Angiotensin II Type 1 Receptor–Mediated Peroxide Production in Human Macrophages

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Abstract—Our previous experiments demonstrated upregulation of the renin-angiotensin system in macrophages, including angiotensin II type 1 (AT1) and type 2 (AT2) receptors, during transformation from monocytes. We investigated the role of angiotensin II in oxidative stress of monocytes/macrophages, which plays a role in the advance of atherosclerosis. THP1, a human monocyctic leukemia cell line, was differentiated to macrophages by adding of phorbol 12-myristate 13-acetate for 24 hours. The intracellular production of peroxide was measured by a cytofluorometric assay with 2′,7′-dichlorofluorescein-diacetate with a flow cytometer scan. Peroxide was detected in monocytes and upregulated during the transformation to macrophages by 3.18±0.52 times in relative fluorescein of peak value (P<0.01). Angiotensin II (1 μmol/L) induced oxidative stress in macrophages, with the peak at 15 minutes by 451±223%, and returned to the control level within 1 hour. EC50 was 5.4×10−9 mol/L. AT1 antagonist (CV11974, 1 μmol/L) significantly decreased angiotensin II–induced oxidative stress in macrophages, but AT2 antagonist (PD123319, 1 μmol/L) did not. Of interest, AT1 antagonist also decreased basal levels of peroxide production in macrophages in a dose-dependent manner. These results suggest that upregulation of the expression of AT1 receptor in macrophages contributes in part to upregulation of peroxide production. AT1 receptor antagonists may be useful to suppress oxidative stress of macrophages in atherosclerotic lesions. (Hypertension. 1999;33[part II]:335-339.)

Key Words: angiotensin II • receptor antagonists • peroxide • atherosclerosis • macrophages

The Survival and Ventricular Enlargement trial revealed that administration of angiotensin-converting enzyme (ACE) inhibitor after myocardial infarction reduced the incidence of recurrence of myocardial infarction.1 We demonstrated that ACE is massively expressed in the rupture sites of human coronary artery from patients of acute coronary syndrome.2 Diet et al3 and our group also demonstrated that vascular ACE expression in human coronary arteries was upregulated during the development of atheromatous plaques and the acute phase of the tissue repair after coronary angioplasty.4 These suggest that the renin-angiotensin system (RAS) is considered to play a role in the development of atherosclerosis and the disruption of plaques.

Recent pathological investigations including our studies showed that most of the macrophages in the atheromatous lesions were positively stained against ACE and angiotensin (Ang) II antibodies.2–5 The preventive effects of ACE inhibitors on the neointimal proliferation are unclear in the baboon6 and human angioplasty7 but are well observed in the rat balloon injury model.8 Furthermore, ACE inhibitors prevent atherosclerosis of cholesterol-fed rabbits9,10 and monkeys,11 whose vascular lesions are associated with massive infiltration of macrophages. Some balance of an activation of RAS in macrophages would be important for the development of atherosclerosis. Keidar et al12 further reported that the Ang II type 1 (AT1) receptor antagonist losartan inhibits atherosclerosis in apolipoprotein E–deficient mice in association with an inhibition of low-density lipoprotein (LDL) lipid peroxidation.

We also demonstrated that macrophages differentiated from THP1, a human monocyctic leukemia cell line, expressed whole components for Ang II generation, such as renin and angiotensinogen, and resulted in the production of Ang II into culture medium.13 The previous reports showed that other types of macrophages also expressed the components of RAS. That is, rat peritoneal macrophages express renin.14 Mouse peritoneal macrophages, J–774 (a cell line of mouse macrophages), and pig peritoneal macrophages present specific Ang II binding sites.15,16 These investigations suggest not only that Ang II induced by macrophages affects neighboring cells such as smooth muscle cells and fibroblasts but also that Ang II plays a role in macrophages per se through the autocrine/paracrine system.

However, the function of Ang II in macrophages per se is poorly understood. Keidar et al17,18 showed many evidences that Ang II affects the uptake of oxidized LDL into macro-
phages through modification of LDL. However, there is only 1 report that presented the function of Ang II mediated through Ang II receptors on macrophages. Scheidegg et al showed that Ang II increased macrophage lipoxygenase activity through AT1 receptor–mediated mechanisms in mouse peritoneal macrophages and J-774 cells. These facts suggest that Ang II in macrophages plays a role in the production of oxidized LDL and modifies atherosclerosis.

In addition to lipoxygenase, many factors such as superoxide, xanthine oxidase, thiol, peroxide, and myeloperoxidase are reported to be involved in oxidation of LDL. Some of these reactive oxygen species are also reported to contribute to atherosclerosis through other pathways. Therefore, in the present study, we further investigated the effects of Ang II on oxidative stress, especially peroxide production, by macrophages through Ang II receptors.

**Methods**

**Materials**

THP-1, as a human monocytic leukemia cell line, was obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). DCFH-DA, 2’,7’-dichlorofluorescein-diacetate, obtained from Eastman Kodak, was dissolved in anhydrous ethanol, 5 mmol/L and kept at 4°C. RPMI 1640 medium was obtained from ICN Biomedicals. Phorbol 12-myristate, 13-acetate (PMA) and EDTA were obtained from Sigma. Ang I, Ang II, Ang III, Ang IV, and Ang (1–7) were purchased from Peptide Institute, Inc. CV11974, an AT1 receptor antagonist, was donated by Takeda Chemical Industries, Ltd. PD123319, an Ang II type 2 (AT2) receptor antagonist, was donated by Park-Davis Pharmaceutical Research.

**Cell Culture**

THP-1 was grown in suspension culture in RPMI 1640 medium supplemented with penicillin G (100 U/mL) and 10% (vol/vol) fetal bovine serum at 37°C in a humidified atmosphere of air/CO2 (19:1, vol/vol). The cells were seeded into 6-cm culture dishes. To induce macrophage phenotype differentiation, 50 ng/mL PMA was added to THP-1 to transform to macrophages by 3.18 ± 0.52 times in the presence of Ang II (AII) on peroxide production in macrophages.

**Flow-Cytometric Assay of DCFH-DA Oxidation**

To measure peroxide levels, we performed flow-cytometric assay with DCFH-DA according to the previous reports. Macrophages were incubated with 5 × 10^{-6} mol/L DCFH-DA in medium for 30 minutes at 37°C. Reaction was stopped by centrifugation for monocytes and rinsing adherent cells with 4°C PBS(–) for macrophages. Macrophages were detached by trypsin. Cells were washed and centrifuged 3 more times with 4°C PBS(–). Cellular fluorescence was determined with a flow cytometry apparatus (FACS-SCAN, Becton-Dickinson). Measurements were done at 510 to 540 nm after excitation of cells at 488 nm with an argon ion laser. During flow-cytometric analysis, individual macrophages were discerned by the combination of low-angle forward-scattered and side-scattered laser light. Aggregated cells and fragments of cells were excluded from the analysis, then 10,000 events were registered for each experiment.

**Statistics**

For all statistical analysis, we used the computer software application StatView (Abacus Concepts, Inc). Values expressed as mean±SEM were compared by Student’s paired t test or 1-way ANOVA followed by Fisher’s protected least significant difference test. A probability value of <0.05 was considered significant.

**Results**

Peroxide was detected in monocytes and upregulated during the transformation to macrophages by 3.18 ± 0.52 times in the peak value of DCF fluorescence intensity (Figure 1, left). Ang II (10^{-6} mol/L) stimulated the production of peroxide in macrophages (Figure 1, right).

Figure 2 (left) shows the time course of an activation of peroxide production and upregulated during the transformation to macrophages by 3.18 ± 0.52 times in the peak value of DCF fluorescence intensity (Figure 1, left).

Ang II (10^{-6} mol/L) stimulated the production of peroxide in macrophages (Figure 1, right).

Figure 2 (left) shows the time course of changes in DCF fluorescence intensity of macrophages by Ang II (All) (10^{-6} mol/L). Values are mean±SEM of 4 experiments. DCFH-DA was added 30 minutes before cell harvest. *P<0.05 vs control (time 0). Right, Dose-dependent effect of Ang II on DCF fluorescence intensity in macrophages. Cells were incubated with DCFH-DA for 30 minutes. Each concentration of Ang II was incubated for the 15 last minutes. Values are mean±SEM of 4 samples. This is a representative example of similar experiments (n=3) carried out under the same conditions. *P<0.01 vs control.
Ang II by themselves. Therefore, Ang II concentrations leading to the conversion of Ang I from circulation and vascular tissue, and THP-1–derived macrophages generate Ang IV, and Ang (1–7) for 15 to 30 minutes. None of them related peptides, we incubated macrophages with Ang III, was mediated by Ang II but not by Ang II metabolites or enhancement of peroxide production by Ang II. AT2 receptor stimulation by Ang II with AT1 or AT2 receptor antagonist (Figure 3, left). AT1 receptor antagonist almost blocked enhancement of peroxide production by Ang II. AT2 receptor antagonist did not show any changes in peroxide production. The IC50 of AT1 receptor antagonist was 3.2×10⁻⁶ mol/L for the peroxide production by 10⁻⁹ mol/L of Ang II (Figure 3, right).

To evaluate whether Ang II–induced peroxide production was mediated by specific Ang II receptors, we incubated macrophages 5 minutes before and 30 minutes through the stimulation by Ang II with AT1 or AT2 receptor antagonist (Figure 3, left). AT1 receptor antagonist almost blocked enhancement of peroxide production by Ang II. AT2 receptor antagonist did not show any changes in peroxide production. The IC50 of AT1 receptor antagonist was 3.2×10⁻⁶ mol/L for the peroxide production by 10⁻⁸ mol/L of Ang II (Figure 3, right).

To evaluate whether Ang II–induced peroxide production was mediated by Ang II but not by Ang II metabolites or related peptides, we incubated macrophages with Ang III, Ang IV, and Ang (1–7) for 15 to 30 minutes. None of them showed any changes in the amount of peroxide in macrophages.

Ang I enhanced peroxide production as well as did Ang II. Percent changes in DCF fluorescence intensity compared with vehicle were 257±44% by Ang I and 360±41% by Ang II (n=4).

To determine if local generation of Ang II could occur, we studied effects of CV11974 and PD123319 on peroxide production. Incubation with CV11974 for 30 minutes in the absence of added Ang II decreased the basal levels of peroxide production in macrophages in a dose-dependent manner (Figure 4). PD123319 did not affect the basal levels of peroxide production.

**Discussion**

The present study demonstrated that Ang II enhanced peroxide production through AT1 receptor in macrophages. The concentrations of Ang II for stimulation of peroxide was relatively high compared with physiological plasma levels of Ang II in humans. However, macrophages express ACE, leading to the conversion of Ang I from circulation and vascular tissue, and THP-1–derived macrophages generate Ang II by themselves. Therefore, Ang II concentrations at tissue macrophages may be close to those required for peroxide stimulation in the present study. Furthermore, endogenous production of Ang II in macrophages also plays a role in peroxide production because AT1 receptor antagonist suppressed not only exogenous Ang II–induced production but also basal levels of peroxide.

Hydrogen peroxide, most of peroxide detected by DCFH-DA oxidation, is known to freely pass through the cell membrane. Therefore, the measurements of intracellular production of peroxide would associate extracellular concentration of hydrogen peroxide, which is a major source of reactive oxygen stress for oxidation of LDL. One of the important roles of intracellular activation of peroxide production may be stimulation of signal transduction, such as activation of the early growth response-1 gene. Hydrogen peroxide is further reported to contribute to atherosclerosis through other pathways, such as the modulation of vascular matrix metalloproteinases and smooth muscle cell apoptosis. These suggest that vascular Ang II plays an important role in atherosclerosis through peroxide production.

Oxidation of LDL is important in formation of foam cells and is a major risk factor for atherosclerosis. Multiple pathways are reported to be involved in oxidation of LDL. Lipoxygenase is one of these pathways and only one pathway that was previously investigated in the relation with Ang II.

**Figure 3.** Left, Effects of Ang II (AII) receptor antagonists on activation of DCF fluorescence intensity in macrophages by Ang II. AT1 antagonist CV11974 (10⁻⁶ mol/L) or AT2 antagonist PD123319 (10⁻⁶ mol/L) was added 20 minutes before stimulation by Ang II (10⁻⁹ mol/L, 15 minutes). DCFH-DA was added 30 minutes before cell harvest. Values are mean±SEM of 4 samples. *P<0.01 vs control, #P<0.01 vs Ang II.

**Figure 4.** Dose-dependent effect of AT1 antagonist CV11974 without extra addition of Ang II on DCF fluorescence intensity in macrophages. Cells were incubated with each concentration of CV11974 for 5 minutes, then DCFH-DA was added. Cells were further incubated for 30 minutes. Values are mean±SEM of 4 samples. This is a representative example of similar experiments (n=3) carried out under the same conditions. *P<0.01 vs vehicle.
The importance of 15-lipoxygenase in oxidation of LDL is indicated by colocalization of 15-lipoxygenase mRNA and protein with epitopes of oxidized LDL in macrophage-rich areas of atherosclerotic lesions.28 Activation of lipoxygenase by Ang II was investigated 4 hours after stimulation, which is delayed to an activation of peroxide in our experiment. Alternatively, receptor-mediated function of Ang II in our present study differs from the direct modification of LDL by Ang II demonstrated by Keider et al.18,29 They showed that Ang II–modified LDL is taken up by macrophages through the scavenger receptor. Although the concentration of Ang II for the experiments were different between the study by Keider et al and our present study (10−7 to 10−8 mol/L vs 10−10 to 10−8 mol/L), Ang II may accelerate atherosclerosis through both pathways.

Our experiment revealed that Ang II was the only potent peptide to stimulate peroxide production through AT1 receptors but not AT2 receptors. Stimulation of peroxide production by Ang I appears to be the result of converted Ang II. It was reported that ACE was upregulated during transformation from monocytes to macrophages and that ACE was significantly expressed in macrophages in human atherosclerotic lesions.32,33 However, non-ACE–dependent pathways for Ang II generation were also reported in macrophages. Chymase immunoreactivity was observed in some of the CD-68–positive macrophages in the infarcted area of human myocardial infarction.31 Cathepsin G is a well-reported neutrophil enzyme that cleaves Ang I and angiotensinogen.32,33 Although it is unknown which enzymes, including ACE, are dominant in the atherosclerotic lesions, AT1 receptor antagonists may be more useful to suppress oxidative stress of macrophages than ACE inhibitors.

Peroxide is a relatively stable metabolite of reactive oxygen species; xanthine oxidase/xanthine and superoxide dismutase contribute to production of peroxide with superoxide, and catalase and myeloperoxidase produce hydroxyl radicals from peroxide. Colocalization of superoxide dismutase34,35 or myeloperoxidase36 with macrophages in the atherosclerotic lesions was reported. Further studies are required to reveal which pathways are activated or suppressed by Ang II.

In summary, the present study demonstrated that modification of oxidative stress in macrophages is an important role of the upregulated Ang II–generating system including ACE and Ang II receptors in macrophages. Since both exogenous Ang II and AT1 antagonist affected peroxide production, not only circulating Ang II but also Ang II generated by macrophages per se contributes to oxidative stress through the autocrine/paracrine system. ACE inhibitors and AT1 receptor antagonists may affect atherosclerosis not only by inhibition of the growth of smooth muscle cells37 and the synthesis of extracellular matrix38 but by suppression of oxidative stress in macrophages.

References

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