Endotoxin Enhances Liver Alcohol Dehydrogenase by Action through Upstream Stimulatory Factor but Not by Nuclear Factor-κB*

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Liver alcohol dehydrogenase (ADH,¹ alcohol:NAD oxidoreductase, EC 1.1.1.1.) is principally responsible for ethanol oxidation. Immobilization stress in rats, which stimulates the hypothalamo-hypophyseal-adrenocortical axis, increases liver ADH activity and ethanol elimination (1). The enzyme is regulated by hormones and increases in the activity of the enzyme, resulting in increased formation of metabolites of ethanol such as acetaldehyde, which are important in the pathogenesis of alcoholic liver disease (2).

CCAAAT/enhancer binding protein β, upstream regulatory factor (USF), and signal transduction and activator of transcription 5b (STAT5b) are transcription factors that bind to and activate the ADH promoter in transfection experiments. C/EBPβ binds principally between −22 and −11 (3), USF between −60 and −52 (4), and STAT5b between −211 and −203 (5) relative to the start site of transcription. C/EBPβ also binds to a second site adjacent to the STAT5b binding site (5). The action of growth hormone (GH) in enhancing the ADH promoter activity is mediated by both C/EBPβ (6) and STAT5b (5).

Endotoxin originating from intestinal bacteria is an important mediator of hepatocellular inflammation in the intragastrointestinal feeding rat model of alcoholic liver disease (7, 8). Lipopolysaccharide (LPS), the endotoxin component of Gram-negative bacteria, leads to the production of a variety of inflammatory cytokines such as tumor necrosis factor α and interleukin-1, the formation of oxygen radicals, and the translocation of nuclear factor-κB (NF-κB) to the cell nucleus (9, 10). LPS activates the hypothalamo-hypophyseal-adrenocortical axis, and this is manifested principally by increases in ACTH and cortisol secretion (11). The effect of LPS on GH is species-dependent with increases in the human but decreases in the rat (11).

The purpose of the study was to determine whether LPS with its associate acute phase metabolic response influences ADH and whether such an effect on ADH is mediated by NF-κB.

EXPERIMENTAL PROCEDURES

Animals and Materials—Male Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). All animals received humane care in compliance with the guidelines from the Animal Care and Use Committee of The Johns Hopkins University. LPS from Escherichia coli was obtained from Sigma. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, and agarose were purchased from Invitrogen. Plastic 75 cm² tissue culture flasks were purchased from BD Biosciences. [α-32P]dATP and [α-32P]dCTP were purchased from PerkinElmer Life Sciences. An oligonucleotide containing the consensus binding site of NF-κB was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Animal Treatment—Rats received intraperitoneal injections of LPS (100 μg/100g body weight) daily for 3 days, while control rats received isovolumetric amounts of saline. The animals were sacrificed 2 h after the last injection. Approximately 400–500 mg of the liver was homogenized in 4 volumes of 0.25 m sucrose in 0.1 m Tris-HCl buffer, pH 7.4, centrifuged at 10,000 × g for 10 min. The resulting supernatant was used for the determination of ADH activity and ADH protein. One section of 1.0–1.2 cm of the liver was processed for RNA isolation, and the remainder of the liver was used for preparation of nuclear protein extracts as described previously (4). The nuclear protein extracts were aliquoted and stored under nitrogen at −100 °C. Protein content of the cytosol and nuclear extract was determined by the method of Lowry et al. (12).

Alcohol Dehydrogenase Activity—ADH activity was determined in the liver cytosol at 37 °C by the method of Crow et al. (13). The reaction mixture was 1.0 ml and consisted of 0.5 m Tris-HCL, pH 7.2, 18 mm ethanol, 2.8 mm NAD⁺, and 0.03 ml of the liver cytosol. One unit of enzyme activity is defined as the formation of 1 μmol NADH per min. Lactate dehydrogenase activity was determined by the method of Plagemann et al. (14).
ADH-E, ADH-U, and ADHwt oligonucleotides are underlined. The C/EBP, USF, and STAT regulatory motifs, respectively, in the ADH-E, ADH-U, and ADHwt oligonucleotides are underlined.

Effect of LPS administration on hepatic alcohol dehydrogenase (ADH) and lactate dehydrogenase (LDH) activities

LPS was administered intraperitoneally in a daily dose of 100 μg/100 gm body weight for 3 days. Enzyme activities are expressed per liver cytosol protein. All values are expressed as means ± S.E. of six animals.

| Treatment | ADH | LDH |
|-----------|-----|-----|
| Control   | 9.40 ± 1.29 | 2.27 ± 0.05 |
| LPS       | 26.96 ± 6.89* | 1.92 ± 0.15* |

* p < 0.02 versus control.

Immunoactive Protein of Alcohol Dehydrogenase—Immunoactive protein of ADH was determined by quantitative enzyme-linked immunosorbent assay as described previously (15).

Plasmids—The full-length cDNA plasmid encoding the rat class I ADH and the rat class I 240ADH-CAT construct, which were obtained from Dr. David W. Crabb of Indiana University School of Medicine (Indianapolis, IN), have been described previously (3). The NF-κB expression vectors RSV-NF-κB (p50) and RSV-Rel (p65) and the empty vector K1 were obtained from Professor Guidalberto Manfioletti, from the University of Trieste, Italy. The STAT5b expression vector pCMX-STAT5b and the empty vector pCMV5 were gifts from Dr. Gregorio Gil of the Medical College of Virginia (Richmond, VA). Luciferase constructs of the ADH promoter were prepared as described previously (5).

Isolation and Quantitation of Messenger RNA—Total cellular RNA was isolated using the procedure of Chomczynski and Sacchi (16). RNA quality and concentration were verified by agarose-formaldehyde gel electrophoresis with ethidium bromide staining. Northern blots for rat liver ADH and mouse γ-actin were performed as described previously (17). The amount of ADH mRNA hybridized was visualized by autoradiography and quantitated by densitometry.

Electrophoretic Mobility Shift Assays (EMSA)—The sequences of the ADH oligonucleotides used for EMSA are shown in Table I. Complementary strands of each oligonucleotide were annealed, and the double-stranded oligonucleotides were labeled with [α-32P]dATP and [α-32P]dCTP using Klenow enzyme according to the method of Feinberg and Vogelstein (18). DNA-protein binding reactions were performed with nuclear extracts (8 μg of protein) following the previously described EMSA procedure (3). For “supershift” EMSA experiments, rabbit polyclonal antibodies to NF-κB p50, NF-κB p65, USF-1, USF-2, STAT5b, and C/EBPβ, obtained from Santa Cruz Biotechnology, Inc., were used. These antibodies were added separately to the reaction at the completion of DNA-protein binding, incubated for an additional 30 min at room temperature, and resolved on an 8% nondenaturing polyacrylamide gel.

Transient Transfection and Luciferase Assay—Transient transfection experiments were carried out in cultured HepG2 cells using the calcium phosphate precipitation method (19). HepG2 cells were seeded on 75-cm² polytretene flasks and allowed to grow to 60–70% confluence in DMEM containing 10% fetal bovine serum. The medium was renewed 1 h prior to transfection. To each flask 10 μg of pGL3-ADH, 5 μg of β-galactosidase vectors, and 5 μg of salmon sperm DNA were added in the form of calcium phosphate precipitates. For each experiment, pGL3-basic and pGL3-control luciferase vectors were used as a negative and a positive control. After overnight incubation, the cells were shocked with 10% Me2SO in DMEM for 3 min and then washed and refed with fresh DMEM containing 10% fetal bovine serum. At 48 h after transfection, the cells were harvested and subjected to one freeze-thaw cycle in 200 μl of the reporter lysis buffer (Promega). Luciferase activity and β-galactosidase activity were determined by respective chemiluminescent assays (20, 21).

Ultraviolet Cross-linking of Nuclear Proteins to Oligonucleotides and Immunoblot Analysis—The binding of nuclear proteins to the oligonucleotide probe was performed as for EMSA. Reactions using 8 μg of nuclear protein and 50 fmol of radioactively labeled oligonucleotides were used. Following the binding reaction, UV cross-linking was performed as described previously (4, 5). The membranes were then incubated with rabbit polyclonal antibody to NF-κB p50, NF-κB p65, USF-1, USF-2, STAT5b, and C/EBPβ, obtained from Santa Cruz Biotechnology Inc. (Santa Cruz Biotechnology Inc.) for one h. After repeated washing for 1 h, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1: 50,000 dilution) (Amersham Biosciences) for one h. The membranes were then washed for 1 h and then...
immersed in lumigen PS-3 acridan substrate solution (ECL Plus, Amersham Biosciences) for 5 min. The antibodies were visualized by exposing the membrane to x-ray film.

Data Analysis—All data points are expressed as means ± S.E. The differences between means of paired groups and between means of more than two paired groups were examined by Student's t test and by two-way analysis of variance plus appropriate multiple comparisons, respectively.

RESULTS

LPS Enhances ADH—The administration of LPS resulted in a mean 2.9-fold increase in liver ADH activity (Table II) but in no significant change in lactate dehydrogenase activity. ADH immunoprotein was also increased after LPS administration from 7.49 ± 4.12 ng/mg cytosol protein in the controls to 10.91 ± 0.70 ng/mg cytosol protein after LPS (p < 0.05). LPS administration did not change liver weight or protein concentration in the liver cytosol. The effect of LPS in increasing ADH activity and protein was accompanied by an increase in ADH mRNA. The relative densitometric readings of ADH mRNA/g actin mRNA ratios for the autoradiographs of the Northern blots were increased to 164 ± 12 (n = 5) after LPS treatment as compared with 100 ± 24 (n = 5) for controls (p < 0.05).

NF-κB Binds to the ADH Promoter and This Binding Activity Is Increased by LPS—LPS increased the binding activity of NF-κB to an oligonucleotide specifying the NK-κB binding consensus sequence (Fig. 1). Furthermore, NF-κB in nuclear extracts from control and LPS-treated rats were increased to 164 ± 12 (n = 5) after LPS treatment as compared with 100 ± 24 (n = 5) for controls (p < 0.05).

TABLE III
Effect of NF-κB on activity of the alcohol dehydrogenase (ADH) promoter

| Expression vector | Luciferase activity expressed as a percentage of the control ADH promoter |
|-------------------|-------------------------------------------------|
| Control           | 100 ± 11                                         |
| RSV NF-κB (p65)   | 24 ± 1b                                          |
| RSV NF-κB (p50)   | 50 ± 5b                                         |
| RSV NF-κB (p65) + | 24 ± 3c                                         |
| RSV NF-κB (p50)   |                                                |

* The mean luciferase activity on transfection of 15 μg of pGL3-ADH was 7657,407 RLU/mg protein.
* p < 0.01 as compared to control.
* p < 0.05 as compared to control.

TABLE IV
Effect of NF-κB (p65) on the activity of the alcohol dehydrogenase (ADH) promoter in the absence and presence of the STAT5b expression vector

| pCMV-STAT5b μg | Luciferase activity expressed as percentage of the control ADH promoter £| NF-κB (p65) |
|----------------|-------------------------------------------------|-------------|
| 0              | 100 ± 11                                         | 74 ± 16     |
| 1              | 123 ± 10                                         | 56 ± 6b     |
| 2.5            | 174 ± 6b                                         | 52 ± 7b     |
| 5.0            | 96 ± 8b                                          | 64 ± 5b     |
| 10.0           | 529 ± 15b                                        | 120 ± 6b    |

£ The mean pGL3-ADH luciferase activity on transfection of 15 μg of pGL3-ADH was 7590,108 RLU/mg protein.
* p < 0.01 as compared with respective value obtained with pCMV-STAT5b alone.
* p < 0.01 as compared with respective control.
tracts from rat liver was found to bind to the oligonucleotide ADHwt, specifying a region from −226 to −194 of the ADH promoter (Table I). EMSA with the nuclear extracts from control rats shows four protein-DNA complexes with the ADHwt oligonucleotide (Fig. 2A, arrows). These complexes were previously identified (5) as binding by STAT5b (upper complex) and C/EBPβ (two middle complexes). Nuclear extract from LPS-treated rats resulted in the formation of two additional protein-DNA complexes with the ADHwt oligonucleotide (Fig. 2A, arrowheads). Antibody to NF-κB p50 decreased the uppermost protein-DNA complex and resulted in the formation of a supershifted complex, whereas antibody to NF-κB p65 resulted in a smaller supershifted complex (Fig. 2B). LPS did not significantly change the binding activity of nuclear STAT5b and C/EBPβ to the ADHwt oligonucleotide from that obtained with nuclear extracts of control rats. LPS, however, resulted in a decrease in the formation of the protein-DNA complex with the ADH-E oligonucleotide (Fig. 3), which represents C/EBPβ binding to the more proximal site (Table I) of the ADH promoter (3).

The NF-κB (p65) and the NF-κB (p50) Expression Vector Inhibit the Activity of the ADH Promoter—To determine whether the action of LPS in increasing ADH activity was mediated by NF-κB, we determined the effects of NF-κB expression vectors on the cotransfected ADH promoter. The NF-κB (p65) and NF-κB (p50) expression vectors resulted in inhibition of the activity of the ADH promoter (pGL-ADH) (Table III). The combination of the NF-κB expression vectors did not result in additional inhibition over that obtained with NF-κB (p65) alone. The NF-κB (p65) expression vector also markedly inhibited the activation of pGL-ADH by the STAT5b expression vector (Table IV).

LPS Increases the Binding of USF Proteins to the ADH Promoter—USF was previously shown to be induced and activate the ADH promoter (4). UV cross-linking with immunoblots was done to further define the effects of LPS on NF-κB and C/EBPβ binding and to determine the effect of LPS on USF binding to ADH oligonucleotides. LPS increased the binding of NF-κB p65 (Fig. 4) but had no effect on the minimal binding of NF-κB p50. LPS decreased the binding of the 35-kDa C/EBPβ to ADH-E (Fig. 5). By contrast, LPS resulted in moderate increases in the binding of the 45-kDa USF-1 and 44-kDa USF-2 proteins and a marked increase in the binding of the 17-kDa USF protein to the ADH-U oligonucleotide (Fig. 6).

DISCUSSION

This study shows that LPS increases the message, protein, and activity of liver ADH. LPS activates NF-κB, and NF-κB in turn is a known regulator of the gene expression of tumor necrosis factor α and other inflammatory cytokines. NF-κB in nuclear protein extracts from rat liver was shown in this study to bind to the ADH promoter. Although LPS resulted in the increased expression of NF-κB and in increased NF-κB binding to the ADH promoter, the effect of LPS in enhancing the ADH was not mediated by NF-κB. Indeed, the NF-κB expression vectors resulted in inhibition of the activity of the ADH promoter. Furthermore, the NF-κB (p65) expression vector inhibited the activation of the ADH promoter by the STAT5b expression vector. Acelatdehyde in vitro was shown to diminish LPS-stimulated degradation of IsBo and to inhibit the nuclear translocation of NF-κB p65, resulting in decreased NF-κB binding to the NF-κB consensus oligonucleotide (22). Hence, it is possible that any inhibitory effect of NF-κB on ADH may be markedly decreased or abrogated by the acetaldehyde produced during ethanol metabolism.

GH enhances ADH, an effect mediated by both C/EBPβ (6) and STAT5b (5). The effect of LPS on increasing ADH, however, is not mediated by GH. LPS increases GH secretion in the human but decreases GH secretion in the rat (11, 23). Furthermore, LPS did not affect the binding of STAT5b and decreased the binding of C/EBPβ to oligonucleotides specifying their binding sites on the ADH promoter. These findings are in agreement with prior observations showing that LPS down-regulated the STAT5-mediated GH-responsive gene serine protease inhibitor 2 (24). Also, LPS decreased the 42-kDa C/EBPβ and the 35-kDa C/EBPβ proteins in nuclear extracts from mice and their binding to the C/EBP binding site of the α1-acid glycoprotein promoter (25). The 20-kDa C/EBPβ and its binding were increased acutely after LPS but decreased to baseline values after 48 h (25).

This study indicates that the effect of LPS in increasing ADH is mediated by increased binding of USF to the ADH promoter. In a previous study, we demonstrated that USF activated the promoter of the rat ADH gene (4). The binding to the ADH promoter of the 43-kDa USF-1 and the 44-kDa USF-2 isoforms, which are principally responsible for transcriptional activity in mammalian cells (26), was increased by LPS. In addition, the binding of a smaller 17-kDa USF polypeptide was also increased by LPS. The 17-kDa polypeptide is recognized by USF-1 antibody and binds to DNA as both a homodimer and a heterodimer with full-length USF-2 (26, 27). Heat shock and LPS were previously found to increase binding of nuclear proteins to the E-box of the murine-inducible nitric oxide gene, and these proteins were supershifted with antibodies to USF-1 and -2. However, mutation of the E-box did not affect the activation of the promoter by LPS (28).

Despite the inhibitory effect of NF-κB on ADH in transfection experiments, LPS administration in vivo resulted in an increase in ADH message, protein, and activity, indicating that activators such as C/EBPβ and USF negate a possible inhibitory effect of NF-κB. Also of note is that in vivo the accumulation of NF-κB p50 homodimers is greater than that of NF-κB p50-p65 heterodimers in rat hepatocytes after LPS administration (29) and, as shown in this study, the inhibitory action of NF-κB p50 was less than that of NF-κB p65 in transfection experiments. The greater increase in ADH activity than in ADH protein and mRNA after LPS administration suggests that the effect of LPS is not only on transcription but possibly also on activation of ADH.

In summary, this study shows that LPS, the endotoxin component of Gram-negative bacteria, increases ADH activity and that this effect is mediated by increased binding of USF to the ADH promoter and not by NF-κB, which has an inhibitory action. An increased rate of formation of acetaldehyde caused by an enhanced ADH activity may contribute to worsening of alcoholic liver injury caused by endotoxin.

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