Dose-Dependent Regulation of NAD(P)H Oxidase Expression by Angiotensin II in Human Endothelial Cells

Protective Effect of Angiotensin II Type 1 Receptor Blockade in Patients With Coronary Artery Disease

Uwe Rueckschloss, Mark T. Quinn, Juergen Holtz, Henning Morawietz

Objective—Angiotensin II (Ang II)–mediated induction of vascular superoxide anion formation could contribute to the development of endothelial dysfunction, hypertension, and atherosclerosis. An NAD(P)H oxidase has been identified as a major endothelial source of superoxide anions. However, the molecular mechanism underlying the regulation of NAD(P)H oxidase activity in response to Ang II is not well understood.

Methods and Results—We investigated the dose-dependent regulation of superoxide anion formation and of NAD(P)H oxidase subunit expression in response to Ang II in human endothelial cells. Ang II regulates superoxide anion formation and the limiting subunit of endothelial NAD(P)H oxidase, gp91-phox, in a dose-dependent manner via Ang II type 1 (AT1) receptor–mediated induction and Ang II type 2 receptor–mediated partial inhibition at higher Ang II concentrations. Furthermore, AT1 receptor blocker therapy before coronary bypass surgery downregulates the gp91-phox expression in internal mammary artery biopsies of patients with coronary artery disease.

Conclusions—Our data support a dose-dependent induction of proatherosclerotic oxidative stress in human endothelial cells in response to Ang II. The expression of NAD(P)H oxidase subunit gp91-phox is critical for endothelial superoxide anion formation. AT1 receptor blockade has an antiatherosclerotic and antioxidative potential by the reduction of oxidative stress in the vessel wall. (Arterioscler Thromb Vasc Biol. 2002;22:1845-1851.)

Key Words: angiotensin ■ atherosclerosis ■ cardiopulmonary bypass ■ endothelium ■ free radicals

Angiotensin II (Ang II) has been suggested to be involved in the development and progression of atherosclerosis.1,2 This hypothesis is supported by recent studies with Ang II receptor type 1 (AT1) inhibitors, which show antatherosclerotic effects in experimental studies3 and reversal of endothelial dysfunction in patients with coronary artery disease.4 Furthermore, ACE inhibitor therapy has a positive effect on the prognosis of patients with coronary artery disease.5

A putative risk factor involved in the proatherosclerotic effects of Ang II is increased oxidative stress by an elevated formation of reactive oxygen species, including superoxide anion (O2−). Animals made hypertensive by chronic Ang II infusion show augmented O2− formation and endothelial dysfunction.6–8 O2− rapidly reacts with NO and reduces the bioavailability of this vasoprotective mediator of endothelial function. Therefore, Ang II–stimulated increase in vascular O2− formation might contribute to the development of endothelial dysfunction and atherosclerosis.9

Several studies have been conducted to elucidate the molecular basis of vascular O2− formation. In every cell type of the vessel wall, an NAD(P)H oxidase similar to the phagocytic enzyme complex has been identified as a major source of O2− formation.10–13 The NADPH oxidase complex in neutrophils and, most probably, endothelial cells (ECs) involves 4 essential subunits. The subunits gp91-phox and p22-phox reside in the plasma membrane.14,15 These subunits bind the components of the electron transport chain heme and FAD, forming cytochrome b558. The cytosolic NADPH oxidase subunits p47-phox and p67-phox are involved in activation of the enzyme complex. After stimulation, p47-phox is phosphorylated by protein kinase C, forming a complex with p67-phox. Subsequent translocation of this complex to the cytochrome b558 induces O2− formation.16

In each vascular cell type, Ang II treatment causes upregulation of O2− formation.12,17–19 The molecular basis for this upregulation of enzyme activity by Ang II is not well understood. In adventitial fibroblasts, induction of p67-phox expression by Ang II was suggested to be responsible for the induced NAD(P)H oxidase-dependent O2− formation.17 The NAD(P)H oxidase subunit p22-phox has been shown to be a critical component in the Ang II–induced hypertrophy in vascular smooth muscle cells (VSMCs).20 Data on the regul-
lation of NADPH oxidase subunit expression by Ang II are lacking for ECs. Furthermore, a dose-dependent bimodal regulation of NAD(P)H oxidase activity has been observed in fibroblasts and ECs. The molecular mechanism for this bimodal regulation of NAD(P)H oxidase activity is unknown. In addition, the influence of ACE inhibitor or AT1 receptor blocker therapy on vascular NAD(P)H oxidase expression in patients with coronary artery disease has not been studied to date.

In the present study, we show a dose-dependent bimodal regulation of expression of the limiting NAD(P)H oxidase subunit (gp91-phox) and of corresponding O2− formation by Ang II in human ECs. In addition, gp91-phox expression was reduced in internal mammary arteries of patients with coronary artery disease by AT1 receptor blocker therapy. These data suggest an antioxidative and antiatherosclerotic potential of AT1 receptor blockade by reduction of endothelial NAD(P)H oxidase-dependent O2− formation.

**Methods**

**Cell Culture**
Primary cultures of human umbilical vein ECs (HUVECs) were isolated by using collagenase IV and grown in medium M199 (Life Technologies) supplemented with 20% calf serum, as described previously. Confluent cell cultures were incubated with medium containing 0.5% calf serum for 24 hours and subsequently treated with Ang II (1 nmol/L to 1 μmol/L) or with Ang II and the AT1 receptor antagonist candesartan (1 μmol/L, AstraZeneca) or the Ang II type 2 (AT2) receptor antagonist PD123319 (1 μmol/L, Parke-Davis).

**Patients**
Distal remnant specimens of left internal mammary artery (arteria thoracica interna) were obtained after informed consent from 23 patients undergoing elective coronary artery bypass grafting (CABG) surgery. The use of human tissue was approved by the local ethics committee. Long-term ACE inhibitor or AT1 receptor blocker therapy before surgery was evaluated in a retrospective manner. The following substances were prescribed: ACE inhibitors, including captopril, lisinopril, quinapril, and ramipril (38±10% of target dose in recent heart failure and endothelial function meagtrials); and AT1 receptor inhibitors, including losartan and valsartan (100% of target dose in recent heart failure meagtrials). Nine consecutive patients without pharmacological intervention in the renin-angiotensin system were matched with patients receiving preoperative ACE inhibitor (n=9) or AT1 receptor blocker (n=5) therapy, according to New York Heart Association functional classification. These groups of patients showed no significant differences in systolic or diastolic blood pressure. In addition, no differences in central venous pressure, heart rate, left ventricular ejection fraction, sex, age, weight, or concomitant therapy with calcium antagonists, β-blockers, diuretics, NO donors, antidiabetics, or lipid-lowering drugs were found (please see online Table 1, available at http://atvb.ahajournals.org).

**Cytochrome c Reduction Assay**
Cells were preincubated for 6 hours with or without Ang II and specific Ang II receptor antagonists in medium containing 0.5% calf serum. Cells were subsequently incubated for 1 to 4 hours at 37°C in an assay buffer consisting of medium M199 without phenol red supplemented with 40 μmol/L cytochrome c and 500 μmol/L NADH-nitro-l-arginine methyl ester to exclude EC NO synthase as a possible source of detected O2− generation. To evaluate NAD(P)H oxidase–derived O2− generation, the flavin-containing enzyme inhibitor diphenylene iodonium (DPI, 100 μmol/L) was included in some experiments. At indicated time points, aliquots of the supernatant were taken, absorption at 550 nm was determined, and blank was subtracted. The amount of O2− generated was estimated by the use of the millimolar extinction coefficient for reduced cytochrome c (295 L·mol−1·cm−1). The O2− generation was normalized versus protein concentration of samples determined with the BCA Protein Assay Reagent (Pierce). DPI-inhibited O2− generation was estimated as the difference between samples with or without DPI in each group.

**RNA Isolation**
Total RNA from primary cultures of HUVECs and from biopsies of internal mammary arteries was isolated by guanidinium thiocyanate/cesium chloride centrifugation as previously described.

**Quantification of NAD(P)H Oxidase Subunit mRNA Expression by Multistandard-Assisted RT-PCR**
For quantification of mRNA expression of NAD(P)H oxidase subunits gp91-phox, p22-phox, p47-phox, and p67-phox in competitive reverse transcription (RT)–polymerase chain reaction (PCR), a common linker primer, PCR-generated, internal-deleted, and in vitro–transcribed multistandard cRNA was generated. The identity of the amplified RT-PCR fragments was confirmed by DNA sequencing. Competitive RT-PCR was performed by using the NAD(P)H oxidase multistandard as previously described. DNA sequences of specific primers and PCR characteristics (PCR protocol: 30 seconds at 95°C, 30 seconds at primer-specific annealing temperature, and 30 seconds at 72°C) are summarized in online Table 2, available at http://atvb.ahajournals.org.

**Protein Isolation and Western Blot Analysis**
Protein isolation and Western blot analysis was performed as described with the use of monoclonal gp91-phox–specific antibodies.

**Statistical Analysis**
Data are given as mean±SEM. An ANOVA followed by the Bonferroni method (multiple comparison) or a Student t test was used for statistical comparison, as appropriate, and correlation was tested by using SigmaStat software (Jandel Corp). A value of P<0.05 was considered statistically significant.

An extended Methods section is available online at http://atvb.ahajournals.org.

**Results**

**Dose-Dependent Regulation of O2− Formation and NAD(P)H Oxidase Subunit Expression by Ang II in HUVECs**
Incubation of primary cultures of HUVECs with 100 nmol/L Ang II for >7 hours resulted in 2-fold induction of endothelial O2− formation (Figure 1A). In contrast, HUVECs treated with 1 μmol/L Ang II compared with control cells showed no significant difference in O2− formation.

Because an NAD(P)H oxidase has been previously shown to be a major source of endothelial O2− formation, mRNA expression of the 4 essential NAD(P)H oxidase subunits (gp91-phox, p22-phox, p47-phox, and p67-phox) was determined by multistandard-assisted competitive RT-PCR. First, expression of the limiting subunit of endothelial NAD(P)H oxidase gp91-phox was analyzed. Stimulation of HUVECs with 100 nmol/L Ang II caused a time-dependent induction of gp91-phox mRNA expression, reaching its maximum after 7 hours (Figure 1B). Further increase of Ang II concentration up to 1 μmol/L resulted in a partial inhibition of augmented gp91-phox expression (Figure 2A). Expression of NAD(P)H oxidase subunits p22-phox, p47-phox, and p67-phox was 2- to 3-fold induced by 100 nmol/L Ang II as well (maximum at
7 hours; please see online Figure IA, available at http://atvb.ahajournals.org). In contrast to gp91-phox, no decrease in the expression of these subunits could be found after incubation with 1 μmol/L Ang II.

The expression of NAD(P)H oxidase subunits in control and Ang II–stimulated HUVECs (10 nmol/L to 1 μmol/L Ang II) after 7 hours was graphically plotted versus DPI-inhibited O$_2^-$ formation at the corresponding Ang II concentrations after 8 hours. The dose-dependent regulation of endothelial gp91-phox mRNA expression and O$_2^-$ formation by Ang II was determined by competitive RT-PCR from primary cultures of HUVECs. Cells were incubated with 100 nmol/L Ang II for >7 hours caused an induction of endothelial O$_2^-$ formation. A higher dose of Ang II (1 μmol/L) had no significant effect on O$_2^-$ formation. B, Time-dependent regulation of gp91-phox mRNA expression by Ang II was determined by competitive RT-PCR from primary cultures of HUVECs. Cells were incubated with 100 nmol/L Ang II for 1, 3, 7, or 24 hours (control without Ang II, open bars). Maximal induction of gp91-phox expression by Ang II (2.6-fold) was found after 7 hours. *P<0.05 and **P<0.01 vs control.

The endothelial gp91-phox protein expression was induced by 100 nmol/L Ang II (8 hours) as well, whereas no induction could be found with the use of 1 μmol/L Ang II (Figure 3).

**Role of Specific Ang II Receptors in Dose-Dependent Regulation of NAD(P)H Oxidase Expression and Activity**

Induction of endothelial gp91-phox mRNA expression by treatment with 100 nmol/L Ang II was inhibited by a specific AT$_1$ receptor antagonist (1 μmol/L candesartan, Figure 4A). A similar effect of AT$_1$ receptor antagonism could be demonstrated on the functional level for Ang II–induced endothelial O$_2^-$ formation (Figure 4B).

The impact of specific AT$_1$ receptor antagonism on reduced gp91-phox expression by higher Ang II dose (1 μmol/L) was evaluated. As shown in Figure 5, concomitant

**Figure 1.** Induction of O$_2^-$ formation and NAD(P)H oxidase subunit gp91-phox expression in human ECs by Ang II. A, DPI-inhibited O$_2^-$ formation of primary cultures of HUVECs was determined by cytochrome c reduction assay in control cells (●) and cells stimulated with 100 nmol/L (○) or 1 μmol/L Ang II (●). Incubation with 100 nmol/L Ang II for >7 hours caused an induction of endothelial O$_2^-$ formation. A higher dose of Ang II (1 μmol/L) had no significant effect on O$_2^-$ formation. B, Time-dependent regulation of gp91-phox mRNA expression by Ang II was determined by competitive RT-PCR from primary cultures of HUVECs. Cells were incubated with 100 nmol/L Ang II for 1, 3, 7, or 24 hours (control without Ang II, open bars). Maximal induction of gp91-phox expression by Ang II (2.6-fold) was found after 7 hours. *P<0.05 and **P<0.01 vs control.

**Figure 2.** Dose-dependent regulation of NAD(P)H oxidase subunit gp91-phox expression by Ang II is correlated with O$_2^-$ formation in HUVECs. A, mRNA expression of NAD(P)H oxidase subunit gp91-phox was quantified by competitive RT-PCR from HUVECs after incubation without (control, open bar) or with Ang II (7 hours at 10 nmol/L, 100 nmol/L, or 1 μmol/L; solid bars). Expression of gp91-phox is induced by 100 nmol/L Ang II. Further increase of Ang II concentration to 1 μmol/L mediates a partial inhibition of augmented gp91-phox expression. B, NAD(P)H subunit gp91-phox mRNA expression in Ang II–stimulated HUVECs (10 nmol/L, 100 nmol/L, and 1 μmol/L Ang II) after 7 hours was plotted graphically vs DPI-inhibited O$_2^-$ formation determined by cytochrome c reduction assay in control and stimulated HUVECs at corresponding Ang II concentrations after 8 hours. CIs are shown by dotted lines. *P<0.05 vs control.
treatment of HUVECs with 1 μmol/L Ang II and 1 μmol/L PD123319, a specific AT2 receptor antagonist, resulted in significant induction of gp91-phox mRNA expression (n = 5, panel A) and O2− formation (panel B). These data suggest an AT2 receptor–mediated downregulation of gp91-phox expression and O2− formation in response to higher doses of Ang II in HUVECs.

Regulation of Vascular gp91-Phox Expression by Therapeutic Intervention in the Renin-Angiotensin System

The impact of preoperative ACE inhibitor or AT1 receptor blocker therapy before CABG surgery on vascular gp91-phox expression was determined in internal mammary arteries of patients with coronary artery disease (Figure 6). Long-term treatment with ACE inhibitors had, at the prescribed dosages, no effect on vascular gp91-phox expression. In contrast, pharmacological treatment with AT1 receptor antagonists resulted in significant reduction of gp91-phox expression. These data suggest an antioxidative and vasoprotective potential of AT1 receptor blocker therapy by downregulation of endothelial NAD(P)H oxidase expression.

Discussion

In the present study, regulation of O2− formation and NAD(P)H oxidase subunit expression in response to Ang II was investigated in HUVECs. Incubation of HUVECs with Ang II resulted in a time- and dose-dependent induction of O2− formation. At higher Ang II concentrations, no significant increase in O2− formation could be found. This bimodal regulation of O2− formation in HUVECs is in agreement with previous studies in ECs from human umbilical arteries and the guinea pig microcirculation and in adventitial rabbit fibroblasts.7

To find a molecular source for this dose-dependent induction of endothelial O2− formation, the expression of NAD(P)H oxidase subunits was studied in response to Ang II in HUVECs. A similar dose-dependent bimodal regulation of NAD(P)H oxidase expression by Ang II (7 hours) is inhibited by treatment with specific AT1 receptor antagonist candesartan (1 μmol/L) in HUVECs (con indicates control without Ang II). These data suggest an AT1 receptor–mediated downregulation of gp91-phox expression and O2− formation in response to higher doses of Ang II in HUVECs.

In further studies, we sought to determine which Ang II receptor mediates the dose-dependent induction or repression of gp91-phox expression by the use of specific Ang II receptor antagonists. An AT1 receptor antagonist attenuated the induction of gp91-phox expression and endothelial O2− formation by 100 nmol/L Ang II. These data provide evidence that induction of gp91-phox expression and O2− formation by Ang II is mediated via AT1 receptor stimulation.
feedback regulation of AT1 receptor expression. However, Ang II concentration might be the consequence of a negative-
gp91-phox expression and O2
induction of gp91-phox expression compared with control or 1 receptors. Therefore, a possible explanation for the reduced
VSMCs, and intact aortic segments. These data are in agreement with previous reports showing that AT1 receptor antagonists are capable of blocking Ang
II–mediated induction of O2
mediation is further induced via AT1 receptor stimulation. Thus, the cell- and vessel-specific ratio of AT1 and AT2 receptors shown in the present study could
supports the view that repression of gp91-phox expression and O2
formation at high Ang II concentration is at least partially mediated by AT2 receptor stimulation. Because a similar affinity to Ang II was shown for AT1 and AT2 receptors, the different threshold levels of AT1 receptor-mediated induction and AT2 receptor–mediated repression of gp91-phox expression might result from differences in receptor density. Because cultured ECs express AT1 receptors in excess compared with AT2 receptors, higher Ang II concentrations might be necessary for AT2 receptor stimulation. Thus, the cell- and vessel-specific ratio of AT1 and AT2 receptor expression might determine an induction or repression of gp91-phox expression at a given Ang II concentration.

The role of the AT2 receptor in the regulation of vascular tone has been analyzed in several studies. Increased blood pressure after chronic infusion of Ang II in rats was augmented by concomitant application of PD123319. Furthermore, an elevated blood pressure even under basal conditions was reported for AT2 receptor–knockout mice. Finally, mice overexpressing the AT2 receptors showed a blunted pressure response to chronic Ang II infusion. Because it is known that elevated vascular O2
formation reduces the bioavailability of NO, the bimodal regulation of endothelial gp91-phox expression and corresponding O2
formation via AT1 and AT2 receptors shown in the present study could contribute to the described changes in blood pressure.

To gain further insight into the regulation of NAD(P)H oxidase expression in vivo, we quantified the expression of gp91-phox in internal mammary arteries of patients undergoing elective CABG surgery. Long-term treatment with ACE inhibitors had no effect on vascular gp91-phox expression. In contrast, long-term therapy with AT1 receptor blockers reduced vascular gp91-phox expression. These data could be the consequence of the bimodal dose-dependent regulation of gp91-phox by Ang II described in the present study in vitro.

In our retrospective study, ACE inhibitor dosages prescribed by the referring physicians were only 38% of the respective...
target dosage in recent clinical megatrials.22–25 As a consequence, local Ang II concentration might be decreased below the threshold of AT₁ receptor–mediated repression, but it remains above the threshold level of AT₁ receptor–mediated induction of gp91-phox expression. Therefore, prescribed ACE inhibitor dosages might be critical regarding gp91-phox expression and corresponding O₂⁻ formation. This is in agreement with clinical studies suggesting beneficial effects of higher doses of ACE inhibitors in patients with heart failure.38 In contrast, AT₁ receptor blocker therapy is effective in reducing gp91-phox expression. This is in agreement with our in vitro data showing an AT₁ receptor–mediated induction of endothelial gp91-phox expression in response to Ang II. Because of the small sample size of internal mammary artery biopsies, we were not able to perform additional protein expression or O₂⁻ measurements in these tissues. However, our data in HUVECs in the present study support a correlation of gp91-phox mRNA expression and O₂⁻ formation. Therefore, the decreased gp91-phox expression by AT₁ receptor blocker therapy suggests a reduced O₂⁻ formation in internal mammary arteries. A critical role of gp91-phox in vivo is further supported by the 2-fold increased O₂⁻ formation by Ang II infusion in the aortas of wild-type animals but not in gp91-phox–knockout mice.39

In summary, we show that the dose-dependent bimodal regulation of endothelial NAD(P)H oxidase by Ang II is exclusively related to the regulation of gp91-phox expression. Induction of gp91-phox is mediated by the AT₁ receptor, whereas a partial inhibition involves AT₂ receptor stimulation. Furthermore, vascular gp91-phox expression in internal mammary arteries of patients with coronary artery disease was reduced in response to AT₁ receptor blocker therapy. These data suggest an antioxidative and vasoprotective potential of AT₁ receptor blocker therapy by downregulation of vascular NAD(P)H oxidase expression.

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