The involvement of oxidative stress in polymorphonuclear leukocytes (PMN) in the pathogenesis of hypertension remains to be elucidated. We analyzed the generation of reactive oxygen species (ROS) by the circulating and peritoneally infiltrating PMN from spontaneously hypertensive rats (SHR) and Wistar Kyoto rats (WKY). Flow cytometric analysis revealed that ROS generation by PMN from SHR was higher than that from WKY before (at 6 weeks of age) and after (at 16 weeks of age) the onset of hypertension. In vivo, ROS generation by PMN from SHR, but not by PMN from WKY, was significantly suppressed by 10-week treatment with 50 mg/kg/day carvedilol, and this treatment did not affect blood pressure. Western blotting analysis revealed that protein kinase C δ (PKC δ), but not PKC ζ I or ζ II, was activated more strongly in PMN from SHR than in PMN from WKY. Furthermore, expression of p47phox of nicotinamide adenine dinucleotide phosphate oxidase, but not of p67phox, in PMN from SHR was higher than that in PMN from WKY. These results suggest that ROS generation by PMN is principally enhanced in SHR through activation of PKC δ and p47phox. (Hypertens Res 2003; 26: 999–1006)

Key Words: genetic hypertension, leukocytes, oxidative stress, fluorescence, kinase

Introduction

It has been postulated that oxidative stress underlies the pathogenesis of hypertension and related cardiovascular diseases (1–7). Recent studies have reported that the generation of reactive oxygen species (ROS) by the circulating polymorphonuclear leukocytes (PMN) was significantly higher in spontaneously hypertensive rats (SHR) than in control Wistar Kyoto rats (WKY) (8, 9). Although the mechanism of ROS generation by PMN has been studied extensively, the pathophysiological significance of the enhanced ROS generation in SHR remains to be elucidated. Because nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is the primary source of ROS generated by PMN (10–13), we here compared the activity of NADPH oxidase and related enzymes in PMN from SHR with those in PMN from WKY.

Phosphorylation followed by translocation of p47phox, a cytosolic component of NADPH oxidase, plays a critical role in the activation of the enzyme (14, 15). Phosphorylation of p47phox by a protein kinase C (PKC) has been shown to stimulate the translocation of this subunit onto the oxidase in plasma membranes (16). The levels of PKC activity in the aorta and erythrocytes from patients with essential hypertension have been shown to be higher than those in healthy subjects (17, 18). Therefore, we studied the possible involvement of PKC and NADPH oxidase in the enhanced generation of ROS by PMN from SHR.

From the Department of Biochemistry and Molecular Pathology and *Department of Cardiology, Osaka City University Medical School, Osaka, Japan.

This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Sports, Science, and Technology of Japan and the grants from the Takeda Science Foundation, the Kimura Memorial Heart Foundation, and the Research Foundation of Community Medicine.

Address for Reprints: Kensaku Maeda, M.D., Department of Biochemistry and Molecular Pathology, Osaka City University Medical School, 1–4–3 Asahimachi, Abeno-ku, Osaka 545–8585, Japan. E-mail: maedahk@med.osaka-cu.ac.jp

Received April 25, 2003; Accepted in revised form September 2, 2003.
Some antihypertensive agents show antioxidant-like activity. If enhanced oxidative stress underlies the pathogenesis of cardiovascular diseases, antihypertensive agents having antioxidant activity would become more important than previously expected. Thus, we also analyzed the effect of the antihypertensive agent carvedilol, a vasodilating β-blocker that has been shown to have antioxidant activity (19), on the generation of ROS by the circulating and peritoneally infiltrating PMN from WKY and SHR before and after the occurrence of hypertension.

Methods

Materials

Catalase, Cu/Zn-superoxide dismutase (SOD), diphenyleneiodonium (DPI), phorbol 12-myristate 13-acetate (PMA), prazosin, atenolol, and ICI 118551 were purchased from Sigma Chemical Co. (St. Louis, USA). Staurosporine, 2’,7’-dichlorodihydrofluorescein diacetate bis-acetoxymethyl ester (CDCFH), and alotinol were purchased from Wako Pure Chemical Co. (Osaka, Japan), Molecular Probe Co. (Eugene, USA), and Sumitomo Pharmaceuticals Co. (Osaka, Japan), respectively. 8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4-(2H,3H)-dione (L-012) was donated by Takeda Chemical Ind. (Osaka, Japan).

Animals

Male SHR (6 and 16 weeks of age) and age-matched WKY were purchased from SLC Co. (Shizuoka, Japan). They were housed in a controlled environment and maintained with free access to a standard laboratory chow and water. All studies were approved by the Institutional Animal Care and Use Committee of Osaka City University Medical School.

Preparation of PMN

Venous blood samples were collected from the femoral vein in polyethylene tubes containing 20 µl of 3.8% sodium citrate, and freshly used for experiments. Peritoneal PMN were obtained from the peritoneal fluid 16 h after intraperitoneal injection of 100 ml/kg of 2% casein containing 5 mmol/l CaCl₂, and freshly used for experiments. Peritoneal PMN were obtained from the peritoneal fluid 16 h after intraperitoneal injection of 100 ml/kg of 2% casein containing 5 mmol/l CaCl₂, and freshly used for experiments. The purity of PMN in the samples, as assessed by microscopy, was higher than 95%.

Chemiluminescence Analysis

L-012, a highly sensitive chemiluminescence probe that shows strong chemiluminescence in the presence of superoxide, hydrogen peroxide, and/or their metabolite(s), was used for the analysis of ROS generation by PMN in fresh blood samples. Fresh blood samples (10–50 µl) and peripheral PMN (1 × 10⁶ cells/ml) were incubated in 0.5 ml of PBS in the presence or absence of 400 µmol/l L-012. After incubation of the mixtures at 37 °C for 3 min, ROS generation was analyzed by the presence or absence of 0.3 µmol/l PMA. During the incubation, chemiluminescence intensity was recorded continuously for 20–40 min using a BLR-201 Luminescence Reader (Aloka, Tokyo, Japan) as described previously (20).

Flow Cytometric Analysis

To distinguish PMN from whole leukocytes, we employed a flow cytometric method using CDCFH, a hydroperoxide-sensitive fluorescent probe that is trapped within viable cells in a nonfluorescent form and converted to fluorescent dichlorofluorescein by hydroperoxides. Generation of ROS by PMN was analyzed using a gated-flow cytometry method with a FACS Calibur (Becton Dickinson, Mountain View, USA) as described previously (21, 22). After incubation of PMN in 500 µl of PBS containing 5 µmol/l CDCFH at 37 °C for 15 min, the fluorescence intensity was determined.

Separation of Membrane Samples from PMN

Precipitated PMN were solubilized in 20 mmol/l HEPES buffer (pH 7.4) containing 250 mmol/l sucrose, 2 mmol/l EDTA, 2 mmol/l EGTA, 10 mmol/l mercaptoethanol, 1 mmol/l leupeptin, and 1 mmol/l PMSF. After sonication at 4 °C for 20 s, the samples were centrifuged at 100,000 × g for 40 min. The precipitated fraction was solubilized in the same buffer containing 0.5% TritonX-100 and centrifuged at 100,000 × g for 40 min. The resulting supernatant was used for experiments.

Western Blotting Analysis

Precipitated PMN were incubated in 1 ml of 0.9% NaCl containing 20% trichloroacetic acid at 4°C for 15 min, and then centrifuged at 10,000 × g for 5 min. The precipitate was dissolved by sonication for 10 s in 80 µl of 9 mol/l urea containing 2% TritonX-100 and 1% dithiothreitol. The samples were mixed with 20 µl of 10% lithium dodecylsulfate (LiDS) containing 0.01% bromophenol blue. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% gel) was carried out after adjusting the pH to 7.4. The electrophoresed proteins were transferred to a nylon membrane, and analyzed by Western blotting using specific antibodies to PKC α, PKC β, PKC βII, p67phox (Santa Cruz Biotechn-
Fig. 1. Properties of ROS metabolites in PMN from WKY and SHR. A: Chemiluminescence intensities of unstimulated and 0.3 \( \mu \text{mol/l} \) PMA-stimulated PMN in fresh blood samples were analyzed using L-012, a highly sensitive chemiluminescence, as described in the text. Values (kcpm/10^6 cells) are the means \( \pm \) SD for 4 animals. *p < 0.05. B: At 16 h after intraperitoneal injection of 2% casein, the chemiluminescence intensities of unstimulated and 0.3 \( \mu \text{mol/l} \) PMA-stimulated PMN in the peritoneal lavage fluid from WKY and SHR were analyzed by using L-012 as described in the text. Values (kcpm/10^6 cells) are the means \( \pm \) SD for 4 animals. *p < 0.05. C: Fluorescence intensities of PMN in the peritoneal lavage fluid from WKY and SHR were analyzed using flow cytometry as described in the text. Values (arbitrary units) are the means \( \pm \) SD for 6 animals. *p < 0.01. Flow cytometry analysis of PMN from WKY (closed peaks) and SHR (open peaks) are shown. The horizontal axis shows the relative intensity of fluorescence, while the vertical axis displays the cell number.
Immunoreactive protein bands were visualized by an enhanced chemiluminescence method.

**Antihypertensive Protocol**

At 6 weeks of age, both WKY and SHR were divided into 2 groups that were respectively treated with vehicle (0.5% carboxymethylcellulose solution) and carvedilol (50 mg/kg/day). These treatments were orally administered by gastric gavage once a day until 16 weeks of age (i.e., for 10 weeks). The systolic blood pressure (SBP) of conscious rats was measured by the tail-cuff method at 6 h after each oral dosing. At 16 weeks of age, ROS generation by peritoneal PMN from these rats was measured using flow cytometry as described above.

**Statistical Analysis**

Statistical analysis of fluorescence intensity was performed by analysis of variance and Student’s t-test. All values are the means ± SD derived from 6 animals. Values of *p* < 0.05 were considered statistically significant.

**Results**

**ROS Generation by PMN**

We examined the properties of ROS generated by PMN in fresh blood samples using L-012, a highly sensitive chemiluminescence probe (20). The chemiluminescence intensities of the control and PMA-stimulated blood samples from SHR were about 2.4- and 12-fold higher than those from WKY, respectively (Fig. 1A). To determine the relationship between the properties of PMN in the circulation and peritoneal fluid, chemiluminescence intensities of control and PMA-stimulated peritoneal PMN were also compared between the two animal groups. The chemiluminescence intensities of the control and PMA-stimulated peritoneal PMN from SHR were about 1.5- and 6.7-fold higher than those from WKY, respectively (Fig. 1B). Furthermore, the CDCFH-dependent fluorescence intensity of the peritoneal PMN from SHR was about 2.5-fold higher than that from WKY (Fig. 1C).

**ROS Generation by PMN before and after the Onset of Hypertension**

To determine whether the oxidative stress elicited by PMN was the cause or the consequence of hypertension, we compared the generation of ROS by PMN before (at 6 weeks of age) and after (at 16 weeks of age) the onset of hypertension. The chemiluminescence intensity of PMA-stimulated PMN from SHR was significantly higher than that from WKY irre-
spective of the age at sampling (Fig. 2). The CDCFH-dependent fluorescence intensity of PMN from SHR was also higher than that from WKY irrespective of the occurrence of hypertension.

Properties of ROS Generated by PMN

To elucidate the biochemical properties of ROS generated by peritoneal PMN, the effects of catalase and SOD on the fluorescence intensity of CDCFH were studied. Although the fluorescence intensity of PMN from SHR was significantly higher than that of PMN from WKY, the presence of catalase and SOD decreased the fluorescence intensity in both animal groups (Fig. 3).

Effects of PKC- and NADPH Oxidase-Inhibitors on ROS Generation

To elucidate the mechanism by which ROS generation by PMN from SHR was enhanced, we examined the possible involvement of PKC and NADPH oxidase using specific inhibitors of the enzymes (Fig. 4). Staurosporine, but not DPI, inhibited the generation of ROS by control PMN from WKY, while the PMA-enhanced ROS generation was inhibited by DPI. In contrast, ROS generation by PMA-stimulated PMN from SHR was inhibited strongly by staurosporine and DPI.

Western Blotting Analysis of PMN Enzymes

To determine the isoform(s) of PKC underlying the enhancement of ROS generation by PMN, Western blotting analysis was carried out using specific antibodies. Figure 5 shows that cellular levels of PKC-α and PKC-βI, but not PKC-βII, were significantly higher with PMN from SHR than with PMN from WKY. The level of p47phox, but not p67phox, was also higher in PMN from SHR than in PMN from WKY. No significant difference in cellular levels of gp91phox, p22phox, p40phox, or rac was found between the two animal groups (data not shown).

We also examined the translocation of the constituents of the two enzymes from the cytosol to the plasma membrane of PMN. The level of membrane-bound PKC-α, but not PKC-βI or PKC-βII, was significantly higher in SHR than in WKY. The level of membrane-bound p47phox, but not p67phox, was also higher in SHR than WKY.
Effects of Adrenoceptor Blockers on ROS Generation by PMN

To clarify which adrenoceptors are involved in ROS generation by PMN, we examined the effect of α- and/or β-adrenoceptor inhibitors on ROS generation by PMN in the two animal groups (Fig. 6). Although carvedilol inhibited the generation of ROS by control and PMA-stimulated PMN from WKY only minimally, it strongly inhibited those from SHR. ROS generation by PMA-stimulated PMN from SHR, but that by PMA-stimulated PMN from WKY, was suppressed slightly by ICI 118551, a specific β2-blocker. Other blockers of adrenoceptors, i.e., prazosin (α-blocker), alotinolol (α, β-blocker), and atenolol (specific β1-blocker), did not affect the generation of ROS by PMN.

Effect of Carvedilol in Vivo

To elucidate whether carvedilol affects ROS generation by PMN in vivo, we examined the fluorescence intensities of CDCFH-treated PMN in SHR and WKY treated with vehicle or treated chronically with carvedilol for 10 weeks using flow cytometry. As shown in Fig. 7, chronic treatment with carvedilol did not decrease blood pressure in SHR, but it significantly suppressed ROS generation by PMN from SHR. On the other hand, blood pressure in 16-week-old WKY was unchanged by treatment with carvedilol (vehicle, 116.4 ± 10.2 mmHg; carvedilol, 123.1 ± 19.2 mmHg), and administration of carvedilol also did not affect ROS generation by PMN from WKY.

Discussion

Although ROS generation by circulating PMN is enhanced in patients with essential hypertension (3–5) and in SHR (7, 23), the involvement of PMN in the pathogenesis of hypertension remains to be elucidated. The present work clearly showed that ROS generation by circulating and peritoneally infiltrating PMN from SHR before and after the occurrence of hypertension was significantly higher than that by such PMN from WKY. The increased ROS generation by PMN from SHR was associated with increased expression of PKC-α and p47phox. ROS generation by PMN from SHR was suppressed by carvedilol more strongly than that by PMN from WKY. The strong suppression of ROS generation by this inhibitor might indicate that PKC is down-regulated in PMN from SHR.

Because the chemiluminescence intensities of unstimulated (control) and PMA-stimulated PMN in fresh blood and those obtained from the peritoneal cavity of SHR were significantly higher than those of WKY, both types of cells from the former generated larger amounts of ROS than those from the latter. Flow cytometric analysis also showed that ROS generation by peritoneal PMN from SHR was higher than that by peritoneal PMN from WKY. Because peritoneal PMN were collected using a casein-stimulated method, they might already have been primed, at least to some degree, to generate ROS. Both the chemiluminescence and fluorescence intensities of PMN obtained from the peritoneal cavity were higher than those of PMN from fresh blood. Thus, oxidative stress in and around PMN of SHR appears to be stronger than that in and around PMN of WKY.

Furthermore, both the chemiluminescence and fluores-
cience intensities of PMN obtained from 6- and 16-week-old SHR were higher than those from age-matched WKY; the ROS-generating activity of PMN was high at the age of 6 weeks, when SHR exhibited normal blood pressure (blood pressure in 6-week-old SHR, 118.3 ± 15.5 mmHg), and remained unchanged through 16 weeks (blood pressure in 16-week-old SHR, 190.3 ± 14.2 mmHg). This observation suggests that the increased ROS generation by PMN from SHR is not the consequence but perhaps the cause of hypertension. Thus, the possible involvement of PMN-elicted oxidative stress in the etiology of hypertension in human subjects should be studied further. Blood pressure has been shown to decrease after administration of antioxidants to patients with hypertension (24, 25). This observation is consistent with our hypothesis that PMN-elicted oxidative stress underlies the pathogenesis of hypertension.

Since both staurosporine and DPI strongly suppressed the increased generation of ROS by PMN from SHR, a PKC-dependent signaling pathway to activate NADPH oxidase might be stimulated in these cells. The PKC isoform responsible for NADPH oxidase activation has been studied extensively (26–29). PKC β and βII isoforms and NADPH oxidase subunits (p47phox, p67phox) have been shown to play important roles in the differentiation of human promyelo-cyctic HL60 cells (26). PKC (α, βII) and NADPH oxidase p47phox also play critical roles in the generation of ROS by human neutrophils (27, 28). On the other hand, PKC α but not PKC βII, is associated with p47phox activation in human monocytes (29). These results suggest that PKC α might be principally responsible for the activation of NADPH oxidase. In fact, Western blotting analysis in the present study demonstrated enhanced expression of PKC α, PKC βII, and p47phox, and the activation of PKC α and p47phox in PMN from SHR. Because phosphorylation of p47phox by PKC is known to activate NADPH oxidase, the present results suggest that PKC α activated in PMN from SHR might enhance the expression of p47phox and its translocation to membranous NADPH oxidase to generate ROS. Although the expression of PKC βII was enhanced in PMN from SHR, the catalytic activity of this isoform remained unchanged. Thus, PKC βII may not be involved in the activation of NADPH oxidase that enhances ROS generation by PMN from SHR.

We speculated that suppression of ROS generation might be associated mainly with β2-adrenoceptor blockade in PMN, but the mechanism of ROS suppression by carvedilol was found to involve not only β2-adrenoceptor blockade, but also suppression of PKC α activation. Because carvedilol more significantly suppressed ROS generation (30, 31) by control PMN from SHR and PMA-stimulated PMN from both animal groups compared with other adrenoceptor blockers, this agent seems to selectively modulate the activated form of PKC α. The carvedilol-induced inhibition of ROS generation by PMN from SHR might reflect not only β2-adrenoceptor blockade but also the suppression of PKC activation in cells. In addition to the direct effect of carvedilol as a β2-adrenoceptor blocker, the apparent decrease by this agent of the oxidative stress elicited by activated PMN may have beneficial effects in patients with hypertension.

There was no difference in oxidative stress in PMN between 6- and 16-week-old SHR, although blood pressure was significantly increased during that period. As shown in Fig. 7, carvedilol, which did not decrease blood pressure, significantly suppressed ROS generation by PMN. The changes in blood pressure were thus not necessarily related to the changes in oxidative stress by PMN in SHR. This result suggested that enhanced oxidative stress by PMN, which did not correlate with blood pressure, was peculiar to SHR and might be one cause of hypertension.

In conclusion, we found that generation of ROS in the circulating and peritoneally infiltrating PMN from SHR was principally higher than in those from WKY, presumably due to enhancement of PKC α and/or NADPH oxidase. The results obtained from the experiments using carvedilol suggest that this antihypertensive agent decreases oxidative stress in hypertensive subjects by suppressing the activity of PKC α, a critical enzyme that activates NADPH oxidase.

References