The effect of XO inhibition by oxypurinol on endothelium-dependent vasomotion is substantially reduced after chronic treatment with the AT1-receptor blocker losartan in patients with coronary disease, compatible with the notion that Ang II–dependent endothelial XO activation contributes to endothelial dysfunction in coronary disease. Furthermore, chronic XO inhibition by allopurinol inhibits endothelium-bound XO activity to a similar extent as compared with losartan treatment and improves endothelium-dependent vasodilation in patients with coronary disease, further supporting the concept that inhibition of endothelial XO contributes to beneficial effects of losartan on endothelial function in patients with coronary disease.

Increased vascular reactive oxygen species production, in particular superoxide, has been suggested as a major cause of endothelial dysfunction in both experimental atherosclerosis and patients with coronary disease. Accumulating evidence suggests that endothelial dysfunction as determined by impaired endothelium-dependent vasodilation is associated with cardiovascular events, likely in part attributable to a loss of vasopro-
ective properties of endothelial nitric oxide. Therefore, there has been a major interest to understand mechanisms leading to increased vascular superoxide production and consequen-

tively endothelial dysfunction in atherosclerosis. Experimental studies have identified endothelial XO as a potential major source of endothelial superoxide in atherosclerosis. Administration of the XO inhibitor allopurinol or its metabolite oxypurinol reduced vascular superoxide production and improved endothelium-dependent vasodilation in hypercholesterolemic rabbits. In addition, Cardillo et al have described that administration of oxypurinol improved endothelium-dependent vasodilation in patients with hypercholesterolemia. A beneficial effect of XO inhibition on endothelial function has also been observed in patients with other cardiovascular risk factors or heart failure. Furthermore, we and others have recently observed that endothelium-bound XO activity is markedly increased in patients with coronary disease, carotid stenosis, or heart failure and is inversely related to endothelium-dependent vasodilation. In addition, serum levels of uric acid, the product of XO, have been suggested as a predictor of cardiovascular mortality. The mechanisms, however, leading to activation of endothelial XO in atherosclerosis remained largely undefined.

In the present study we provide evidence that Ang II represents a potent stimulus of endothelial XO protein levels and XO-dependent endothelial superoxide production in vitro. Of note, the conversion of xanthine dehydrogenase to XO that is promoting superoxide formation by the enzyme has been shown to be stimulated by reactive oxygen species, in particular peroxynitrite. Moreover, McNally et al have demonstrated increased XO-dependent superoxide production in tissue cultures and increased levels in response to peroxynitrite. However, no increase of XO protein levels was observed in these studies. In contrast, in the present study there was a marked increase of endothelial XO protein levels, but not xanthine dehydrogenase protein levels in response to Ang II. Furthermore, the present study suggests that NAD(P)H oxidase and subsequently hydrogen peroxide and peroxynitrite contribute to increased endothelial XO protein levels in response to Ang II.

Notably, in coronary arteries from patients with coronary disease an increase of XO, but not xanthine dehydrogenase protein levels has recently been observed. The present study suggests, as discussed below, that Ang II is a major stimulus leading to increased vascular XO protein levels in coronary disease. Importantly, we have observed that endothelium-bound XO activity as determined by ESR-spectroscopy is markedly reduced after chronic AT1-receptor blocker therapy with losartan in patients with coronary disease, but not after placebo treatment. This could represent a novel mechanism whereby AT1-receptor blocker therapy improves endothelial function in patients with coronary disease. To further evaluate this concept we have determined the effect of the XO inhibitor oxypurinol on endothelium-dependent vasodilation before and after chronic AT1-receptor blocker therapy in patients with coronary disease. These studies revealed a marked reduction of the effect of oxypurinol on endothelium-dependent vasodilation in patients with CAD after AT1-receptor blockade, compatible with the concept that Ang II-dependent XO activation contributes to endothelial dysfunction in coronary disease.

We cannot, however, exclude the possibility that improved endothelium-dependent vasodilation after losartan therapy may have contributed to a reduced effect of oxypurinol on endothelium-dependent vasodilation after losartan therapy in patients with coronary disease. To further address this issue we have studied the effect of 4 weeks of allopurinol therapy on endothelium-bound XO activity and endothelial function in patients with coronary disease. Allopurinol treatment resulted in a similar reduction of endothelium-bound XO activity as compared with losartan treatment, and improved endothelium-dependent vasodilation, further suggesting that reduced endothelial XO activation after losartan therapy contributes to beneficial effects on endothelium-dependent vasodilation. Of note, other mechanisms in addition to XO inhibition are likely to contribute to beneficial effects of losartan therapy on endothelial function as well, because the effect of losartan therapy on endothelium-dependent vasodilation appeared to be more pronounced as compared with allopurinol treatment, despite a similar reduction of endothelium-bound XO activity. These include prevention of NAD(P)H oxidase-dependent superoxide production, increased scavenging of superoxide by extracellular superoxide dismutase, and increased activation of the AT1 receptor after blockade of the AT1 receptor.

Study Limitations

The number of patients in the placebo group of the clinical part of the present study represents a potential limitation. Because the novel ESC guidelines do recommend ACE inhibitor therapy for patients with coronary disease, our ethics committee did not permit the study of further patients on placebo treatment. However, in this regard, it must be emphasized that the measurements of the endothelial release of XO were performed by ESR spectroscopy at multiple time points after heparin bolus injection to have a particular high accuracy of these analyses.

In addition, the present study was not powered to assess the impact of losartan treatment on uric acid serum levels, the product of XO: However, a recent analysis of the LIFE study has shown an attenuation of the increase of serum uric acid levels over 4.8 years of losartan treatment, which was not observed with atenolol therapy, and this has been suggested to explain up to 29% of the treatment effect on the primary composite end point. It is therefore tempting to speculate that inhibition of endothelium-bound XO may contribute to prevention of an increase of uric acid serum levels over time in addition to an uricosuric effect of losartan.

In summary, the present study describes a novel mechanism of endothelial oxidant stress in response to Ang II, ie, redox-sensitive activation of endothelial XO (Figure 5). We further provide evidence that Ang II–dependent stimulation of endothelial XO activity plays a major role for increased endothelium-bound XO activity in patients with coronary disease, because endothelium-bound XO activity was markedly reduced after chronic AT1-receptor blockade as determined by ESR spectroscopy. In addition, our findings are compatible with the notion that Ang II–dependent XO activation contributes to endothelial dysfunction in patients with coronary disease.
II. Ang II results in endothelial NAD(P)H oxidase activation via the AT₁-receptor, which in turn increases endothelial NMD-mediated superoxide production thereby promoting oxidant stress in the endothelium.

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Disclosures
None.

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Angiotensin II induces endothelial xanthine oxidase activation: role for endothelial dysfunction in patients with coronary disease

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METHODS

Endothelial Cell Culture: Bovine aortic endothelial cells (BAECs; Clonetics, East Rutherford, NJ) were cultured in microvascular endothelial cell medium (EGM-MV; Clonetics, Solingen, Germany) supplemented with endothelial growth factors (EGM-MV BulletKit, Clonetics) and 5% fetal calf serum (FCS). BAECs between passages 3 and 6 were used for experiments. Human aortic endothelial cells (HAECs; PromoCell, Heidelberg, Germany) were cultured in endothelial cell growth medium 2 (PromoCell) supplemented with endothelial growth factors (SupplementPack, PromoCell) and 2% FCS. HAECs were used between passages 3 and 5.

For experiments, endothelial cells were stimulated with $10^{-7}$ mol/L of angiotensin II, since this concentration has been shown to result in a profound increase of endothelial cell superoxide production. In previous studies, a higher concentration of angiotensin II did not result in a more pronounced stimulation of endothelial cell superoxide production, that has been suggested to be a consequence of AT$_2$ receptor activation. Endothelial cells were stimulated for 12 hours with angiotensin II, a time point that was selected according to the time course of xanthine oxidase protein levels in response to angiotensin II (Figure 1 A).

Two structurally different XO-inhibitors, i.e. oxypurinol ($10^{-6}$ mol/l) and tungsten ($10^{-6}$ mol/l) were used, and endothelial cells were pretreated with these inhibitors 12 hours prior to exposure to angiotensin II. To examine the role of the AT$_1$ and AT$_2$ receptor for angiotensin II dependent effects on endothelial xanthine oxidase, endothelial cells were pretreated with
the AT\textsubscript{1}-receptor antagonist losartan (10\textsuperscript{-6} M) or the AT\textsubscript{2}-receptor antagonist PD 123319 (10\textsuperscript{-7} M) 12 hours prior to exposure to angiotensin II. These concentrations of losartan and PD 123319 have been shown previously to effectively prevent responses mediated by the AT\textsubscript{1} or AT\textsubscript{2} receptor, respectively.\textsuperscript{4,5} NAD(P)H oxidase activation was inhibited in endothelial cells by pre-treatment (12 hours) with apocynin (500 µmol/L), that prevents association of the cytosolic subunit p47\textsubscript{phox} with the membrane-bound catalytic subunit of NADPH oxidase\textsuperscript{6} or siRNA transfection for p47phox as described below.

**Measurements of Superoxide Production in Cultured Endothelial Cells:** Endothelial cell superoxide (O\textsubscript{2}\textsuperscript{-}) production was measured by using electron-spin resonance spectroscopy (ESR) and the spin trap 1-hydroxy-3-carboxy-pyrrolidine (CP-H; Alexis Corporation, Lausen, Switzerland) as described in detail previously.\textsuperscript{7,9} In brief, cells were rinsed in 50 mmol/L PBS buffer (pH 7.4; 37°C) and removed from the plate by gentle scrapping. After centrifugation (8000 U/min; 8 minutes), cells were resuspended in 400 µL PBS buffer (37 °C) containing an antiproteolytic cocktail (Complete mini; Merck, Darmstadt, Germany) and kept at 37°C until measurement. To inhibit iron-catalyzed oxidation of the spin trap, DTPA (0.2 mmol/L) was added to all samples. ESR measurements were performed in 50-µL glass capillaries (Corning, NY, USA). The ESR spectra were recorded using a MiniScope ESR spectrometer (MagnetTech, Berlin, Germany). O\textsubscript{2}\textsuperscript{-} formation was determined by following the oxidation of CP-H to paramagnetic 3-carboxy-proxyl (CP).\textsuperscript{10-12}

The ESR instrumental settings were as follows: field sweep, 108 G; microwave frequency, 9.78 GHz; microwave power, 20 mW; modulation amplitude, 2 G; conversion time, 656 ms; time constant, 656 ms; 4096 points resolution and receiver gain, 1x10\textsuperscript{5} (74 dB), for plasma 1x10\textsuperscript{-3}. Time scans were recorded using 1312 ms conversion time, 5248 ms time constant, and monitoring the ESR amplitude of low-field component of ESR spectrum of carboxy-proxyl nitroxide for 300 s.
**Immunoblot Analysis:** Protein (30 µg) of endothelial cell lysates was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to membranes, and immunoblotted with a monoclonal anti-XO antibody (NeoMarkers Laboratory Vision, Fremont, CA, USA). The anti-XO antibody was used at a dilution of 1:200 and the secondary antibody was used at a dilution of 1:2000. Protein was visualized with an enhanced chemiluminescence detection system.

**Transfection of BAECs with p47phox-specific small interference RNA (siRNA):** To knock-down p47phox expression in BAECs, cells were transfected with small interference RNA specific for bovine p47phox according to the manufactures' instructions (Qiagen; “4 for silencing” package). Small interference RNA was designed by targeting the p47phox sequence 5'AAAGCCAGAGACATACCTGAT 3', according to the siRNA design guidelines (www.qiagen.com, Qiagen Inc, Fremont, CA, USA). The sequence 5'AATTCTCCGAACGTGTCACGT 3' was used as the scrambled siRNA control; it does not match any mammalian sequences currently available in online databases. Transfections were performed when the cells were 60-70% confluent according to the manufacturer’s instruction. In preliminary experiments, we found that >80% of endothelial cells were transfected, as judged by transfection with fluorescein–labeled siRNA. More importantly, expression of p47phox was inhibited by >70% as estimated by western analysis (data not shown).

**Measurement of Endothelium-bound XO Activity In Vivo by using ESR Spectroscopy:** XO is localized and bound to the surface of endothelial cells by glycosaminoglycans\textsuperscript{13} and is released from endothelium into plasma by heparin bolus injection,\textsuperscript{7, 8, 14} allowing determination of eXO activity in humans in vivo. Determination of endothelium-bound XO-activity was performed as described in detail previously with some modifications.\textsuperscript{7, 8} In brief, for measurement of plasma XO at baseline, 2 arterial (brachial artery) and 2 venous (antecubital vein) blood samples were drawn. Then, 5000 IU of heparin were injected into the
brachial artery, and blood samples were drawn from the antecubital vein of the same arm (3, 5, 7, and 10 minutes after heparin) into EDTA-containing vacuum tubes. Blood samples were centrifuged immediately (1500g for 15 minutes at 4°C), and plasma was stored at -80°C. The instrument settings for ESR measurements and the reagents used were the same as described above for in vitro measurements. The intensity of ESR spectra was quantified, and the ESR signal of plasma without xanthine (obtained for each sample) was subtracted. The activity of eXO (O2·−/µl plasma per min) was calculated as area under the curve of the increase of plasma XO activity within 10 minutes after heparin bolus injection. In preliminary studies we have observed that inhibition of SOD did not change XO measurements in plasma samples, suggesting that the amount of SOD released after heparin bolus injection is too low to interfere with XO measurements (data not shown).

**Measurement of flow-dependent, endothelium-mediated vasodilation (FDD):** Radial artery diameters were measured using a high-resolution ultrasound system (ASULAB, Marin, Switzerland). A wrist arterial occlusion (8 minutes) was performed, and FDD in response to reactive hyperemic blood flow was assessed. Sodium nitroprusside was infused (SNP; 10 µg/min IA; 5 minutes) to assess endothelium-independent vasodilation. This method is well established in our laboratory, has an excellent reproducibility and variability, and was used as described in detail previously. All measurements were recorded, and vessel diameters were subsequently analyzed by 2 investigators unaware of the interventions.

Blood flow velocity was recorded continuously, radial artery diameter was determined every 30 seconds until stable baseline conditions were obtained (approximately 30 minutes). Then a wrist arterial occlusion (8 min.) was performed and FDD in response to reactive hyperemic blood flow was assessed. When radial artery diameter and blood flow had returned to
baseline values the XO-inhibitor oxypurinol was infused (600µg/min; 10 min.; brachial artery) followed by determination of FDD.

REFERENCES:


SUPPLEMENTAL FIGURE LEGENDS

**Figure I:**  
*A,* Effect of the AT₁ receptor antagonist losartan (10⁻⁶ mol/l) and the AT₂ receptor antagonists PD 123319 (10⁻⁷ mol/l) on endothelial xanthine oxidase protein levels in response to angiotensin II (BAECs). A representative western blot of XO protein levels is shown. n=3-6.  
*B,* Effect of losartan and PD 123319 on endothelial superoxide production in response to angiotensin II. n=4-8.

**Figure II:**  
Mechanisms of redox-sensitive xanthine oxidase activation by angiotensin II. Effect of pre-treatment of endothelial cells with N-acetyl-cysteine (10⁻⁶ mol/L), the hydrogen peroxide scavenger catalase (10⁻⁶ mol/L), catalase + PEG-SOD (150 U/mL) and the NO synthase inhibitor L-NAME (10⁻⁴ mol/L) on angiotensin II induced xanthine oxidase protein levels is shown. n=3-6.
Fig. I

A

90 kDa

XO protein levels

P < 0.05

P < 0.05

Ang II

Losartan

PD-123319

B

Superoxide production

nmol O₂⁻ x µg⁻¹ protein

P < 0.01

P = n.s.

Ang II

Losartan

PD-123319

Ang II

Losartan

PD-123319
Fig. II

% Change of XO protein levels

- AngII
- AngII + NAC
- AngII + Catalase
- AngII + Cat. + SOD
- AngII + L-NAME

P < 0.05
P < 0.01
P < 0.05