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Cardiovascular Research 44 (1999) 215–222

Cardiovascular Research

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Angiotensin II-induced superoxide anion generation in human vascular endothelial cells: Role of membrane-bound NADH-/NADPH-oxidases

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Received 15 December 1998; accepted 10 May 1999

Abstract

Background: Angiotensin II (ANG II) mediated hypertension accelerates atherosclerosis (AS) and thereby increases the incidence of myocardial infarction (MI). On the other hand, superoxide anion (O_2^-) is involved in the modification of low density lipoproteins, inhibition of prostacyclin (PGI_2) formation and breakdown of nitric oxide. These events finally lead to rapid progression of AS and MI. In the present study, we investigate whether ANG II can induce O_2^- release from human vascular endothelial cells (HVECs) and the possible mechanisms involved. **Methods and Results:** The expression of ANG receptors subtype-1 (AT-1) and subtype-2 (AT-2) were identified by using reverse transcription polymerase chain reaction and sequence analysis. The O_2^- production was dose-dependently increased in HVECs treated with ANG II (10^{-7}–10^{-9} M) and with a maximum rate after 1 h of incubation. This event was significantly inhibited by pretreatment of cells with the specific AT-1 blocker losartan (10^{-7} M) and to a lesser extent by the specific AT-2 receptor blocker PD123319 (10^{-7} M). The combined incubation of both receptor blockers was even more effective. In addition, our lucigenin-enhanced chemiluminescence assay showed that the activity of plasma membrane-bound NADH-/NADPH-oxidases derived from ANG II-treated cells was also significantly increased, this effect was reduced in cells pretreated with losartan or to lesser extent by PD123319. However, the activity of xanthine oxidase remained unchanged in response to ANG II. Furthermore, the basal O_2^- release from HVECs was inhibited in cells treated with angiotensin-converting enzyme (ACE) inhibitor, Lisinopril (10^{-6} M), and this event could be reversed by ANG II. **Conclusion:** ANG II induces O_2^- release in HVECs via activation of membrane-bound NADH-/NADPH-oxidases, an effect, that is mediated by both AT-1 and AT-2 receptors. This suggests that acceleration of AS and MI in ANG II-mediated hypertension may at least be due to ANG II-induced O_2^- generation from vascular endothelial cells. In this case, the ACE inhibitors and the ANG receptor antagonists may act as causative ‘‘antioxidants’’. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Angiotensin II; Superoxide anion; Endothelial cell; NADH-/NADPH-oxidase

1. Introduction

Angiotensin II (ANG II) has numerous effects on the cardiovascular system, i.e., induction of vascular smooth muscle cells (VSMCs) proliferation and myocardial hypertrophy. It contributes to the development and progression of hypertension [1,2]. These events cause an acceleration of atherosclerosis [3,4] or coronary vasospasm and can eventually lead to myocardial infarction [5]. It has been reported that hypertensive patients with elevated plasma renin and ANG II levels show a increased incidence of myocardial infarction [4,6,7]. In addition, it has been found that ANG II increases the generation of superoxide anions (O_2^-) in VSMCs and aortic rings derived from ANG II-induced hypertensive rats by direct stimulation of NADH-/NADPH-oxidases [8,9]. ANG II also induces macrophage-mediated oxidation of low density lipoproteins (LDL) an event that involves the action of cellular NADPH-oxidase [4]. Up to now, there is no

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Time for primary review 28 days.

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PII: S0008-6363(99)00183-2

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evidence that ANG II influences the O_2^- release from human vascular endothelial cells (HVECs).

Oxygen free radicals, such as O_2^-, play an important role in the development of cardiovascular disease [10]. They can be generated by neutrophils, endothelial cells, VSMCs, and macrophages [11,12]. Under pathologic conditions, stimulated O_2^- production may contribute to the inhibition of prostacyclin formation and accelerated breakdown of endothelial-derived nitric oxide (NO), a known potent endogenous vasodilator and inhibitor of platelet aggregation [13]. O_2^- is also involved in oxidative modification of LDL to oxidized LDL. The latter has been found to be involved in atherosclerotic lesion [14], and LDL of hypertensive patients is more susceptible to oxidation than LDL from normotensives [4]. In VSMCs, the membrane-bound component of the NADH-/NADPH-oxidases system has been characterized as being a cytochrome b558 α-subunit $p22^{phox}$ that appears to be essential for the ANG II-induced O_2^- generation [15]. The mechanismS of basal and stimulated O_2^- formation by endothelial cells are not fully understood, but may be similar to those previously reported in VSMCs [16,17].

The functions of ANG II are mediated by two angiotensin receptor subtypes, type 1 (AT-1) and type 2 (AT-2) which have been cloned and characterized in several species and cell lines [18,19]. In contrast to VSMC where in physiological states only the AT-1 subtype is present, both subtypes of the receptor are expressed in rat coronary endothelial cells [20,21], and mediate its functions in response to ANG II in a different manner.

In the present study, we investigated the effect of ANG II on O_2^- release in HVECs as well as the possible involvement of oxidases and angiotensin receptor subtypes. In addition, we tried to find out whether there is an effect of angiotensin-converting-enzyme (ACE) inhibitor on the basal O_2^- release from HVECs. These results may lead to better understanding the mechanism of ANG II-induced hypertension and atherosclerosis.

2. Methods

ANG II, NADH, NADPH, hypoxanthine, superoxide dismutase, endothelial cell growth supplement, monoclonal anti-von Willebrand factor VIII, ferricytochrome C, heparin, lucigenin, aprotinin, leupeptin, pepstatin, phenylmethylsulfonyl fluoride were purchased from Sigma, Germany. Medium 199, fetal calf serum (FCS), penicillin and streptomycin were purchased from Biochrom, Germany. Polymerase chain reaction (PCR) primers were synthesized by Pharmacia, Germany. Dispase II, TRIzol reagent, Taq DNA polymerase, AMV (avian myeloblastosis virus) reverse transcriptase, dNTP (deoxynucleosidetriphosphate) were purchased from GibcoBRL, Germany. Losartan was offered by MSD-Merck and PD123319 was a gift from Dr. Gohlke, University of Kiel, Germany.

2.1. Cell culture

Endothelial cells were isolated from human umbilical artery by digestion with 2.4 U/ml dispase II for 20 min at 37°C in a shaking waterbath. Cells were grown to confluence in medium 199 containing 20% FCS, 50 μg/ml endothelial growth supplement, 2 mM glutamin, 100 U/ml penicillin, 100 μg/ml streptomycin and 5 U/ml heparin. Cells were kept at 5% CO_2/95% air in a humidified atmosphere. The purity of the endothelial cells was identified by their "cobblestone" morphology and by positive immunofluorescence using monoclonal anti-von Willebrand factor antibody. The second passage of cells was used in all the experiments. Cell viability was assessed by 0.1% trypan blue exclusion.

2.2. Measurement of O_2^- release in intact endothelial cells

HVECs were cultured in a 24-well plate. After growing to confluence, the complete medium was removed and the cell monolayer was washed three times with Hank's balanced salt solution (HBSS). A 250 μl volume of serum and phenol-red free medium 199 with 0.25% bovine serum albumin were added to each well. After 2 h of equilibration, 250 μl of the same medium containing 140 μM ferricytochrome C and ANG II were added to each well. In some wells, the cells were pretreated with different angiotensin receptor antagonists for 30 min before ANG II was added. The plate was kept in the cell culture incubator. After different periods of incubation time, the supernatant from each reaction was pipetted out and analyzed by using spectrophotometer at a wavelength of 550 nM. The amount of O_2^- release was calculated by dividing the difference in absorbency of the samples with and without SOD (superoxide dismutase) by the extinction coefficient for reduction of ferricytochrome C to ferrocytochrome C (ϵ= 21.1 cm^{-1} M). The detection limit of this method is nanomolar.

2.3. Measurement of NADH-/NADPH-oxidases activity from subcellular fractions

HVECs were grown to confluence in a six-well culture plate. Cells were then stimulated as described above in condition medium without ferricytochrome C. Afterward, the cell monolayer was washed three times with ice cold HBSS, and cells were scraped from each well in 500 μl ice cold HBSS pH 7.4 containing 10 μg/ml aprotinin, 1μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM EGTA ([ethylenebis-(oxyethylene-nitrilo)] tetracetic acid, 1 mM phenylmethylsulfonyl fluoride. The cells were destroyed by sonicating and subcellular fractions of cells were separated by centrifugation at 14 000 *g*, 4°C for 20 min. Both cell membrane and cytosolic fractions were saved and the cell membrane fractions were resuspended in 500 μl of the

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same lysis buffer. The protein concentration was adjusted to 200 μg/ml. A 250 μl volume of cell membrane suspension or cell cytosolic fraction was mixed with 250 μl HBSS containing 500 μM lucigenin and kept in Multi-Biolumat (Berthold, LB 9505C) at 37°C and 10 min for equilibration. The NADH-/NADPH-oxidases assays were started by adding 10 μl concentration of 100 μM NADH or 100 μM NADPH respectively. The photon emission was measured for 10 min continuously and the respective background counts were subtracted. Neither subcellular fractions alone nor NADH and NADPH alone evoked any lucigenin chemiluminescence signal. The detection limit of this method is picomolar.

2.4. Reverse transcription (RT)-PCR analysis of angiotensin receptor subtype

HVECs were grown to confluence in 50 ml culture flash and were rinsed twice with phosphate buffered saline. Total RNA of HVECs was isolated by using TRIzol reagent. One μg of total RNA was reversibly transcripted to cDNA in a reaction condition of 25 mM Tris–HCl (pH 8.3), 5 mM $MgCl_2$, 50 mM KCl, 2 mM DTT (dithiothreitol), 1 U/μl, 1 mM dNTP each, 40 μg/ml primer dT_{15} and 200 U/ml AMV reverse transcriptase in a final volume of 25 μl and incubated for 40 min at 42°C. RT was terminated by heating at 95°C for 5 min. Five percent of the cDNA was used as template for PCR. These reactions were performed in 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM each dNTP, 0.5 mM each primer, and 1.25 U of Taq polymerase in a final reaction volume of 50 μl. For AT-1 primers, we used 5′-ACTATTACGCTTCAGCCACG as sense and 5′-CGGCTGTATGCCAATATCTAC as antisense. For AT-2 primer, we used 5′-AGTCCGCATGCAAACTTG as sense and 5′-CGACTGAGCATAAGCCCTCGCG as antisense. The primers are predicted to amplify 532 base pairs (bp) and 469 bp DNA fragment respectively. The PCR reactions were done at 94°C 45 s, 62°C 45 s, 72°C 1 min for 35 cycles. The PCR products were analyzed on a 1.5% agarose gel and stained by ethidium bromide. Afterward, the sequences of the PCR products were analyzed by ALFexpress DNA Sequencer and Fragment Analysis System from Pharmacia Biotech.

2.5. Statistical analysis

Data are expressed as mean±SD from duplicate determinations of six separate experiments. The comparison between groups was performed by unpaired Student's *t*-test, Bonferroni's correction for multiple comparisons was used to determine the level of significance of the *P*-value. Tucket's test was also employed where multiple comparisons were made. Statistical significance was defined as $p<0.05$.

3. Results

After exposure of HVECs to ANG II, the O_2^- production was significantly increased in a dose-dependent manner (10^{-7}–10^{-9} M) and reached a maximal level at a concentration of 10^{-7} M after 1 h stimulation. However, the O_2^- production was reduced when ANG II 10^{-6} M was used (Fig. 1). The reason may be that ANG II 10^{-6} M leads to activation of ecNOS (endothelial cell nitric oxide synthase). Recently one group reported that treating bovine endothelial cell with ANG II 10^{-6} M increased cNOS

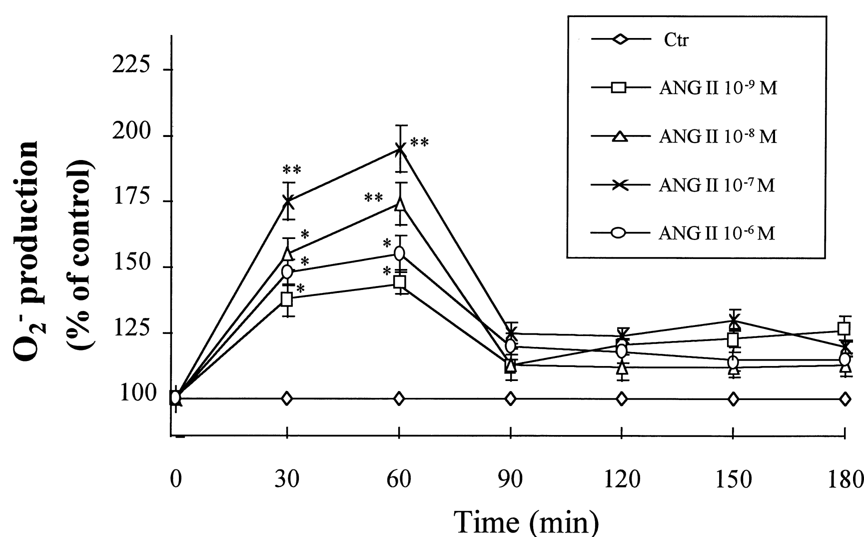

Fig. 1. ANG II induces O_2^- generation in HVECs. HVECs were grown to confluence in 24-well culture plate, then stimulated with ANG II (10^{-6}–10^{-9} M) up to 3 h. The assay of O_2^- release was performed by measurement of superoxide dismutase-inhibitable reduction of ferricytochrome C as described in Methods. The data (mean±SD) are presented as percentage of control from duplicate determinations of six different experiments. * $P<0.05$ vs. control, ** $P<0.01$ vs. control.

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(inducible nitric oxide synthase) protein, mRNA level and NO production [22].

In order to investigate the subcellular location of xanthine-, NADH- and NADPH-oxidase activity in HVECs, the membrane and cytosolic fractions of homogenized HVECs were prepared. The oxidase's activities were measured by using hypoxanthine, NADH or NADPH as a substrate. We found that almost all the lucigenin signals produced by these oxidases were from the cell membrane fraction and only very low lucigenin signals were detected in the cell cytosolic fraction (Fig. 2A). The incubation of HVECs with ANG II (10^{-7} M for 1 h) increased both the NADH-/NADPH oxidase activities in the membrane fractions as compared with untreated cells. The activity of xanthine-oxidase that presented in cell membrane fractions remained unchanged (Fig. 2B).

In addition, when NADH was used as substrate, the O_2^- generation from cell plasma membrane was about three-times higher than when NADPH was used as substrate (Fig. 2A). The stimulation effect of ANG II was more pronounced on the NADH-oxidase than on the NADPH-oxidase. These results suggest that NADH-oxidase is the main source of O_2^- production in HVECs.

To identity the receptor subtypes of ANG II on HVECs, VSMCs and human polymorphonuclear neutrophils (HPMNs), the mRNA of these three cell lines were reversibly transcripted to cDNA. The specific AT-1 and AT-2 receptor primers were used for PCR amplification. PCR products were analyzed by 1.5% agarose gel electrophoresis (Fig. 3). In contrast to VSMCs where only the AT-1 subtype is present, both AT-1 and AT-2 PCR fragments of HVECs cDNA were found and confirmed by sequence analysis. However, neither AT-1 nor AT-2 PCR fragment was found by using HPMNs cDNA as template. These suggest that both angiotensin receptor subtypes AT-1 and AT-2 are expressed on HVECs, but not on HPMNs.

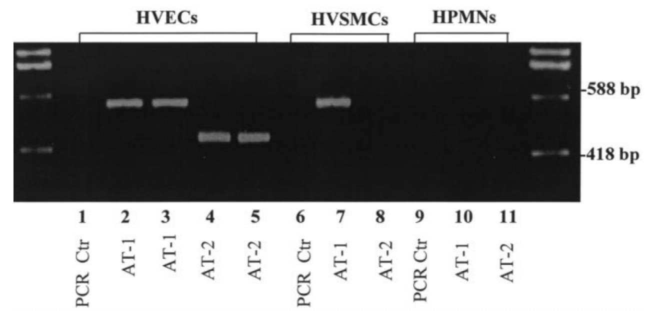

Fig. 3. RT-PCR analysis of angiotensin receptor subtypes. The total RNA of HVECs, HVSMCs and HPMNs were isolated. The mRNA was reversibly transcribed to cDNA. Two primers were designed to amplify angiotensin receptor subtype 1 (AT-1) or subtype 2 (AT-2), respectively. The PCR produces were analyzed on a 1.5% agarose gel electrophoresis.

The ANG II induced O_2^- production was significantly inhibited by pretreatment of cells with the specific AT-1 blocker losartan (10^{-7} M) in a dose-dependent manner and to a lesser extent by the specific AT-2 receptor blocker PD123319 (10^{-7} M) (Figs. 4 and 5). The coincubation of both receptor blockers was even more effective. In addition, the ANG II-stimulated increase in NADH-/NADPH-oxidases activity was significantly blocked by preincubation of cells with either losartan (10^{-7} M) or to a lesser extent by PD123319 (10^{-7} M) (Fig. 6).

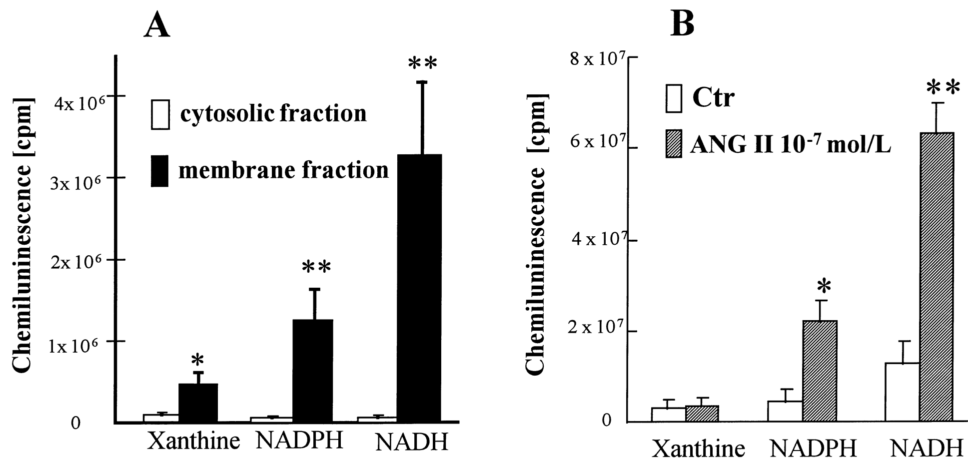

Fig. 2. ANG II activates membrane-bound NADH-/NADPH-oxidases in HVECs. (A) In order to study the subcellular location of xanthine-, NADH-/NADPH-oxidase activity, HVECs were homogenized, the cell membrane and cytosolic fractions were separated. Both cell membrane and cytosolic fractions were used for measurement of xanthine-, NADH-/NADPH-oxidases activity by using lucigenin-enhanced chemiluminescence. Hypoxanthine, NADH or NADPH was used as substrate. The data (mean±SD) represent duplicate determinations from six separate experiments. * $P<0.05$ vs. cytosolic fractions, ** $P<0.01$ vs. cytosolic fractions. (B) After stimulation of HVECs with ANG II 10^{-7} M for 1 h, the cell plasma membrane was prepared. The membrane bound NADH-/NADPH-oxidases and xanthine oxidase were measured as described in Methods. NADH, NADPH or hypoxanthine was used as substrates, respectively. The data (mean±SD) are presented from duplicate determinations of six different experiments. * $P<0.05$ vs. control, ** $P<0.01$ vs. control.

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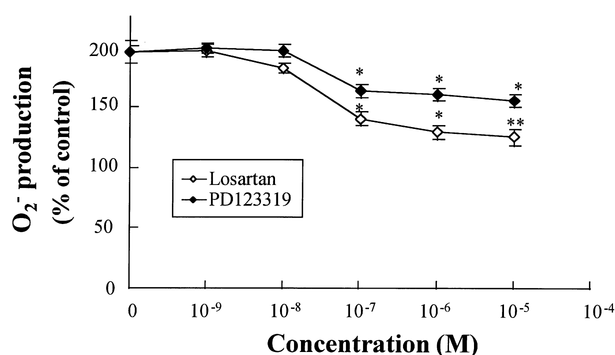

Fig. 4. Dose response course of losartan and PD123319 in ANG II-induced O_2^- production in HVECs. Cells were grown to monolayer in a 24-well culture plate, then stimulated with ANG II (10^{-7} M) for 1 h with or without pretreatment with losartan (10^{-9}–10^{-5} M) or PD123319 (10^{-9}–10^{-5} M), respectively. The assay of O_2^- release was performed by measurement of superoxide dismutase-inhibitable reduction of ferricytochrome C as described in Methods. The data (mean±SD) represent duplicate determinations from six separate experiments. * $P<0.05$ vs. ANG II stimulation alone, ** $P<0.01$ vs. ANG II stimulation alone.

In the following experiment (Fig. 7), ANG I (10^{-6} M) was added in the cell culture medium as substrate in either control cells or the cells were treated for 1 h with the ACE inhibitor Lisinopril (10^{-6} M) and both ANG receptor blockers, respectively. The O_2^- production was significantly reduced by Lisinopril. The effect of Lisinopril could be reversed by ANG II (10^{-7} M) (Fig. 7).

The other two ANG peptides ANG (1–7) (10^{-7} M) and ANG IV (10^{-7} M) was also used to test the possible effect on O_2^- formation; we have not seen a significant effect in response to both peptides. Furthermore, we have not seen a significant effect on O_2^- production in cells treated with an aminopeptidase inhibitor, amastatin (10^{-6} M) or in cells coincubated with amastatin and ANG II together (Fig. 8).

4. Discussion

Blood vessels are covered by a single layer of endothelial cells that release a variety of bioactive substances that play a central role in the regulation of vascular tone. ANG II is one of these substances, and it has been implicated as an important factor in various cardiovascular diseases such as hypertension.

In the present study, we found that ANG II significantly stimulates O_2^- release from HVECs in a dose-dependent manner and this stimulation was maximal after 1 h of incubation. It has been reported that NADH-/NADPH-oxidases represent the most important source of O_2^- in both VECs and VSMCs [8,23]. Our experiments show that most O_2^- was produced by HVECs membrane preparation, whereas almost no signal is detectable in cell cytosolic fractions. This suggests that in HVECs both oxidases are membrane bound proteins.

In addition, the ANG II-stimulated O_2^- production in the membrane fraction was about three-times higher when NADH was used for substrate as compared with the electron donor NADPH. However, the chemiluminescence signals did not change after adding hypoxanthine to the plasma membrane suspension derived from either control cells or ANG II-stimulated cells. These results suggest that the ANG II-stimulated O_2^- generation in HVECs is predominantly mediated by NADH-oxidase and to a lesser

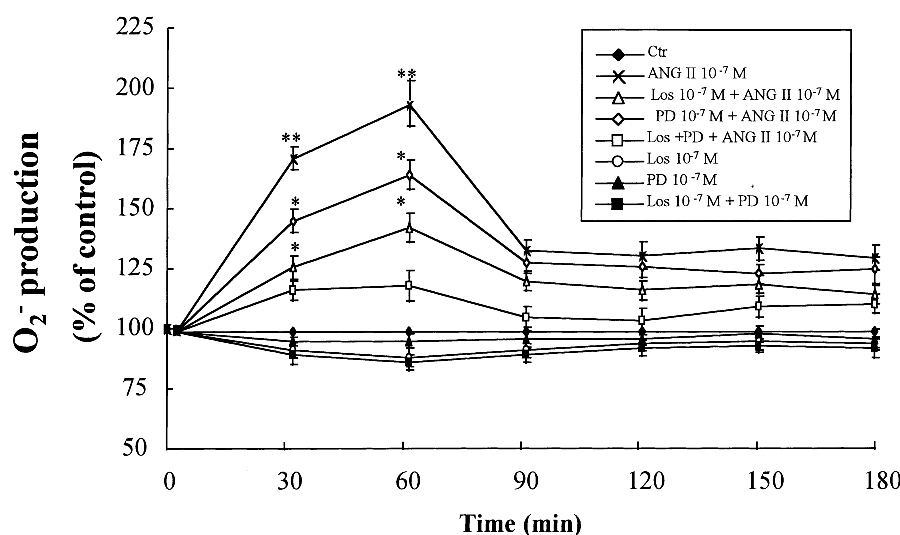

Fig. 5. Influence of angiotensin receptor blockers on O_2^- production in HVECs. Cells were grown to monolayer in a 24-well culture plate, then stimulated with ANG II (10^{-7} M) for 1 h with or without pretreatment with losartan (10^{-7} M) and/or PD123319 (10^{-7} M). The assay of O_2^- release was performed by measurement of superoxide dismutase-inhibitable reduction of ferricytochrome C as described in Methods. The data (mean±SD) represent duplicate determinations from six separate experiments. * $P<0.05$ vs. control, ** $P<0.01$ vs. control

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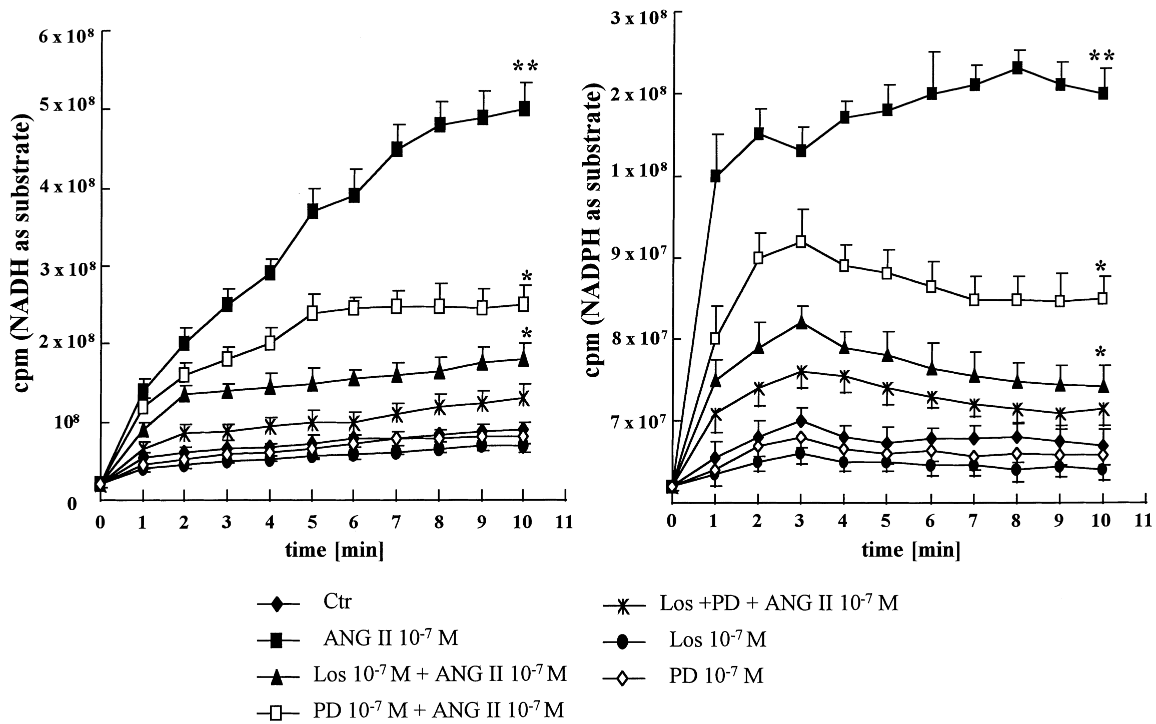

Fig. 6. Effect of ANG receptor blockers on ANG II-induced NADH-/NADPH-oxidases activity in HVECs. HVECs were stimulated with ANG II (10^{-7} M) for 1 h with or without pretreatment with losartan (10^{-7} M) and PD123319 (10^{-7} M), respectively. Cell plasma membranes were prepared and NADH-/NADPH-oxidases activity was measured as described in Methods. NADH (A) or NADPH (B) was used as substrate, respectively. The lucigenin signal was counted in a luminometer. The data are presented as mean±SD from duplicate determinations of six different experiments. * $P<0.05$ vs. control, ** $P<0.01$ vs. control.

extent by NADPH-oxidase, but xanthine-oxidase is not involved in this event.

Some groups reported that ANG II increases O_2^- in cultured VSMCs and in ANG II infusion of rat vascular ring that is mediated by AT-1 receptors, because this effect could be abolished by losartan [8,9]. In contrast to cultured VSMCs where only AT-1 receptors have been found [20], our results demonstrate that both angiotensin receptor subtypes, AT-1 and AT-2 mRNA are expressed in HVECs. The ANG II-induced O_2^- production and both NADH-/NADPH-oxidases activation were significantly inhibited by the specific AT-1 receptor blocker losartan and to a

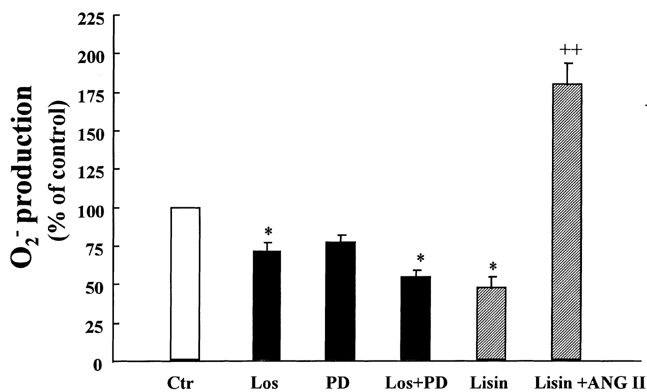

Fig. 7. Influence of ACE inhibitor and ANG II receptor blockers on basal O_2^- production. Cells were treated with losartan (10^{-7} M) and PD123319 (10^{-7} M) for 1 h, or with ANG II (10^{-7} M) for 1 h with or without pretreatment with Lisinopril (10^{-6} M). All these experiments were performed in presenting ANG I (10^{-6} M) in the culture medium. The O_2^- releases were measured by using cytochrome C assay as described in Methods. The data are presented as mean±SD from six separate experiments. * $P<0.05$, ** $P<0.01$ vs. control, ++ $P<0.01$ vs. Lisinopril group.

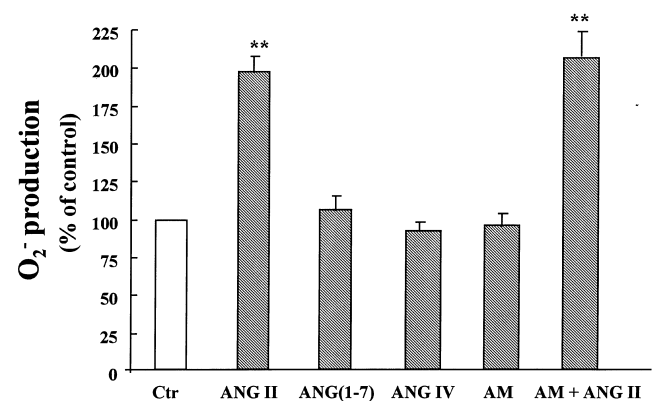

Fig. 8. Influence of ANG IV, ANG (1–7) and amastatin on O_2^- production. Cells were treated with ANG IV (10^{-7} M) and ANG (1–7) (10^{-7} M) for 1 h, or with ANG II (10^{-7} M) for 1 h with or without pretreatment with amastatin (10^{-6} M). The O_2^- releases were measured by using cytochrome C assay as described in Methods. The data are presented as mean±SD from six separate experiments. ** $P<0.01$ vs. control.

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lesser extent by the AT-2 receptor antagonist PD123319. The coincubation of both receptor blockers was even more effective. These results suggest that ANG II-induced O_2^- generation in HVECs is mediated by both AT-1 and AT-2 receptors.

The signal transduction pathway by which ANG II stimulates O_2^- formation in VSMCs or HVECs is not fully understood. AT-1 receptor was reported to stimulate G protein-coupled phospholipase C and hydrolyze membrane phosphoinositides thus leading to activate protein kinase C, MAP (mitogen-activated protein) kinase and elevate intracellular calcium level [24]. Both PKC (protein kinase C) and Ca^{2+} have been found to activate NADPH-/NADH-oxidase and increase O_2^- formation [8,25,26]. In contrast to the AT-1 receptor, the signal transduction cascade in response to ANG II-induced AT-2 receptor stimulation has not been well established. AT-2 has been associated with cell adhesion processes. On vascular endothelial cell, AT-2 stimulates adhesion molecule expression and increases leukocyte adhesion [24] and O_2^- does the same [27]. Oxygen-derived free radicals play a major role in atherogenesis and in the pathogenesis of reperfusion arrhythmias, stunned myocardium, etc. They can directly destroy VECs and are involved in the oxidative modification of LDL to oxidized-LDL that is thought to be involved in the initiation and progression of the atherosclerotic process. Cells of the arterial wall can induce LDL oxidation [28]. So antioxidation is an important strategy in preventing and treating atherosclerosis.

The O_2^- generation system of vascular cells seems to be different from that of HPMNs. HPMNs produce micromolar amounts of O_2^- within short periods of time during respiratory bursts in response to different stimuli, and most of the O_2^- is produced by NADPH-oxidase [17]. In contrast to PMNs, our results indicate that NADH acts as the main source of O_2^- generation in HVECs and continuously produces nanomolar amounts of O_2^-. It was reported that VSMCs and HVECs produce more than 1000-times less concentration of O_2^- than neutrophils even upon stimulation [29]. Other groups found that the vascular oxidases in spite of all the apparent difference to the neutrophil/macrophage NADPH-oxidase, may use some of the same components as the neutrophil oxidases. The VSMCs derived NADH-oxidase contains a spectrally detectable cytochrome b588, similar to the electron transport component of the neutrophil NADPH-oxidase. $p22^{phox}$ Protein, the subunit of cytochrome b558 shares more than 90% homology between VSMCs and neutrophils [9,30]. By using RT-PCR, the expression of $gp91^{phox}$, $p22^{phox}$, $p67^{phox}$, and $p47^{phox}$ have been recently demonstrated in endothelial cells [16]. However, no detection of O_2^- release from HPMNs in response to ANG II was found in our observation (data not shown). Furthermore, there is no expression of angiotensin receptors on neutrophils. These results suggest that the O_2^- is mostly produced by vascular cells and not from PMNs during high plasma renin–angiotensin levels. However, it might also be produced by macrophages [31]. Thus, ANG II-mediated cardiovascular diseases might be partially due to continuous generation of O_2^- from HVECs.

ACE is widely distributed in the cardiovascular system, particularly in endothelial cells [32]. It catalyzes both the conversion of Ang I to ANG II and the degradation of bradykinin [33]. Our results show that the O_2^- production was suppressed by ACE inhibitor and ANG II receptor blockers when ANG I was added in HVECs culture as substrate. The effect of ACE inhibitor can be reversed by ANG II. This data further confirms that ANG II contributes to increase O_2^- level in cardiovascular system.

ACE inhibitors have been found to attenuate atherosclerosis in some hypercholesterolemic animal models [34], and hypercholesterolemia increases endothelial O_2^- production [35]. This suggests that the renin–angiotensin system in the pathogenesis of atherogenesis may at least be partially due to ANG II-induced O_2^- production from vascular cells. ACE inhibitors may block this effect because of the reduction of ANG II synthesis from HVECs. ACE inhibitors also have been found to reduce ischemic events in coronary artery disease, to repress LDL lipid preoxidation and the development of early lesions in the apoE (apolipro-protein E) deficient atherogenic model [36]. So chronic treatment with ACE inhibitors may reduce arteriosclerosis and myocardial infarction.

In summary, we found that ANG II induces O_2^- generation in HVECs. This is predominantly mediated by membrane-bound NADH-oxidase and to a lesser extent by NADPH-oxidase, but not by xanthine-oxidase. Both AT-1 and AT-2 receptors are expressed on HVECs. They are involved in the activation of NADH-/NADPH-oxidases and the O_2^- production in response to ANG II. ACE inhibitors reduce ANG II synthesis that leads to reduce O_2^- production from HVECs. These results indicate that especially in ANG II-mediated hypertension accelerated atherosclerosis may at least be partially due to ANG II-induced O_2^- generation from VECs. Thus, besides the well known antihypertensive effect of ACE inhibitors and ANG receptor antagonists, they may also be seen as causative antiatherosclerotic agents since they act as a kind of ‘‘antioxidants’’ due to altering the ANG II synthesis or blockade of the ANG receptor thus causing a decrease of O_2^- release in the circulation.

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