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Lipopolysaccharide Inhibition of Rat Hepatic Microsomal Epoxide Hydrolase and Glutathione S-Transferase Gene Expression Irrespective of Nuclear Factor-κB Activation

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ABSTRACT. Lipopolysaccharide (LPS) is an endotoxin involved in septic shock syndrome and potentiates toxicant-induced liver injury. The effects of LPS on the constitutive and inducible expression of hepatic microsomal epoxide hydrolase (mEH) and glutathione S-transferase (GST) genes were studied in rats. Northern blot analysis showed that treatment of rats with LPS caused suppression in mEH and GST gene expression. The mEH mRNA level was decreased in a time-dependent manner following a single dose of LPS (1 mg/kg, i.v.), resulting in levels of 52%, 22%, 17%, and 94% of those in untreated animals at 2, 6, 12, and 24 hr, respectively. The levels of rGSTA2 and rGSTA3 mRNA were suppressed in response to an LPS injection to the similar extents as observed in mEH mRNA, whereas rGSTM1 and rGSTM2 mRNA levels were less affected. LPS inhibited mEH gene expression at the doses of 1 μg or greater. Whereas treatment of rats with allyl disulfide (ADS), oltipraz (OZ) or pyrazine (PZ) at the dose of 50 mg/kg caused increases in the mEH mRNA level at 12 hr, a concomitant LPS injection (1 mg/kg) resulted in 80%–95% suppression of the inducible gene expression. The inducible rGSTA2, rGSTA3, rGSTM1, and rGSTM2 mRNA levels were also 50%–90% decreased at 12 hr after LPS treatment, with the relative change in rGSTA being greater than that in rGSTM. Three consecutive daily treatments with LPS (10 μg/kg/day) resulted in significant decreases of the constitutive and PZ (50 mg/kg/day, i.p. for 3 days)-inducible mEH and GST mRNA levels, which were consistent with those in the protein levels. Gel shift retardation analysis showed that LPS substantially activated the hepatic nuclear p65/p50 nuclear factor-κB (NF-κB) complex with the maximal effect observed at 1 hr at the doses of 1 μg/kg or greater. LPS-induced activation of nuclear NF-κB (1 μg/kg, i.v.) failed to be inhibited by concomitant treatment with the mEH and GST inducers, including ADS (300 mg/kg, p.o.), OZ (300 mg/kg, p.o.), and PZ (300 mg/kg, i.p.), indicating that NF-κB activation was not required for suppression of the gene expression by LPS. In contrast, $GdCl_3$, an inhibitor of mEH and GST expression, inhibited LPS-induced activation of the p65/p50 NF-κB. These gel shift analyses provided evidence that LPS-induced activation of the NF-κB was not responsible for alterations in the gene expression. In summary, the results of this research demonstrate that LPS effectively inhibits constitutive and inducible mEH and GST expression with decreases in their mRNA levels, and that LPS suppression in the expression of the detoxifying enzymes is not mediated with its activation of NF-κB. BIOCHEM PHARMACOL **56**;11:1427–1436, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. microsomal epoxide hydrolase; glutathione S-transferase; gene expression; lipopolysaccharide; NF-κB activation

LPS† [1] is an endotoxin involved in septic shock syndrome. LPS also potentiates toxicant-induced liver injury, and thus contributes to hepatotoxicity [1, 2]. Intravenous administration of LPS to rats results in the accumulation of neutrophils in the liver and the development of midzonal hepatocellular necrosis. LPS stimulates mononuclear phagocytes to generate tumor necrosis factor (TNF)-α, interleukin-1 (IL-1), IL-6, prostanoids, leukotrienes, and nitric oxide [3]. Thus, Kupffer cells in the liver are the major source of eicosanoids and cytokines and are associated with the increased oxygen uptake after stimulation. Pathologic alteration of the liver induced by toxicants is related, at least in part, to Kupffer cells [4–6]. The latter are also likely to be involved in the metabolic activation of xenobiotics as well as in inflammatory injury.

LPS strongly activates the nuclear NF-κB in endotoxemia. Activation of NF-κB involves the phosphorylation and proteolysis of inhibitor of NF-κB, and the subsequent translocation of NF-κB to the nucleus leads to the tran-

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† *Abbreviations:* ADS, allyl disulfide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione S-transferase; mEH, microsomal epoxide hydrolase; LPS, lipopolysaccharide; OZ, oltipraz; PKC, protein kinase C; PZ, pyrazine; SSC, standard saline citrate; NF-κB, nuclear factor-κB.

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scriptional activation of several genes [7]. Studies have shown that the activation of NF-κB is controlled by reactive oxygen species as well as by the intracellular redox state [8–11]. It has been suggested that the NF-κB transcription factor is potently and rapidly activated by reactive oxygen intermediates and that *N*-acetyl cysteine and other thiol compounds, scavengers for reactive oxidative intermediates, block the activation of NF-κB by TNF-α and LPS [12, 13]. Thus, the possibility has been raised that the activation of NF-κB is associated with a common mechanism involving the production of reactive oxygen intermediates. This was further supported by the potent blocking of NF-κB in the presence of dithiocarbamates and metal chelators [14].

The intracellular thiol level has been implicated in the regulation of transcriptional expression of a variety of genes in association with the activation of NF-κB. The gene of NAD(P)H: quinone oxidoreductase, a detoxifying antioxidant enzyme, is transcriptionally activated in response to hypoxia and OZ, apparently in association with NF-κB activation [15]. A recent study also demonstrated that OZ increased manganese-dependent superoxide dismutase gene expression and enhanced the binding of NF-κB at early times in primary-cultured rat hepatocytes [16]. It has been suggested that NF-κB activated by oxidative stress can interact synergistically with various transcription factors for the expression of the GSTP1 gene, which possesses a response element for NF-κB [10, 17]. An NF-κB-like element of the gene appeared to be responsible for suppression of GSTP1 gene expression in MCF7 cells [18]. NF-κB has also been implicated in the expression of rGSTA2 in association with oxidative stress, and the induction of AP-1 and NF-κB nuclear factors are likely to affect the antioxidant-inducible GST gene expression in hepatoma cells [10, 11, 18].

It has been shown that hepatic cytochrome P450 levels are decreased after LPS treatment, whereas expression of certain P450 is elevated [19–21]. The down-regulation of cytochrome P450 expression seemed to be mediated by an inflammatory cytokine response after endotoxin treatment [19]. Nonetheless, the effect of LPS on mEH and major GST gene expression and the underlying mechanism for alteration of the gene expression have not been studied yet. The present study was designed to investigate the LPS effect on gene expression in the rat liver. Given the regulation of NF-κB by the intracellular thiol levels and redox state, and the possibility of NF-κB-mediated alteration of mEH and GST expression, this study was extended to determine the role of NF-κB activation in the suppression of mEH and GST expression by LPS.

MATERIALS AND METHODS

Materials

LPS (*Escherichia coli* 026:B6; LD50, 7.33 mg/kg, i.p., in mouse) was obtained from Difco Laboratories. PZ and ADS were purchased from Aldrich Chemical Co. OZ was a gift from Rhône-Poulenc Rorer. [α-^{32}P]dATP (3000 mCi/mmol) and [γ-^{32}P]ATP (3000 mCi/mmol) were purchased from Amersham. Random prime labeling and 5′-end-labeling kits, biotinylated goat anti-rabbit IgG and streptavidin-conjugated horseradish peroxidase were purchased from Life Technologies. The consensus sequence of NF-κB was provided by Promega Corporation, whereas an antibody for the p65 subunit (Rel A) of NF-κB was obtained from Oncogene Research Products. Form-specific polyclonal rabbit anti-rat rGSTA1/2 and rGSTM1 antibodies were purchased from Biotrin International. Most reagents for the molecular studies were purchased from Sigma Chemical Co.

Animal Treatment

Male Sprague–Dawley rats (200–250 g) were obtained from the Korea Food and Drug Administration (Seoul, Korea) and maintained at a temperature of 20 to 23° with a relative humidity of 50%. Animals were caged under a supply of filtered, pathogen-free air. Cheiljedang rodent chow (Seoul, Korea) and water were available *ad lib.* unless specified. Rats (200–250 g) were treated with each of the inducing agents (50 or 300 mg/kg/day, 1 or 3 days) prior to injection and fasted 16 hr before sacrifice. LPS was injected through the tail vein at the doses of 1–1000 μg/kg. To determine the effects of LPS on the inducible expression, OZ, ADS, and PZ were administered as described previously [22, 23]. LPS was given at the same time as the xenobiotics. ADS and OZ were administered by gavage with the former dissolved in corn oil as a vehicle and the latter suspended in a 0.1% carboxymethylcellulose solution. PZ was intraperitoneally administered in an aqueous solution.

Subcellular Fractionation

Hepatic microsomal and cytosolic fractions were prepared by differential centrifugation [24]. Briefly, microsomes were washed in pyrophosphate buffer and stored in 50 mM Tris acetate buffer (pH 7.4) containing 1 mM EDTA and 20% glycerol. The hepatic cytosol was prepared from homogenates in 0.1 M Tris acetate buffer (pH 7.4) containing 0.1 M potassium chloride and 1 mM EDTA, by centrifugation at 10,000 *g* for 30 min and then at 100,000 *g* for 90 min. Microsomal and cytosolic preparations were stored at −70° until use.

Immunoblot Analysis

Immunoblot analysis was performed according to previously published procedures [24–26]. Microsomal and cytosolic proteins were separated by 8% and 11% SDS-PAGE, respectively, and electrophoretically transferred to nitrocellulose paper [24]. The nitrocellulose paper was incubated with either rabbit anti-rat mEH or rabbit anti-rat GST antibodies. Immunoreactive protein was visualised by incu-

bation with streptavidin-horseradish peroxidase, followed by addition of both 4-chloro-1-naphthol and hydrogen peroxide.

cDNA Synthesis and PCR Amplification

Specific cDNA probes for GST genes were amplified by the reverse transcription-polymerase chain reaction (PCR) using the selective primers for each gene, as described previously [24, 27]. A cDNA for mEH was prepared as described previously [25]. PCRs were performed for 40 cycles under the following conditions: denaturation at 94° for 1 min, annealing at 50° for 1 min and elongation at 72° for 3 min.

RNA Blot Analysis

Northern blot analysis was carried out according to the procedures described previously [24–27]. Total RNA (20 μg) isolated from rat livers was resolved by electrophoresis in a 1% agarose gel containing 2.2 M of formaldehyde and was then transferred to nitrocellulose paper by capillary transfer followed by hybridisation [28, 29]. The nitrocellulose paper was baked in a vacuum oven at 80° for 2 hr. Blots were incubated in hybridisation buffer containing 50% deionized formamide, 0.1% SDS, 200 μg/mL of sonicated salmon sperm DNA, 6× standard saline/phosphate/EDTA (1× standard saline/phosphate/EDTA contains 0.15 M NaCl, 10 mM NaH_2PO_4, and 1 mM Na_2EDTA, pH 7.4), and 5× Denhardt's solution [0.1% Ficoll, 0.1% polyvinylpyrrolidine and 0.1% BSA (Pentex fraction V)] at 42° for 1 hr without probe. Hybridisation was performed at 42° for 18 hr with a heat-denatured probe, which was random-prime labeled with [α-^{32}P]dATP, as described previously [24–27]. Filters were washed in 2× SSC (1× SSC contains 0.15 M NaCl and 0.015 M sodium citrate, pH 7.4) and 0.1% SDS for 10 min at room temperature twice and in 0.1× SSC and 0.1% SDS for 10 min at room temperature twice. Filters were finally washed in the solution containing 0.1× SSC and 0.1% SDS for 60 min at 60°. The stripped membranes were hybridised with a labeled GAPDH probe and/or $poly(dT)_{16}$, which was end-labeled with [γ-^{32}P]ATP, to quantify the amount of mRNA loaded onto the agarose gel and transferred to the nitrocellulose paper. Films were exposed at −70° for 12 to 48 hr using intensifying screens. Northern blot analysis was carried out with the liver samples pooled utilising at least two animals. Each data point represents mean ± standard error from independent measurements of three different animal experiments.

Gel Retardation Assay

A double-stranded DNA probe for the consensus sequence of NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′) was used for gel shift analyses after end-labeling of the probe with [γ-^{32}P]ATP and T_4 polynucleotide kinase. Nuclear extracts were obtained by a modification of the procedure published previously [30, 31]. The reaction mixtures contained 2 μL of 5× binding buffer containing 20% glycerol, 5 mM $MgCl_2$, 250 mM NaCl, 2.5 mM EDTA, 2.5 mM dithiothreitol (DTT), 0.25 mg/mL of poly dI-dC and 50 mM Tris-Cl (pH 7.5), 3.5 μg of nuclear extracts, and sterile water in a total volume of 10 μL. Incubations were carried out at room temperature for 20 min by the addition of 1 μL of probe (10^6 cpm), following 10-min preincubations. An antibody for the p65 subunit was added to the completed gel shift reaction to examine the specificity of NF-κB binding. Samples were loaded onto 4% polyacrylamide gels at 100 V. The gels were removed, fixed and dried, followed by autoradiography.

Scanning Densitometry

Scanning densitometry was performed with a microcomputer imaging device, model M1 (Imaging Research). The area of each lane was integrated using MCID software (version 4.20, revision 1.0), followed by background subtraction.

Data Analysis

Data were analysed using computer programs for pharmacological calculations [32]. One-way analysis of variance procedures were used to assess significant differences among treatment groups. For each significant effect of treatment, the Newmann–Keuls test was used for comparison of multiple group means. The Student's *t*-test was used to determine whether two population means differed significantly. The criterion for statistical significance was set at $\alpha = 0.05$.

RESULTS

Effects of a Single Dose of LPS Injection on the Constitutive Gene Expression

The effect of LPS on the constitutive mEH and GST gene expression was examined in the liver (Fig. 1A and 1B). Northern blot analyses revealed that LPS suppressed the expression of mEH and GST genes. mEH mRNA levels were decreased in a time-dependent manner after LPS treatment. The relative mEH mRNA levels were suppressed to 52%, 22%, 17%, and 94% of those in untreated animals at 2, 6, 12, and 24 hr, respectively, after a single dose of LPS (1 mg/kg, i.v.). The maximal reduction of the mEH mRNA level was noted at 6–12 hr post-treatment, followed by a rebound at 24 hr.

The expression of representative GST genes, rGSTA2 and rGSTA3 in class alpha family, and rGSTM1 and rGSTM2 in class mu family was examined. Many subunits in each GST class can form heterodimers [18]. Expression of rGSTA2 was suppressed by 78%–92% at 6 to 12 hr after a single injection of LPS with the mRNA level being suppressed up to 24 hr. The change in the rGSTA3 mRNA level was quite similar to that of mEH (Fig. 1A and 1B).

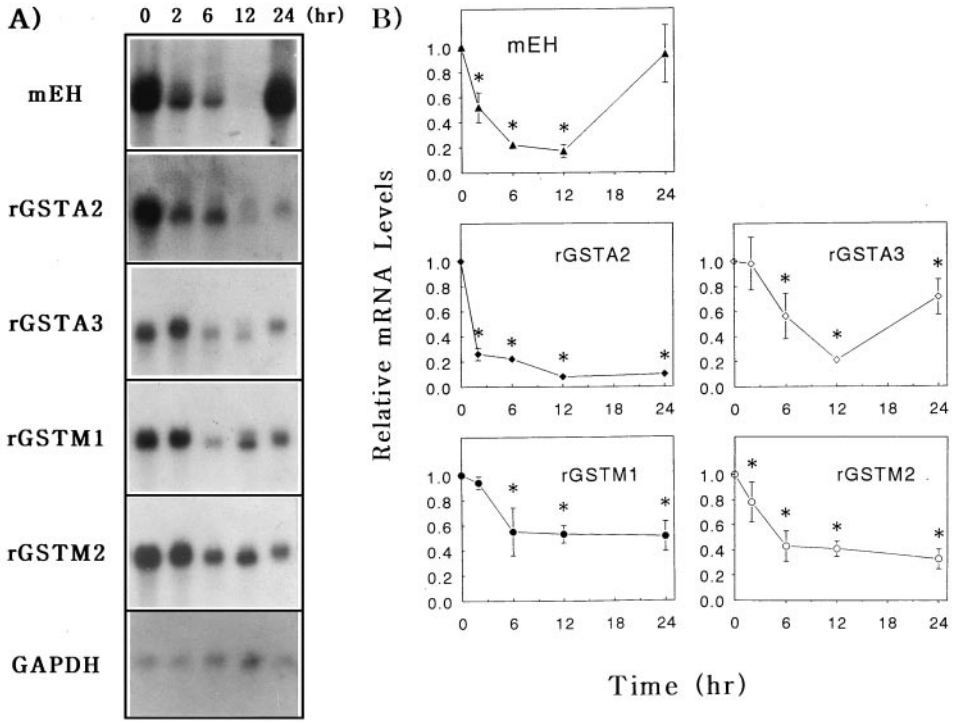

FIG. 1. Effects of LPS on the constitutive mEH and rGST mRNA levels in the liver. (A) RNA blot analyses of hepatic mEH, rGSTA2, rGSTA3, rGSTM1, and rGSTM2 mRNA. Northern blot analysis was performed to examine mRNA levels in total RNA fractions (20 μg each) isolated from rats at 0, 2, 6, 12, and 24 hr after an i.v. injection of LPS at the dose of 1 mg/kg (0, no treatment). The RNA was fractionated in a 1% agarose gel containing 2.2 M of formaldehyde, transferred to a nitrocellulose paper and hybridized with a ^{32}P-labeled cDNA probe. The amount of RNA loaded in each lane was confirmed by rehybridisation of the stripped membrane with a labeled GAPDH probe and/or a ^{32}P-labeled poly(dT)$_{16}$(only GAPDH was shown). (B) Relative changes in the mEH, rGSTA2, rGSTA3, rGSTM1 and rGSTM2 mRNA levels, as compared to that in untreated rats. The mRNA levels were assessed by scanning densitometry of the blots followed by normalisation. Each point represents the mean ± SEM of three experiments. Data were analysed with one-way analysis of variance followed by Newmann–Keuls test for comparison with untreated animals (*$P < 0.05$).

Thus, LPS was effective in substantially inhibiting the mRNA levels of rGSTA. The mRNA levels for rGSTM1 and rGSTM2 were suppressed to lesser extents by LPS treatment, resulting in 45%–70% decreases at 6 to 24 hr post-treatment.

Dose–response studies were carried out to determine whether the effects of LPS could be reproduced at lower doses of the endotoxin. The relative mRNA level of mEH was assessed at 6 hr after an injection of LPS (Fig. 2). The constitutive mEH mRNA levels were 70%–80% inhibited at doses from 1 μg through 1 mg/kg of LPS.

Effects of a Single Dose of LPS on the Inducible Gene Expression

Structurally different organic inducers were used in subsequent experiments to determine whether LPS was capable of inhibiting the inducible expression of the genes. ADS, OZ, and PZ were used to assess the effect of LPS on the xenobiotic-inducible expression. Because a substantial decrease in the mRNA level was noted at 12 hr after an LPS injection followed by a rebound at 24 hr, the effect of LPS on the xenobiotic-inducible mEH and GST mRNA levels was assessed at 12 and 24 hr. Whereas treatment of rats with ADS, OZ or PZ at the dose of 50 mg/kg caused 9-, 10-, and 2-fold increases in the mEH mRNA level at 12 hr after treatment, respectively, as compared to that in untreated animals, a concomitant LPS injection at the dose of 1 mg/kg resulted in 2-, 0.5- and 0.3-fold relative changes (Fig. 3A and 3B). Thus, LPS-mediated inhibition of the mEH gene expression was a common phenomenon irrespective of the chemical structures of inducers.

Studies were further extended to determine whether LPS suppressed the inducible expression of major GST genes. Northern blot analysis revealed that the rGSTA2 mRNA level was 2- to 3-fold increased at 12 hr after treatment with the inducers, whereas a single dose of concomitant LPS inhibited the inducible rGSTA2 mRNA levels by 70%–90% (Fig. 3B). Inducible expression of rGSTA3 was also suppressed to a similar extent (Fig. 3A and 3B). The rGSTM1 and rGSTM2 mRNA levels were also 50%–90% decreased at 12 hr after administration of LPS. Relative decreases in rGSTA mRNA levels in response to LPS were greater than those of rGSTM.

Expression of mEH and GST Genes after Consecutive LPS Treatment

Because LPS can induce a state of hyporesponsiveness to its own effect, we determined the alterations of constitutive

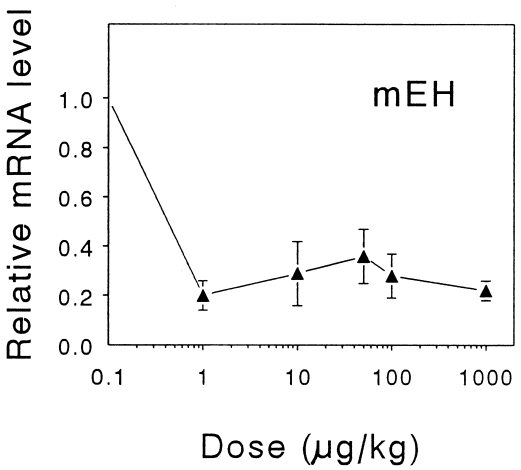

FIG. 2. Relative mEH mRNA levels after an injection of LPS at a variety of doses. Northern RNA blot analyses performed with hepatic total RNA isolated from rats at 6 hr after an injection of 1, 10, 50, 100, or 1000 μg/kg of LPS. The mRNA levels were assessed by scanning densitometry of the blots followed by normalisation. Each point represents the mean ± SEM of three experiments. One-way analysis of variance showed no significant changes.

and inducible gene expression after consecutive 3-day treatment with LPS (Fig. 4A and 4B). The 12-hr time point after the last treatment was chosen on the basis of the effects observed in the study after a single dose of LPS. The relative mEH, rGSTA2, and rGSTM1 mRNA levels were suppressed by 10%, 40%, and 72% after consecutive 3-day treatment with LPS (10 μg/kg/day, i.v.), respectively. The effect of multiple LPS administration on the PZ-inducible mEH and rGST expression was also determined. Whereas treatment of rats with PZ at the dose of 50 mg/kg per day for 3 days (i.p.) caused 11-, 15-, and 9-fold increases in mEH, rGSTA2, and rGSTM1 mRNA levels as compared to that in untreated rats, animals exposed to both PZ and LPS (10 μg/kg) resulted in 2.7-, 7.6-, and 2.8-fold increases in the mRNA levels (Fig. 4B). Thus, mEH, rGSTA2 and rGSTM1 mRNA levels were 50%–75% suppressed after multiple LPS treatment, indicating that the inhibitory extents after multiple administration were less than those after a single injection.

Immunoblot Analysis of mEH and rGST

Immunoblot analyses were carried out to determine hepatic mEH, rGSTA2, and rGSTM1 protein levels in rats after treatment with LPS for 3 days (Fig. 5). Although decreases in the constitutive protein levels were not marked after LPS treatment, PZ-inducible mEH and rGSTA2 protein levels were reduced to 60% and 20% of PZ alone, respectively, after concomitant LPS administration (mean ± SEM, $N = 3$, $P < 0.05$). The inducible rGSTM1 protein level was only 20% suppressed by LPS. These data showed that LPS suppressed the inducible mEH and rGSTA2 protein expression.

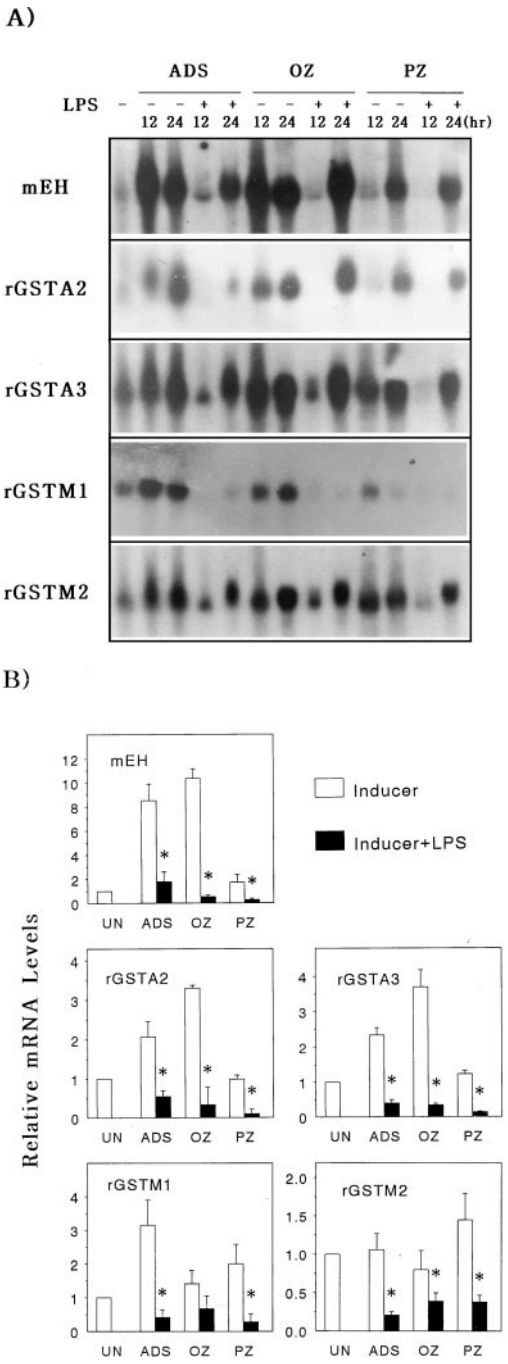

FIG. 3. Effects of LPS on the inducible mRNA levels for mEH and major GST subunits. (A) Northern blot analyses for mEH, rGSTA2, rGSTA3, rGSTM1, and rGSTM2 mRNA levels. Northern RNA blot analyses were carried out with the hepatic total RNA (20 μg each) isolated at 12 and 24 hr after a single dose of ADS (50 mg/kg, p.o.), OZ (50 mg/kg, p.o.) or PZ (50 mg/kg, i.p.) treatment with (+) or without (−) an LPS injection (1 mg/kg, i.v.). (B) Shown are the relative changes in the mRNA levels at 12 hr after ADS, OZ or PZ administration with or without an LPS injection, as compared to that in untreated rats (UN, relative mRNA level of untreated animals = 1). Relative changes in the mRNA levels were assessed by scanning densitometry of the blots followed by normalisation. Each point represents the mean ± SEM of three experiments. Data were analysed with one-way analysis of variance followed by Newmann–Keuls test for comparison with the respective xenobiotic without LPS (*$P < 0.05$).

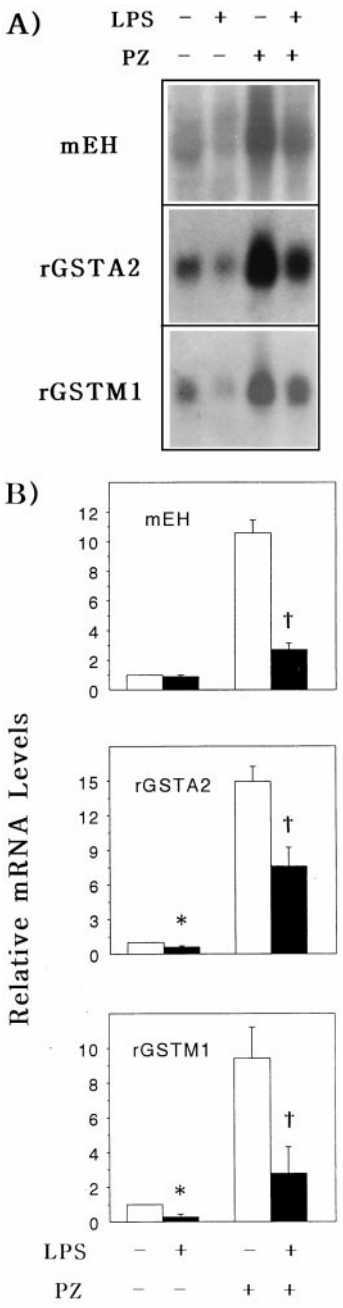

FIG. 4. mEH and GST mRNA levels in the liver after consecutive LPS treatment. (A) RNA blot analyses of hepatic mEH, rGSTA2 and rGSTM1 mRNA after consecutive daily administration of PZ (50 mg/kg/day, i.p., 3 days) with (+) or without (−) LPS injections (10 μg/kg/day, i.v., 3 days). The RNA was isolated at 12 hr after the last treatment. Northern blot analysis was performed as described above. (B) LPS effect on the relative mEH and rGST mRNA levels. The mRNA levels were assessed by scanning densitometry of the blots followed by normalisation (relative mRNA level of untreated animals = 1). Each point represents the mean ± SEM of three experiments. The criterion for statistical significance as assessed by Student's *t*-test was set at $P < 0.05$ for comparison with untreated animals (*) or with PZ alone (†).

LPS-Inducible NF-κB Activation

To determine whether the NF-κB level after LPS treatment was associated with the suppression of mEH and GST gene expression, gel shift assays were performed using the NF-κB consensus sequence. Treatment of rats with LPS at the dose of 1 mg/kg resulted in a substantial increase in the nuclear p65/p50 NF-κB complex. The level of NF-κB appeared to be maximally activated at 1 hr after an injection of LPS (1 mg/kg, i.v.) (Fig. 6A). The supershift was clearly detected in the completed gel shift reaction added with an antibody for the p65 subunit (Rel A), which was carried out to examine the specificity of NF-κB binding to the consensus sequence (Fig. 6B).

The activation of NF-κB was assessed at various doses of LPS. Nuclear p65/p50 NF-κB complex was greatly activated at the dose of 1 μg/kg or greater (Fig. 7A). To further monitor the level in the nuclear p65/p50 NF-κB complex in response to the chemical inducers, rats were treated with the mEH and GST inducers including ADS (300 mg/kg, p.o.), OZ (300 mg/kg, p.o.), and PZ (300 mg/kg, i.p.) at 2 hr prior to an LPS injection (1 μg/kg). The inducers failed to suppress the activation of NF-κB by LPS (Fig. 7B). Rather, the inducers might slightly increase the activation of NF-κB by LPS. Treatment of rats with the inducers alone failed to activate NF-κB (data not shown). The effect of $GdCl_3$, an inhibitor of mEH and GST expression, on the LPS-induced activation of NF-κB was monitored to examine whether these agents antagonised each other in a common intracellular pathway. Interestingly, $GdCl_3$ in particular blocked the LPS activation of nuclear p65/p50 NF-κB at 1 hr post-treatment (Fig. 7B). Taken together, these results provided evidence that the activation of NF-κB by LPS was not responsible for its alteration of mEH and GST gene expression.

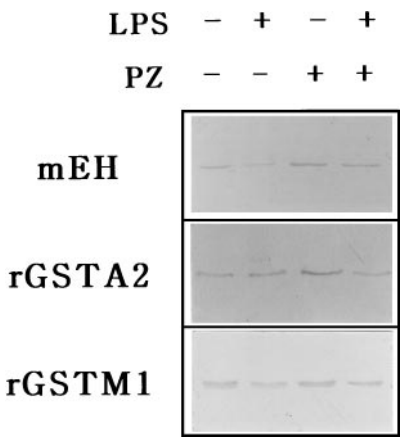

FIG. 5. Representative immunoblot analyses of hepatic mEH, rGSTA2 and rGSTM1 after LPS treatment. These blots exhibit hepatic mEH and rGST protein levels in untreated rats (−) or LPS (10 μg/kg/day, i.v.)-treated rats with (+) or without (−) PZ (50 mg/kg/day, i.p., 3 days). These changes were confirmed by multiple immunoblottings.

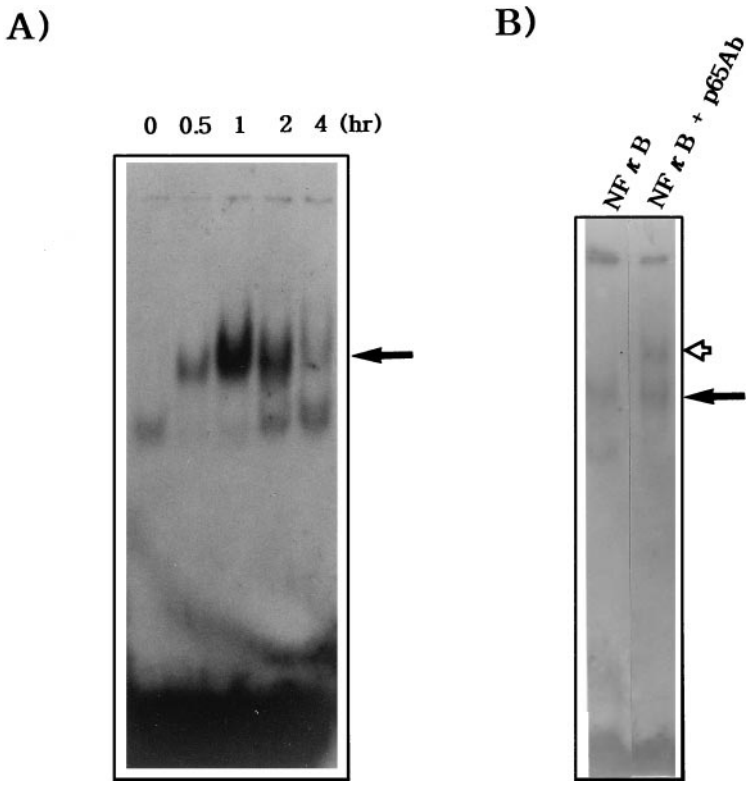

FIG. 6. Gel shift analysis of nuclear NF-κB transcription complexes in hepatic nuclear extracts from LPS-treated rats. (A) Hepatic nuclear extracts were isolated from rats at 0 (no treatment), 0.5, 1, 2, and 4 hr after an LPS injection (1 mg/kg, i.v.). All lanes contained 3.5 μg of nuclear extract. The lower bands are p50 homodimers. (B) Supershift of NF-κB profile in the presence of 2 μg of p65 (Rel A) NF-κB antibody. The lane represents a hepatic nuclear extract from a rat at 1 hr after an LPS injection (1 μg/kg, i.v.). The closed arrow shows p65/p50 NF-κB. The open arrow indicates supershifted NF-κB.

DISCUSSION

The expression of drug-metabolising enzymes has been shown to be modulated by cytokines [19–21]. The level of hepatic cytochrome P450 is suppressed during inflammatory responses [19, 21, 33]. For example, the expression of cytochrome P450 2C11 in male rat liver is transcriptionally decreased by endotoxin treatment through the inflammatory cytokines such as IL-1, IL-6, TNF-α, and interferons [19]. Because Kupffer cells in the liver play a role in secreting cytokines, which may modulate the expression of hepatic enzymes, mEH and GST expression is likely to be affected by changes in secretion of certain cytokines following LPS challenge. These inflammatory mediators are also involved in tissue injury such as hepatic ischemia and reperfusion injury.

The present study demonstrated for the first time that mEH and major GST mRNA levels were substantially altered by LPS. LPS was effective in suppressing the expression of mEH and GST at the doses of 1 μg/kg or greater. The doses that we used were chosen on the basis of the preliminary experiments and the previous studies on septic shock [34]. Suppression of xenobiotic-metabolising enzymes by LPS was not due to its cytotoxicity, which was supported by unaltered transcription of the GAPDH gene. The mEH and GST mRNA levels were decreased as early as 2 hr post-treatment with LPS. A single dose of LPS suppressed mEH and rGSTA2 expression to a maximal extent at 12 hr. The time-course in suppression of the xenobiotic-inducible expression was comparable to that of the constitutive expression. The comparable extent of LPS inhibition in the constitutive and inducible expression of mEH and GST despite the difference in chemical structures of the inducers and the similar time point for a maximal inhibition indicates that LPS might block certain common step(s) in the expression of the detoxifying enzymes and in the cellular signaling pathway. The result in this study was partly consistent with the time-course of cytokine production from mononuclear phagocytes after endotoxin treatment. Suppression of mEH and GST expression by LPS was also observed after multiple daily treatments, although a certain extent of tolerance was noted. The reduced inhibition of the mRNA levels after a 3-day treatment in this study might result from the hyporesponsiveness after multiple challenges with LPS [35]. A recent study has shown that endotoxin tolerance involves the lack of endotoxin-inducible inhibitor of NF-κB kinase activity [35].

Although many effects of LPS are considered to be mediated by cytokines derived from other cells, association of LPS with liver parenchymal cells raises the possibility that some LPS-induced hepatic effects result from direct alterations in hepatocytes. LPS can directly affect hepatocytes and suppress the mEH and GST expression, as was observed in the potentiation of inducible nitric oxide synthase production [3, 36]. This remains to be further established. It has been shown that tyrphostins, inhibitors of protein tyrosine kinase, protected animals against LPS-induced lethal toxicity through blockage of LPS-induced TNF-α production [34]. Preliminary studies showed that pretreatment of rats with tyrphostin AG126 failed to reverse LPS suppression of mEH and GST expression

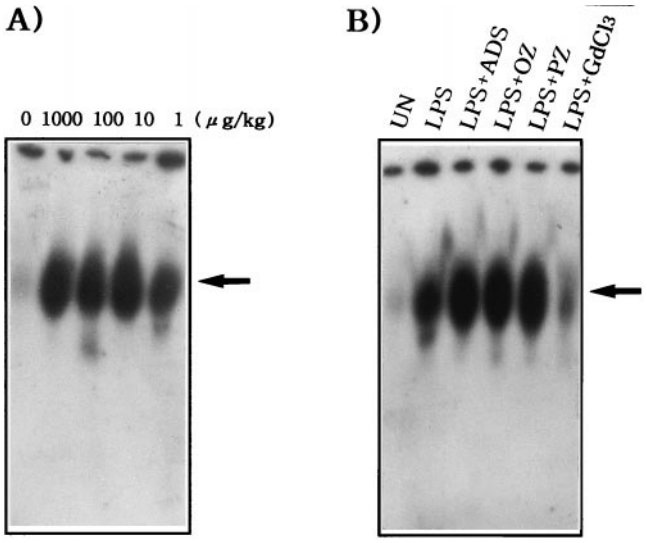

FIG. 7. Gel shift analysis of p65/p50 NF-κB transcription complex in hepatic nuclear extracts after an LPS injection. (A) Gel shift analysis was performed with hepatic nuclear extracts isolated from rats at 1 hr after an i.v. injection of 1000, 100, 10, or 1 μg/kg of LPS (0, no treatment). All lanes contained 3.5 μg of nuclear extract. The arrow indicates p65/p50 NF-κB. (B) NF-κB profiles after treatment of rats with LPS + xenobiotics. Nuclear extracts were isolated 1 hr after an LPS injection (1 μg/kg, i.v.) with or without xenobiotics. Rats were treated with ADS (300 mg/kg, p.o.), OZ (300 mg/kg, p.o.), PZ (300 mg/kg, i.p.) or gadolinium chloride (10 mg/kg, i.v.) in combination with LPS. UN represents no treatment. The inducers were administered 2 hr prior to an LPS injection, whereas gadolinium chloride was injected at the same time as LPS.

despite its blockage of LPS activation of nuclear NF-κB, suggesting the possible direct effect of LPS on the hepatocytes.

LPS substantially inhibited induction of the detoxifying enzymes by various agents in this study. Previous studies in this laboratory have shown that organosulfur compounds including ADS and propyl sulfide transcriptionally induce mEH, rGSTA, and rGSTM [24, 37]. Certain nitrogen- and sulfur-containing heterocycles, but not aromatic or aliphatic hydrocarbons, are also active in stimulating the gene expression [26]. A number of studies have shown that administration of OZ, a cancer chemopreventive agent, to laboratory animals resulted in increases in the GSH level in the various organs and enhanced the expression of several enzymes involved in the detoxification of carcinogens including mEH and GSTs [38]. The cellular response after OZ administration involves modulation of the expression levels of metabolic enzymes that protect the cell from mutagenic and toxic species [38–40]. A recent study has shown that OZ is converted to a reactive intermediate in vitro producing activated oxygen species in the presence of free sulfhydryl (e.g. β-mercaptoethanol and glutathione) [41]. OZ appears to be bioactivated, leading to the production of activated oxygens which contribute to the transcriptional activation of the genes [38, 39, 41]. Thus, the anticarcinogenic effect of oltipraz is considered to be mediated by the induction of phase II enzymes.

The intracellular redox state may affect the induction of certain GSTs as well as NF-κB activation. Increases in cellular GSH levels by *N*-acetyl-L-cysteine derivatives cause blocking of NF-κB activation and subsequently inhibit the release of cytokines from macrophages [8, 9, 42]. Certain mEH and GST inducers including OZ elevate the cellular GSH levels and alter the cellular redox state [18]. In the present study, the chemical inducers including OZ failed to inhibit the LPS activation of NF-κB at the dose of 300 mg/kg, although these agents serve as strong inducers for the expression of mEH and GST. These agents might enhance the LPS activation of NF-κB, if significant, probably because the inducers serve as pro-oxidants, leading to generation of reactive oxygen species [15, 16]. It has been suggested that NF-κB may be involved in the altered gene regulation of phase II enzymes by OZ [40, 41]. OZ rapidly increased the nuclear NF-κB binding in primary-cultured rat hepatocytes [16]. The fact that chemical inducers failed to antagonise the LPS-induced activation of NF-κB despite their reversal of LPS suppression of the mRNA levels at the particular dose (i.e. 300 mg/kg) provided evidence that NF-κB activation was not one of the events responsible for the suppression of hepatic mEH and GST genes by LPS [11].

The previous study from this laboratory clearly showed that suppression of mEH and GST mRNA levels by $GdCl_3$ appeared to involve its competitive inhibition of intracellular calcium influx [22]. In spite of the suppressive effect of mEH and GST gene expression, $GdCl_3$ blocked NF-κB activation induced by LPS in the present study, indicating that LPS activation of NF-κB was not required for its suppressive effect of the gene expression. The inhibition of LPS-induced NF-κB activation by $GdCl_3$ may result from its blocking of Ca^{2+} influx into hepatocytes [22, 43]. Alternatively, the $GdCl_3$ effect might involve the inactivation of Kupffer cells and subsequent alteration in the production of cytokines. It has been shown that the LPS-induced down-regulation of cytochrome P450 enzyme activities in Kupffer cell co-cultured hepatocytes was attenuated by the presence of $GdCl_3$ [44].

LPS regulates the expression of several genes through cell surface receptors and intracellular signaling pathways [3]. Activation of calcium and phospholipid-dependent PKC has been implicated after exposure of cells to LPS [45–47]. The signaling pathway associated with LPS-induced alteration in cell function involves membrane-associated G protein activation, which is coupled to the intracellular transduction system. The effects of LPS in this study may also be mediated by PKC-dependent activity in the induction pathway(s) in association with the change in the intracellular Ca^{2+} level [3, 47]. Because PKC activity is dependent on the intracellular Ca^{2+} level, $GdCl_3$ blocking of Ca^{2+} influx into hepatocytes might affect the activity of PKC, which could result in changes in the expression of the xenobiotic-metabolising enzymes and NF-κB activation.

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