Effect of Methylprednisolone on Plasma Lipid Peroxidation Induced by Lipopolysaccharide

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ABSTRACT—The effects of methylprednisolone succinate (MP) on plasma lipid peroxidation, plasma SOD activity and superoxide production in polymorphonuclear leukocytes (PMNs) induced by lipopolysaccharide (LPS) were examined in rats in vivo and in vitro. In rats subjected to LPS treatment, plasma phosphatidylcholine hydroperoxide (PCOOH) levels significantly increased, and the plasma Cu,Zn-SOD activity decreased by about 75%. When rats were given 30 mg/kg of MP intravenously, MP suppressed the elevation of plasma PCOOH levels and partially inhibited the decrease in plasma Cu,Zn-SOD activity. MP also suppressed PMA-induced superoxide production in PMNs primed by LPS. In in vitro experiments, low concentrations of MP had no effect on NADPH-dependent lipid peroxidation, but 4 mM MP produced 50% inhibition. MP had little effect on PMA-induced superoxide production in PMNs primed by LPS. Moreover, MP had no radical-trapping effect on superoxide, hydroxyl radical and stable DPPH radical. These results suggest that the suppressive effect of plasma lipid peroxidation by MP is not due to radical-trapping effects or preventive anti-oxidation, but may involve the suppression of the lipid chain reaction in liver membrane resulting from PMA-induced superoxide anions generated by PMNs.

Keywords: Lipopolysaccharide, Methylprednisolone, Lipid peroxidation, Plasma phosphatidylcholine hydroperoxide, Polymorphonuclear leukocyte

Although lipopolysaccharide (LPS, endotoxin) from Gram-negative bacteria has been shown to induce increases in lipid peroxidation, just how LPS acts as an oxidant stressor has yet to be determined (1). Injection of LPS causes lung and liver lipid peroxidation in a number of animal models, measured indirectly by increased levels of malondialdehyde (MDA) or conjugated dienes (2-4). Lipid peroxidation appears to play a significant role in oxidative pathology (5-7). In addition, the administration of antioxidants has been reported to decrease the physiological, biochemical and histological abnormalities caused by LPS. For example, coenzyme Q10 or a-tocopherol were able to function in cooperation with endogenous antioxidants to prevent tissue damage caused by lipid peroxidation in endotoxemia (8). CV-3611, an antioxidant, increased the survival rate of mice subjected to endotoxin (9). There appears to be a cause-and-effect relationship between LPS administration and the resulting overproduction of reactive oxygen species. Reactive oxygen species, including free radicals (for example, superoxide anion and hydroxyl radical) and other toxic oxygen metabolites, such as hydrogen peroxide and hypochlorous acid, have been postulated to be important mediators in several models of tissue injury (10, 11).

Methylprednisolone succinate (MP) has often been used in cases of endotoxemia and consequent endotoxemic shock, since glucocorticoids are believed to prevent endotoxic shock in animals through the stabilization of the lysosomal membrane, the restoration of blood glucose and other mechanisms (12). Kawamura et al. (13) indicated that glucocorticoids enhanced the endogenous activity of glomerular antioxidant enzymes, instances of renal injury caused by reactive oxygen species and attenuated lipid peroxidation. Moreover, Marzatico et al. (14) showed that methylprednisolone treatment decreased the products of lipid peroxidation in all areas of the brain.

In the present study, we investigated the beneficial effects of MP against plasma lipid peroxidation induced by LPS and attempted to clarify the relationship between reactive oxygen species and LPS-induced lipid peroxidation.
MATERIALS AND METHODS

Animals
Male Wistar rats, weighing 200 to 400 g, were housed in an environmentally controlled room (20–25°C, 50–60% humidity, illuminated from 7:00 to 19:00 hr) with food and water available ad libitum for 5 days prior to our experiments. The rats were then starved for 16 hr before blood sampling.

Administration of LPS and MP
Rats were divided into three groups: A (saline), B (LPS only) and C (LPS + MP). Rats from Group A were injected intraperitoneally with 1 ml/kg of 0.9% saline. Rats from Group B were injected intraperitoneally with 5 mg/kg of LPS (from Salmonella typhphumurium). Rats from Group C were injected intravenously with 30 mg/kg of MP immediately after LPS treatment. Blood was obtained under light ether anesthesia 12 hr after the injection of LPS.

Assay of plasma PCOOH
Plasma lipids were extracted in a test tube filled with N₂ gas as described by Folch et al. (15). The lipids from each 1-ml aliquot of plasma were extracted with 8 ml of a chloroform/methanol (3:1, v/v) solution containing 0.005% BHT. The mixture was agitated for 3 min and then centrifuged at 700 x g for 10 min at 10°C. The lower layer was collected, dried under N₂ gas at 33°C and redissolved in 100 μl of a chloroform/methanol (1:1, v/v) solution. Twenty microliters of the final solution, containing lipids extracted from 200 μl of plasma, were injected into the chemiluminescence (CL)-HPLC equipment for the hydroperoxide assay. Plasma phosphatidylcholine hydroperoxide (PCOOH) was determined by the method of Takayama et al. (16).

The CL-HPLC system consisted of an injector (Rheodyne 7161; Rheodyne Inc., Cotati, CA, USA), column (Jasco Fine Pack SIL, 5 μm, 250 × 4.6 mm; Japan Spectroscopic Co., Tokyo), pumps (Jasco 880-PU and Shimadzu LC10AS; Shimadzu Co., Kyoto), a UV detector (Jasco 875UV), a CL detector (Jasco 825), and two integrators (Shimadzu Chromatopack C-R6A). The mobile phase for HPLC was acetonitrile/methanol/water (5.5:3:1.5, v/v). The CL reagent was prepared by dissolving 1 μg/ml of luminol (3-aminophthaloyl hydrazine, Wako Pure Chemical Co.) and 10 μg/ml of cytochrome c (from horse heart type VI, Sigma Chemical Co.) in 20 mM of borate buffer, pH 10, which was saturated with N₂ gas and contained 1% methanol. The flow rate was 1 ml/min.

Plasma SOD activity
SOD (superoxide dismutase) activity was measured by an ESR spin-trapping method utilizing 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) as a spin-trapping agent. The measurement is based on the theory of detecting superoxide anions (O₂⁻) as DMPO spin adducts. O₂⁻ radicals were generated by the reaction of hypoxanthine (HX) and xanthine oxidase (XOD). Briefly, 15 μl of 9.2 M DMPO was mixed with 50 μl of 2 mM HX in a phosphate buffer solution (PBS), pH 7.4, and 35 μl of 5.5 mM diethylenetriamine pentaacetic acid (DETAPAC) in PBS, and samples (50 μl) were added. Then 50 μl of 0.4 U/ml XOD was also added, and the solution was mixed. The reaction mixture was transferred into a quartz cell and the ESR spectra of DMPO-OOH, the spin-trapped adduct of the superoxide anion, were measured with the ESR-spectrometer. ESR measurement was started at room temperature using JES RE1X (Jeol Ltd., Tokyo). The spectrometer was run with microwave power set at 8 mW, center field set at 334.8±5 mT, a modulation width of 0.1 mT, an amplitude of 2.5×100, a time constant of 0.3 sec and a sweep time of 2 min. To measure Mn-SOD activity, 1 mM KCN was added to the reaction mixture. The Cu,Zn-SOD activity was calculated by subtracting the amount of Mn-SOD activity from the total SOD activity.

Assay of NADPH-dependent lipid peroxidation
To determine NADPH-dependent lipid peroxidation, we used a medium consisting of 0.02 M phosphate buffer (pH 7.4), 0.4 mM NADPH, 0.1 mM ferric pyrophosphate and 1.6 mg of liver homogenate protein, in a total volume of 5 ml. The incubation mixture was placed in a shaking water bath at 37°C, and 0.5-ml aliquots were removed at designated times to assay the formation of MDA. MDA was measured by using a modification of the method of Talcott et al. (17). Aliquots were transferred to screw-capped tubes containing 2.5 ml of 20% TCA and mixed for 15 sec. After adding 1.0 ml of 0.67% thiobarbituric acid, the tubes were capped, put in boiling water for 15 min, cooled and then extracted with 4.0 ml of n-butanol. The tubes were then centrifuged (700 x g, 10 min), and the optical density of the n-butanol layer was read at 532 nm. Kinetics were analyzed by the Lineweaver-Burk plot, and a range of 1 to 20 mM NADPH was used to obtain linear results.

Superoxide production in PMNs
Rat blood was collected from the inferior vena cava with a heparinized syringe. The blood was sedimented in 3% dextran, and the supernatant was centrifuged (300 × g, 10 min). Then the pellet was suspended in Hank’s balanced salt solution, layered on a Ficoll-Conrey density gradient and then centrifuged at 500 × g for 30 min. The separated neutrophils were washed twice after hypotonic lysis of residual erythrocytes and suspended in
Hank's solution. The concentration of neutrophils in Hank's solution was then adjusted to $3 \times 10^7$ cells/ml. Superoxide anion production was measured using the reduction of ferricytochrome c, as described by Babior et al. (18). The reaction mixture, containing 400 $\mu$1 of Krebs Ringer phosphate buffer (pH 7.4) with 5.4 mM glucose, 20 $\mu$1 of 2 mM ferricytochrome c, and 100 $\mu$1 of cell suspension ($3 \times 10^6$ cells) was preincubated at 37°C for 2 min in a cuvette with a 1-cm light path, and 10 $\mu$1 phorbol myristate acetate (PMA) was then added. The increase in optical density at 550 nm was then measured using a dual beam spectrophotometer (model 557; Hitachi, Tokyo).

Radical-trapping ability

For determining the superoxide anion-trapping ability of MP, 15 $\mu$1 of 9.2 M DMPO was mixed with 50 $\mu$1 of 2 mM HX in PBS, 35 $\mu$1 of 5.5 mM DETAPAC in PBS and various concentrations of MP (50 $\mu$1). Then 50 $\mu$1 of 0.4 U/ml XOD was added and the solution was mixed. The ESR spectra of DMPO-OOH was measured with the ESR-spectrometer. To determine hydroxyl radical-trapping ability, 20 $\mu$1 of 0.92 M DMPO was mixed with 75 $\mu$1 of 1 mM FeSO4 and 1 mM DETAPAC in PBS, 75 $\mu$1 of 1 mM hydrogen peroxide and various concentrations of MP. The ESR spectra of DMPO-OH, the spin-trapped dact of the hydroxyl radical, was also measured with the ESR-spectrometer. The radical-trapping ability of 1,1-diphenyl-2-picrylhydrazyl (DPPH), a stable free radical, was also estimated. Various concentrations of MP were mixed with 1 mM DPPH in ethanol, and the DPPH adduct was observed with the ESR-spectrometer.

Protein concentrations

Protein concentrations were determined according to the method of Lowry et al. (19), using bovine serum albumin as a standard.

Chemicals

MP was a gift from Japan Upjohn Co., Ltd. (Tokyo). Betamethasone sodium phosphate was donated by Shionogi Pharmaceuticals Co., Ltd. (Osaka). LPS, 2-thiobarbituric acid, DETAPAC and XOD (from butter milk) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka). NADPH, PMA, ferricytochrome c, XO, and SOD (from bovine erythrocytes) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). DMPO was obtained from Labotec Co., Ltd. (Tokyo). Other reagents were of analytical grade.

Statistics

Data were expressed as the mean±S.E. The significant difference between means was determined by Student's t-test. P values <0.05 were considered to indicate a significant difference.

RESULTS

Effect of MP on plasma PCOOH levels

After administration of 5 mg/kg LPS, i.p., the plasma PCOOH level was determined by CL-HPLC. The PCOOH levels of the LPS-treated group were significantly higher than those of the saline group. However, treatment with MP 30 mg/kg, i.v. significantly inhibited the increase in the plasma PCOOH level induced by LPS treatment (Fig. 1).

Plasma SOD activities

Plasma SOD activities were determined by ESR. As shown in Fig. 2, plasma Cu,Zn-SOD activity in the LPS-treated group decreased to about 25% of the activity seen in the saline group. Administration of 30 mg/kg of MP significantly diminished the decrease of plasma Cu,Zn-SOD activity induced by LPS treatment. In contrast, no significant change in plasma Mn-SOD activity was observed in any of the three groups (Fig. 2).

Effect of MP on PMA-induced superoxide production (in vivo)

The effect of MP on PMA-induced superoxide production in rat polymorphonuclear leukocytes (PMNs) was examined in vivo. As shown in Table 1, PMA-induced superoxide production was increased with the addition of LPS at 5 mg/kg, i.p. When MP was intravenously injected, PMA-induced superoxide production in LPS-treated
PMNs was significantly suppressed.

**Effect of MP on PMA-induced superoxide production (in vitro)**

The effects of MP on PMA-induced superoxide production in LPS-treated PMNs were investigated in vitro. As shown in Fig. 3, PMA-induced superoxide production was suppressed by the addition of MP at concentrations of $10^{-1}$ to $10^{-2}$ M. The addition of succinate resulted in an identical, suppressed curve, while betamethasone had no effect on PMA-induced superoxide production.

**NADPH-dependent lipid peroxidation (in vitro)**

The effect of the incubation period on NADPH-dependent lipid peroxidation of liver homogenate in the presence of 8 mM, 4 mM, 2 mM and 20 µM MP is shown in Fig. 4. The formation of MDA induced by NADPH-dependent lipid peroxidation was completely inhibited by the addition of 8 mM MP. Concentrations of 4 mM MP produced about 50% inhibition, while neither 2 mM nor 20 µM MP had any effect on MDA formation. A

### Table 1. The in vivo effect of MP on PMA-induced superoxide production in rat PMNs 12 hr after LPS injection

| Treatments   | $O_2^-$ generation activity (nmol/min/10^6 cells) |
|--------------|-----------------------------------------------|
| Saline       | 1.11 ± 0.32                                   |
| LPS only     | 15.79 ± 1.47                                  |
| LPS + MP     | 9.27 ± 1.35                                   |

Rats were given saline, LPS or LPS + MP as described in Methods. Each value represents the mean ± S.E. from five experiments. *P < 0.01.
Lineweaver-Burk plot was used to investigate the kinetics of inhibition of NADPH-dependent lipid peroxidation caused by MP. Inhibition at high concentrations of MP was not due to competition with NADPH (data not shown).

\textit{Radical-trapping ability}

To determine if the suppressive effects of MP were due to direct radical scavenging, the radical-trapping ability of MP for superoxide, hydroxyl and DPPH radicals was examined by the ESR method. MP at a concentration of 1 mM exhibited no radical-trapping activity toward the above three radicals (Fig. 5).

\section*{DISCUSSION}

Gram-negative bacterial LPS over-stimulates mononuclear cells, resulting in the synthesis and release of cytokines, arachidonic acid metabolites and other mediators (20–22). Other biological effects of LPS include the production of anaphylatoxin that results in the release of chemical mediators such as histamine, serotonin and kinin through activation of the alternative pathway (23); moreover, these effects include the activation of protein kinase C (24), lipid peroxidation (12) and direct cellular damage (25). In the present study, we examined lipid peroxidation by LPS, the participation of reactive oxygen species, and the inhibitory effects of MP on LPS-induced lipid peroxidation.

Many studies have reported the induction of lipid peroxidation by LPS in various organs, particularly in the liver. Bautista et al. (10) demonstrated in vitro that hepatic Kupffer cells produce the superoxide anion (O$_2^-$) when stimulated by LPS. The O$_2^-$ then reacts with H$_2$O$_2$ to produce the toxic hydroxyl radical (OH·), which causes lipid peroxidation (26, 27). Saito also suggested that the O$_2^-$ from PMNs stimulated by LPS is related to lipid peroxidation in the liver (28). PCOOH has recently gained attention as a primary peroxidative product of phosphatidylcholine (PC), the most important functional lipid in the cell membrane (29). It has been reported that the PCOOH level in liver increases with an increase in the duration of ischemia and also increases in proportion to the duration of reperfusion (30). In the present study, we also observed an increase in the plasma PCOOH level upon administration of LPS. Moreover, the addition of LPS resulted in an increase in PMA-induced superoxide production in PMNs and a simultaneous decrease in plasma Cu,Zn-SOD activity. It appears that plasma Cu,Zn-SOD may be used to consume the excess superoxides generated in LPS-treated PMNs. These results indicate that the increase in plasma PCOOH levels may be due to reactive oxygen species.

MP had suppressive effects on the plasma PCOOH level and on PMA-induced superoxide production resulting in...
from LPS treatment. In addition, MP partially restored the plasma Cu,Zn-SOD decrease in activity resulting from LPS administration.

To determine the role of superoxide anions in these interactions, we investigated the effects of MP on superoxide generating systems in vitro. High concentrations of MP inhibited PMA-induced superoxide production in PMNs. However, betamethasone sodium phosphate had little effect on superoxide production, regardless of concentration, while succinate had similar effects as those of MP. Succinate caused a dose-dependent inhibition of superoxide production in vitro. Similar results have been obtained from the effect of succinate on the generation of the respiratory burst by neutrophils, although this phenomenon is markedly pH-dependent (31). These results suggest that one of the suppressive effects of MP on superoxide production in PMNs is due to the presence of succinate in MP, and that the effects are not due to the direct action of glucocorticoid.

MP did not have direct radical-trapping effects on superoxide, hydroxyl or DPPH radicals as determined by ESR methods. Moreover, MP did not inhibit NADPH-dependent lipid peroxidation at concentrations of 2 mM or less. It is considered that MP does not appear to display anti-oxidation effects in in vivo experiments. These results suggest the existence of additional mechanisms for the protective effects of MP on endotoxemia.

We observed reciprocal changes in the values of Cu,Zn-SOD activity and superoxide production in PMNs. Thus, it appears that LPS induced superoxide production in PMNs and that the superoxide produced was then trapped by Cu,Zn-SOD. Excess superoxide caused cell membranes to promote lipid peroxidation, thus increasing plasma PCOOH. The administration of MP inhibited the elevation of LPS-induced plasma PCOOH levels and appears to be related to the inhibitory effects on superoxide production in PMNs and the consequent restoration of Cu,Zn-SOD levels. However, the effects of MP were not due to radical-trapping effects or to direct anti-oxidation.

Recently, it has been reported that MP inhibited the production of TNFα, IL-1α and IL-6 (32). In addition, glucocorticoids have been reported to inhibit the production of cytokines in LPS-stimulated monocytes, endothelial cells, and fibroblasts (33–35). Thus, MP appears to suppress the synthesis or release of these molecules, resulting in the consequent suppression of superoxide production in PMNs and biomembrane lipid peroxidation by oxygen-derived free radicals generated by phagocytes. Further studies are needed to confirm this hypothesis.

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