Reactive Oxygen Species and Cyclooxygenase 2–Derived Thromboxane A2 Reduce Angiotensin II Type 2 Receptor Vasorelaxation in Diabetic Rat Resistance Arteries

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Abstract—Angiotensin II has a key role in the control of resistance artery tone and local blood flow. Angiotensin II possesses 2 main receptors. Although angiotensin II type 1 receptor is well known and is involved in the vasoconstrictor and growth properties of angiotensin II, the role of the angiotensin II type 2 receptor (AT2R) remains much less understood. Although AT2R stimulation induces vasodilatation in normotensive rats, it induces vasoconstriction in pathological conditions involving oxidative stress and cyclooxygenase 2 expression. Thus, we studied the influence of cyclooxygenase 2 on AT2R-dependent tone in diabetes mellitus. Mesenteric resistance arteries were isolated from Zucker diabetic fatty (ZDF) and lean Zucker rats and studied using in vitro using wire myography. In ZDF rats, AT2R-induced dilation was lower than in lean rats (11% versus 21% dilation). Dilation in ZDF rats returned to the control (lean rats) level after acute superoxide reduction (Tempol and apocynin), cyclooxygenase 2 inhibition (NS398), or thromboxane A2 synthesis inhibition (furegrelate). Cyclooxygenase 2 expression and superoxide production were significantly increased in ZDF rat arteries compared with arteries of lean rats. After chronic treatment with Tempol, AT2R-dependent dilation was equivalent in ZDF and lean rats. Chronic treatment of ZDF rats with NS398 also restored AT2R-dependent dilation to the control (lean rats) level. Plasma thromboxane B2 (thromboxane A2 metabolite), initially high in ZDF rats, was decreased by chronic Tempol and by chronic NS398 to the level found in lean Zucker rats. Thus, in type 2 diabetic rats, superoxide and thromboxane A2 reduced AT2R-induced dilation. These findings are important to take into consideration when choosing vasoactive drugs for diabetic patients. (Hypertension. 2010;55:339-344.)

Key Words: type 2 diabetes mellitus ■ angiotensin II ■ angiotensin II type 2 receptor ■ cyclooxygenase 2 ■ oxidative stress ■ thromboxane A2 ■ resistance arteries

Resistance arteries play a key role in vascular homeostasis. They possess a basal tone modulated by neurohormonal systems, among which the renin-angiotensin system has a major role. This basal tone is modified in hypertension, ischemic disease, myocardial infarction, and diabetes mellitus. In diabetes mellitus, blockade of the renin-angiotensin system efficiently reduces vascular damage.1 In addition, in the microcirculation, angiotensin-converting enzyme inhibition has a dual positive effect by increasing postischemic angiogenesis and reducing retinal microvascular damage induced by diabetes mellitus.2 Angiotensin II (Ang II) acts on 2 receptors, the type 1 receptor (AT1R) and the type 2 receptor (AT2R).3 Stimulation of the AT1R is associated with vasoconstriction and vascular cell growth,4 and its blockade explains a large part of the positive effects of targeting of the renin-angiotensin system in diabetes mellitus. Because AT2R has been shown to induce vasodilatation and stimulate anti-growth effects,5 its stimulation may exert an additional positive effect when used in conjunction with AT1R blockers. In fact, AT1R blockade induces AT2R upregulation6 and increases circulating Ang II.6 Nevertheless, we have shown previously that AT2R stimulation induces vasodilatation in normotensive rats but vasoconstriction in hypertensive animals.5,7,8 In human gluteal resistance arteries, AT2R-dependent dilation is reduced in diabetic hypertensive patients. Nevertheless, AT2R-dependent dilation is greatly increased in hypertensive type 2 diabetic patients who have been chronically treated with an AT1R blocker compared with patients treated with the β-blocker atenolol. This suggests that AT2R-dependent dilation could produce a beneficial effect in patients treated with an AT1R blocker.9 Thus, we hypothesized that AT2R stimulation might initiate a different pathway in type 2 diabetes mellitus leading to less vasodilation (as in diabetic patients) or to vasoconstriction (as seen in hypertension6 or aging9).
In diabetes mellitus, excess oxidative stress affects vascular tone, and AT2R-dependent dilation in the aorta is reduced by reactive oxygen species (ROS) generation in type 1 (streptozotocin-treated) diabetic rats. We have shown previously that the balance between NO and cyclooxygenase (COX) derivatives is modified in hypertension and may involve the inducible form of the enzyme, COX-2. Thus, in this study, we investigated the possible roles of ROS and COX-2 in alterations of AT2R-dependent dilation in resistance arteries from type 2 diabetic rats.

Materials and Methods

For an expanded Materials and Methods section, see the online Data Supplement at http://hyper.ahajournals.org.

Animals

Three-month-old male Zucker diabetic fatty (ZDF) and lean (LZ) control rats (Janvier, Le Genest Saint Isle, France) were divided into 3 groups. One was treated with 4-hydroxy-2,2,6,6-tetramethylpiperidinoxy (Tempol; 10 mg/kg per day in drinking water), whereas the other received tap water (n=10 per group). After 21 days, rats were anesthetized (isoflurane), the femoral artery blood pressure was measured, and blood glucose was determined as described previously. The mesentery was then removed and placed in ice-cold physiological salt solution. Several arterial segments from each rat were used for functional, biochemical, and histological studies, as described below.

In a separate series of experiments, rats were treated with the COX-2 inhibitor NS398 (25 mg/kg per day by forced feeding) for 3 weeks (n=6 per group). The procedure followed in the care and euthanasia of the study animals was in accordance with the European Community standards on the care and use of laboratory animals (authorization No. 00577).

Vascular Response to Exogenous Ang II in Isolated Mesenteric Arteries

Segments of mesenteric resistance arteries (MRAs) were mounted on a wire myograph (DMT), as described previously, and bathed in a physiological salt solution maintained at 37°C and pH 7.4 (PO2: 160 mm Hg; PCO2: 37 mm Hg). The physiological salt solution contained candesartan (10 nmol/L) throughout the protocol to block the AT1R. In separate experiments, Ang II (10 nmol/L) was then added to the bath before and after each of the following: Nω-nitro-L-arginine methyl ester (L-NAME); 100 μmol/L; indomethacin (10 μmol/L); the COX-2 blocker NS398 (10 μmol/L); the thromboxane A2 (TXA2) and prostaglandin H2 (PGH2) receptor blocker SQ29548 (10 μmol/L); the TXA2 synthesis inhibitor furegrelate (10 μmol/L); the AT2R blocker PD123319 (10 μmol/L); the AT1R/AT2R inhibitor saralasin (10 μmol/L); the ROS remover Tempol (10 μmol/L); or the ROS generation inhibitor apocynin (10 μmol/L).

Western Blot Analysis of β-Actin, AT2R, gp91phox, p67phox, Cu/Zn-SOD, and Mn-SOD Expression

Western blot analysis was performed as described previously.

Immunostaining of COX2

As described previously, segments of MRA were mounted in embedding medium (Tissu-Tek, Miles, Inc), frozen in isopentane precooled in liquid nitrogen, and stored at −80°C. Transverse cross-sections (7 μm thick) were used for COX-2 immunostaining. Fluorescence staining was visualized using confocal microscopy and image analysis (Histolab, Microvision).

Detection of ROS Using Confocal Microscopy in Resistance Arteries

ROS detection was performed on transverse cross-sections 7 μm thick using dihydroethidine (DHE) and microfluoroscopy, as described previously.

Blood Thromboxane B2 Measurement

Thromboxane B2 (TxB2; the stable metabolite of TxA2) was measured in the rat plasma, as described previously, using a commercially available kit (Cayman).

Statistical Analysis

Results were expressed as mean±SEM. Significance of the differences between groups was determined by ANOVA: 2 factor ANOVA analysis on the whole curve or 1-way ANOVA analysis followed by the Bonferroni test. P values <0.05 were considered to be significant.

Results

Rat body weight was significantly higher in ZDF rats (408±20 g) than in LZ rats (355±15 g). Chronic Tempol (401±17 g in ZDF rats; 342±10 g in LZ rats) or NS398 (412±19 g in ZDF rats; 348±14 g in LZ rats) did not significantly affect body weight. Similarly, blood glucose was significantly higher in ZDF rats (302±23 mg/dL) than in LZ rats (112±13 mg/dl) and was not modified by chronic Tempol (298±20 mg/dL in ZDF rats; 117±10 mg/dL in LZ rats) or NS398 (320±28 mg/dL in ZDF rats; 115±8 mg/dL in LZ rats). Mean blood pressure was not significantly different in ZDF rats (106±6 mm Hg) compared with LZ rats (98±4 mm Hg). Chronic Tempol (105±5 mm Hg in ZDF rats; 98±4 mm Hg in LZ rats) or NS398 (103±4 mm Hg in ZDF rats; 100±3 mm Hg in LZ rats) did not significantly affect blood pressure.

In the presence of candesartan, the acute stimulation of the AT2R with Ang II induced relaxation of the MRA (Figure 1A). After this initial experiment, Ang II at a concentration of 10 nmol/L was used. Ang II (10 nmol/L) induced a maximal dilation (Figure S1, please see the online Data Supplement at http://hyper.ahajournals.org). AT2R-dependent relaxation was significantly lower in ZDF control rats than in LZ control rats. Removal of the endothelium (Figure 1B) and blocking NO production with l-NAME (data not shown) suppressed AT2R-dependent dilation in both strains. Nevertheless, the precontraction level in the presence of l-NAME was sub-maximal, and this is likely to mask a possible AT2R-dependent contraction. The experiment was, thus, repeated in the absence of precontraction. In this condition and in the presence of l-NAME, Ang II induced contraction in ZDF rats (0.85±0.18 mN in ZDF rats; no detectable response in LZ rats). AT2R blockade with PD123319 and AT1R/AT2R blockade with saralasin abolished AT2R-dependent tone in ZDF and LZ rats. AT2R expression level was not different in ZDF and LZ rats (Figure 1B).

To assess the involvement of ROS in AT2R-mediated relaxation, we tested the effects of Tempol and apocynin on MRAs. When acutely applied, Tempol and apocynin did not significantly affect AT2R-dependent relaxation in LZ rats, but both substances increased AT2R-dependent dilation in ZDF rats to the level found in LZ rats (Figure 2A). Furthermore, in arteries from ZDF rats chronically treated with Tempol, AT2R-dependent relaxation was similar to that found in LZ rats (Figure 2B). The arterial ROS level measured using DHE staining was significantly higher in control ZDF rats than in control LZ rats. In LZ and ZDF rats chronically treated with Tempol, ROS were undetectable (Figure 2C).
inhibitor furegrelate on AT2R-mediated relaxation. In arteries from ZDF rats, SQ29548 and furegrelate did not significantly affect AT2R-dependent relaxation, whereas in arteries from ZDF rats SQ29548 and furegrelate significantly increased AT2R-dependent relaxation to the level found in LZ rats (Figure 4C). In ZDF and LZ rats chronically treated with Tempol or with NS398, SQ29548 and furegrelate did not modify AT2R-dependent relaxation (Figure 4B and 4C). Endothelium-independent relaxation to sodium nitroprusside was not affected by diabetes mellitus or by Tempol (data not shown).

Discussion

This study identified a major defect in AT2R-dependent relaxation in MRAs isolated from type 2 diabetic ZDF rats. AT2R-mediated relaxation was reduced because of excessive production of ROS and because of the production of TxA2 by COX-2. Chronic treatment with an antioxidant (Tempol) restored AT2R relaxation to the control level without suppressing COX-2 activity.

The presence of AT2R in the adult vasculature is now well recognized, although its role remains a matter of controversy.21–23 We have shown previously that Ang II does not induce AT2R desensitization22 and that the AT2R plays an important role in hypertension in rats treated with AT1R blockers.5 In fact, AT1R blockers induce an AT2R overexpression and increase circulating Ang II, thus reinforcing the beneficial effect of the AT1R blockade.23 Nevertheless, in untreated hypertensive rats or in old rats, AT2R stimulation induces vasoconstriction in MRAs.5,10 These observations led us to investigate AT2Rs in diabetes mellitus, another critical situation for resistance arteries.

We found that AT2R-dependent relaxation in lean-rat MRAs depended on the presence of the endothelium and was inhibited by NO synthesis blockade, whereas COX-1 and COX-2 inhibition with indomethacin had no effect. These findings are in agreement with our previous reports in the same arterial bed5,7,8,24 and in other arteries.23 AT2R-mediated relaxation was mainly endothelium dependent in both lean and diabetic rats. Indeed, AT2R was located mainly in the endothelium, and AT2R-mediated relaxation was abolished by endothelium removal (Figure 1).

A main new finding of the present study is that AT2R-dependent dilation in type 2 diabetic ZDF rats was strongly reduced compared with that of LZ rats. This is in agreement with a previous study performed in human gluteal resistance arteries in which it was found that AT2R-dependent dilation is reduced in diabetic patients and improved after chronic AT1R blockade.9 The present study brings new insights into the mechanisms leading to reduced AT2R-dependent dilation in type 2 diabetes mellitus. Because endothelium (NO)-dependent dilation in ZDF is reduced,20,25 we tested the effect of blocking NO synthesis on AT2R-mediated relaxation. Nevertheless, the hyporesponsiveness found in ZDF rats was not attributed to a defect in NO synthesis (l-NAME suppresses the relaxation in both LZ and ZDF rats) but to a concomitant overproduction of ROS and to the synthesis of vasoconstrictor agents derived from COX-2. Endothelium-independent relaxation (sodium nitroprusside) was not af-

Figure 1. Typical recordings (A) showing AT2R-dependent relaxation obtained in MRAs isolated from LZ and ZDF rats with endothelium (black tracing) or without endothelium (red tracing). Ang II (1 and 10 nmol/L) was applied to arteries in the presence of the AT1R blocker candesartan to stimulate only the AT2Rs. AT2R expression level was assessed using Western blot analysis after immunoprecipitation and histological localization with fluorescent Ang II (B). Mean±SEM is shown (n=10 rats per group). PE indicates phenylephrine.

The NAD(P)H oxidase subunit gp91phox and p67phox expression levels were significantly increased in ZDF rats compared with LZ rats. On the other hand, Mn-SOD and Cu/Zn-SOD expression levels were increased in ZDF rats. Chronic Tempol treatment increased gp91phox and p67phox expressions, although this was significant only in LZ rats for gp91phox. Chronic Tempol reduced Mn-SOD and Cu/Zn-SOD expression levels in ZDF rats (Figure S2).

COX (indomethacin) and COX-2 (NS398), blockade or the combination of indomethacin plus Tempol, did not affect AT2R-dependent relaxation in LZ rats. In ZDF rats, indomethacin, NS398, and a combination of indomethacin plus Tempol significantly increased AT2R-dependent relaxation to the level found in LZ rats (Figure 3A). The presence of COX-2 was evidenced in the arteries of ZDF rats but not in LZ rats using immunostaining (Figure 3B) or Western blot (Figure S3).

Plasma level of TxB2 was significantly higher in ZDF rats than in LZ rats (Figure 4A). Chronic Tempol treatment of LZ rats did not significantly affect TxB2 plasma levels, whereas in ZDF rats the same treatment significantly reduced plasma TxB2 (Figure 4A).

We then tested the effects of the TxA2/PGH2 receptor blocker SQ29548 (10 μmol/L) and the TxA2 synthesis.
fected by diabetes mellitus or by Tempol, which is in agreement with previous studies.26,27

In ZDF rats chronically treated with Tempol, AT2R-dependent relaxation was equivalent to that found in LZ rats. Thus, ROS production had a key role in the reduction of AT2R-dependent relaxation in diabetic rats. This was confirmed by using Tempol and apocynin acutely in isolated arteries. Both substances, applied acutely to the arteries before Ang II, restored AT2R-dependent relaxation in ZDF rats to the control level. This experiment suggests the in-
volvement of ROS in the reduction in relaxation observed in ZDF rats.

To confirm the presence of an excessive level of ROS in the arterial wall of ZDF rats, we used DHE staining on isolated arteries. Staining was higher in ZDF rats than in control animals and was suppressed by chronic Tempol treatment. In fact, the reduction in DHE staining was correlated with the effect of acute Tempol or apocynin on isolated arteries (more staining was observed when AT2R-dependent relaxation was reduced, with less staining being observed when the relaxation was restored). This excessive ROS production involved in the reduction of AT2R-dependent relaxation may arise from a higher expression level in gp91phox and p67phox, as suggested by our data and by our previous study in similar arteries.28,29

Previous studies have shown that ROS may activate COX-2 expression, enabling the production of both vasodilator and vasoconstrictor agents, such as prostaglandin E2, prostaglandin E3, and TxA2.13 We, thus, tested the effect of a COX-2 inhibitor (NS398) on AT2R-dependent relaxation. NS398, applied acutely to the arteries, restored AT2R-dependent relaxation in ZDF rats to the control level. Furthermore, blockade of the TxA2/PGH2 receptor with SQ29548 and blockade of thromboxane synthase with fureregrelate16 produced the same effect, suggesting that TxA2 (and/or PGH2) was produced in response to AT2R stimulation in diabetic rats. In fact, in the ZDF rat plasma, TxB2 (the TxA2 metabolite) was increased. Interestingly, in ZDF rats treated chronically with Tempol, the TxB2 plasma level alone was reduced compared with untreated ZDF rats. This finding, together with the positive effect already noted for Tempol, NS398, SQ29548, and fureregrelate on relaxation, reinforces the assumption that TxA2-mediated contraction is involved in the reduction of AT2R-induced relaxation in ZDF rats.

Finally, AT2R-induced relaxation in ZDF rats was restored to the control level after chronic treatment with NS398. The effect of COX-2 on AT2R-dependent relaxation was selective as acetylcholine-mediated relaxation, reduced in ZDF, was not improved by NS398 applied either acutely to the arteries or given chronically to the rats.

Our observations are in agreement with a previous study that showed that high glucose levels induce ROS production in human endothelial cells, with consequent COX-2 expression and TxA2 production.30 However, it is important to differentiate between acute and chronic ROS production. Diabetes mellitus is associated with a chronic high basal ROS production, as shown in several vascular beds, including resistance arteries (present study and Reference31). This high basal ROS production reduces endothelium-dependent dilation and enhances contractility.11 In diabetes mellitus, oxidative stress probably induces COX-2 expression, as shown previously,30 and as suggested by our data.

**Perspectives**

We identified an important change in AT2R function in diabetes mellitus. Furthermore, we have shown recently that, in aging, AT2R stimulation induces contraction, which reduces flow-mediated dilation and, in turn, reduces the ability of the endothelium to regulate local blood flow. These observations are especially important because the occurrence and severity of vascular diseases are largely related to vascular aging.31 In addition, diabetes mellitus and aging are both associated with ROS overproduction. Thus, AT2R-dependent dilatation may be more rapidly lost in older diabetic patients and, of course, the vascular consequences of diabetes mellitus may be worse in older patients. Nevertheless, the use of AT1R blockers in diabetic patients could be especially beneficial, because AT2R expression may be increased, with a corresponding improvement of the ability of AT2R to induce dilation. We have shown previously the positive effect of these drugs on AT2R expression and function in hypertensive rats.5 Thus, Ang II and the balance between AT2R and AT1R may play critical roles in subjects submitted to major risk factors, such as aging and diabetes mellitus. Finally, our study provides 2 possible therapeutic tools for the restoration of the vascular response to AT2R stimulation. First, an antioxidant therapy might be efficient, because both Tempol and apocynin restored the response to the control level. Second, COX-2 inhibition had the same effect, but
COX-2 inhibitors have also been shown to have negative effects on the cardiovascular system. Nevertheless, our study highlighted a possible role of the AT2R in type 2 diabetes mellitus.

In conclusion, we found a selective vascular defect in the renin-angiotensin system in type 2 diabetes mellitus in the rat. The defect in AT2R-dependent relaxation is related to excessive oxidative stress and inflammation, inducing COX-2 expression and excessive TXA2 production. Therapeutic tools for diabetic patients may, therefore, be especially beneficial if they are active on both oxidative stress and the AT2R expression level.

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Disclosures

None.

References

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Extended Material and Methods:

Vascular response to exogenous Angiotensin II in isolated mesenteric arteries.
Two-millimeter long segments of mesenteric resistance arteries (MRA) were mounted on a wire-myograph (DMT, Aarhus, DK) as previously described. Two tungsten wires (40 µm in diameter) were inserted into the artery lumen and fixed to a force transducer and a micrometer, respectively. The arteries were bathed in a physiological salt solution (PSS) maintained at 37°C, pH 7.4 (PO₂, 160 mmHg; PCO₂, 37 mmHg). The PSS contained candesartan (10 nmol/L) throughout the protocol in order to block the AT1Rs. Wall tension was applied as described previously, and vessels were then allowed to stabilize for one hour. Artery viability was tested using a potassium rich solution (80 mmol/L, 80K PSS), and the arteries were then precontracted with phenylephrine (PE, 3 µmol/L) and serotonin (1 µmol/L) in order to obtain sustained contraction. Angiotensin II (Ang II, 10 nmol/L) was then added to the bath. After washout, Ang II (10 nmol/L) was again added to the bath in the presence of one of the drugs cited below. Several equivalently-sized segments of MRA were used per rat in order to test the effect of the following factors: endothelium removal; nitric oxide (NO) synthesis blockade (using L-NAME, 100 µmol/L); cyclooxygenase blockade (using indomethacin, 10 µmol/L); cyclooxygenase-type 2 blockade (using NS398, 10 µmol/L); thromboxane A2 (TxA₂) receptor blockade (SQ29548, 10 µmol/L); thromboxane A2 synthesis inhibition (furegrelate, 10 µmol/L), AT2R blockade (PD 123319, 10 µmol/L); AT1R/AT2R inhibition (saralasin, 10 µmol/L); and ROS removal using tempol (100 µmol/L) or apocynin (100 µmol/L).

In a preliminary series of experiments, AT2R-dependent dilation (using Ang II, 1 and 10 nmol/L) was measured in the presence or in the absence of endothelium. The endothelium was removed as previously described.

In a separate series of experiments, the involvement of ROS in U46619 (a stable TxA₂ mimetic) action was assessed by repeating consecutive contractions to U46619 with or without incubation of the arteries with the SOD-mimetic, tempol (100 µmol/L), and with the NAD(P)H-oxidase inhibitor, apocynin (100 µmol/L).

Western Blot Analysis of AT2R expression

Western blot analysis of AT2R was performed in MRAs as previously described. Arterial segments were homogenized using a lysis buffer (1% SDS, 10 mmol/L Tris-HCl [pH 7.4], 1 mmol/L sodium orthovanadate, and a cocktail of protease inhibitors). Extracts were incubated at 4°C for 30 minutes and then centrifuged (14,000g, 15 minutes, 10°C). Protein concentration was determined using the Micro BCA Protein Assay Kit (Pierce Biotechnology). Proteins (100 µg) were incubated with 10 µg of anti-AT2R polyclonal antibody (H-143, Santa Cruz Biotechnology) at 4°C overnight and precipitated by addition of 10 µl of protein A/G-agarose (Santa Cruz Biotechnology). Immunoprecipitates were collected after 4 centrifugations at 3,000g for 1 minute at 4°C with washing of pellet between each centrifugation. The immunoprecipitate was resuspended in Laemmli sample buffer and denaturated at 95°C for 5 minutes. Samples were separated on a 9% polyacrylamide gel and transferred to nitrocellulose membranes (Amersham). Membranes were blocked with 5% bovine serum albumin (BSA) in T-TBS (20 mmol/L Tris, pH 8.0, 150 mmol/L NaCl, and 0.1% Tween-20) for 60 minutes, and were then incubated with rabbit anti-AT2R polyclonal antibody (dilution 1:100, H-143, Santa Cruz Biotechnology) in BSA 5% in T-TBS overnight at 4°C. After extensive
washing in T-TBS at room temperature, membranes were incubated with the anti-rabbit horseradish peroxidase antibody (1:20,000 from Pierce Biotechnology) for 90 minutes at room temperature. After 3 washes with T-TBS, immunocomplexes were visualized using the SuperSignal West Femto Maximum Sensitivity Substrate Kit (Pierce Biotechnology) using a computer-based imaging system (Fuji LAS 3000 plus, Fuji Medical System). Quantification was performed by densitometric analysis.

Specificity of the anti-AT2R antibody was controlled as follows:

1- Direct Western-blot with the antiAT2R (H143) with or without blocking peptide:

2- Immunoprecipitation (IP) with a different anti-AT2R antibody (C18, Santa-Cruz) or with H143 followed by Western-blot (WB) with H143 or C18 with or without blocking peptide:
Western Blot Analysis of beta-actin, gp91phox, p67phox, Cu/ZnSOD and MnSOD expression

Other MRA segments were homogenized using a lysis buffer (1% SDS, 10 mmol/L Tris-HCl [pH 7.4], 1 mmol/L sodium orthovanadate, and a cocktail of protease inhibitors). Extracts were incubated at 4°C for 30 minutes and then centrifuged (14,000g, 15 minutes, 10°C). Protein concentration was determined using the Micro BCA Protein Assay Kit (Pierce Biotechnology). After denaturation at 95°C for 5 minutes, equal amounts of proteins (15 µg) were loaded on a 9% polyacrylamide gel and transferred to nitrocellulose membranes (Amersham). Membranes were blocked with 5% bovine albumin (BSA) in T-TBS (20 mmol/L Tris, pH 8.0, 150 mmol/L NaCl, and 0.1% Tween-20) for 60 minutes and then incubated with rabbit anti-Cu/ZnSOD (1:1,000 from Stressgen), anti-MnSOD (1:5,000 from Stressgen) or mouse anti-gp91phox (1:1,000 from BD Transduction laboratories), anti-p67phox (1:500 from BD Transduction laboratories), anti-beta-actin (1:1,000 from Sigma-Aldrich) antibody in BSA 5% in T-TBS overnight at 4°C. After extensive washing in T-TBS at room temperature, membranes were then incubated with the anti-rabbit horseradish peroxidase antibody (1:10,000 from Pierce Biotechnology) or with the anti-mouse horseradish peroxidase antibody (1:10,000 from Pierce Biotechnology) for 90 minutes at room temperature. After 3 washes with T-TBS, immunocomplexes were detected by chemiluminescent reaction (SuperSignal West Femto, Pierce Biotechnology) using a computer-based imaging system (Fuji LAS 3000 plus, Fuji Medical System). Quantification was performed by densitometric analysis.

Histological detection of the AT2R

As previously described, segments of MRA were mounted in embedding medium (Tissue-Tek, Miles, Inc), frozen in isopentane pre-cooled in liquid nitrogen, and stored at -80°C. Transverse cross-sections (7 µm thick) were incubated with candesartan (30 min, 10 nmol/L, 25°C), then with fluorescent Angiotensin II (angiotensin II Alexa Fluor® 488 conjugate, 30 min, 10 pmol/L, 25°C, reference: A13439, Molecular Probes). Control experiments were performed after incubation with non-fluorescent Angiotensin II or in the presence of PD 123319. Fluorescence staining was visualized using confocal microscopy (Nikon, Eclipse TE2000S and Solamere Technology, Salt Lake City, UT, USA). Image analysis was performed using Histolab (Microvision, France).

Immunostaining of COX2

As previously described, segments of MRA were mounted in embedding medium (Tissue-Tek, Miles, Inc), frozen in isopentane pre-cooled in liquid nitrogen, and stored at -80°C. Transverse cross-sections (7 µm thick) were obtained and COX-2 was detected with primary goat anti-COX-2 polyclonal antibodies (1:200 from Santa Cruz Biotechnology), followed by the fluorescent secondary antibody (1:200 from Fluoroprobes) as previously described. Fluorescence staining was visualized using confocal microscopy (Nikon, Eclipse TE2000S and Solamere Technology, Salt Lake City, UT, USA). Image analysis was performed using Histolab (Microvision, France). In negative control experiments, the primary antibody was omitted. A positive control was obtained with arterial segments from lipopolysaccharide-treated rats.

Detection of ROS using confocal microscopy in resistance arteries

ROS detection was performed on 7 µm thick transverse cross-sections incubated with dihydroethydine (DHE, Sigma-Aldrich), as previously described. DHE in the presence of superoxide is oxidized to fluorescent ethidium bromide. Ethidium bromide is trapped by intercalation with DNA, and the number of fluorescent nuclei indicates the relative level of superoxide production. Positive staining was analyzed using confocal microscopy and image analysis.
References:


Extended Results:

Supplement Figure S1: AT2R-dependent dilation in mesenteric resistance arteries isolated from LZ and ZDF rats. Increasing concentrations of angiotensin II were added to the bath containing the arteries in the presence of the AT1R blocker candesartan. Mean±SEM is shown (n=10 rats per group).
*P<0.01, ZDF versus LZ rats
Supplement figure S2:
Expression level of the NAD(P)H-oxidase subunits (gp91phox and p67phox, A) and of the 2 SOD, Cu/ZnSOD and MnSOD was determined using Western blot analysis in mesenteric resistance arteries isolated from LZ and ZDF rats chronically treated with tempol or solvent (Solv) for 21 days. Mean±SEM is shown (n=10 rats per group).
* P<0.01, ZDF versus LZ rats
& P<0.01, effect of chronic tempol
**COX-2 expression level**

[Bar chart showing COX-2 mRNA levels in LZ and ZDF rats]

**Supplement figure S3:**
Expression level of COX-2 determined using Western blot analysis in mesenteric resistance arteries isolated from LZ and ZDF rats. Mean±SEM is shown (n=10 rats per group).

*P<0.01, ZDF versus LZ rats