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Lipopolysaccharide Induces *CYP2E1* in Astrocytes through MAP Kinase Kinase-3 and C/EBPβ and -δ*

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Cytochrome P450 2E1 (CYP2E1) is highly inducible in a subset of astrocytes *in vivo* following ischemic or mechanical injury and *in vitro* by lipopolysaccharide (LPS) or interleukin-1β. We have studied the mechanism of induction, and found that transcriptional activation of *CYP2E1* occurred within 3 h, and CYP2E1 dependent catalytic activity was induced more than 4-fold within 5 h. The induction was sensitive to several tyrosine kinase inhibitors, and was further modulated by inhibitors of p38 MAP kinase. MAP kinase kinase-3 (MKK3) was phosphorylated in response to LPS, and expression of constitutively active MKK3, but not the MAP kinase kinases MEKK1 or MKK1, activated *CYP2E1*. Transcriptional activation was mediated through a C/EBPβ and -δ binding element situated at −486/−474, and appeared to involve activation of prebound factors as well as recruitment of newly synthesized C/EBPβ and -δ. It is thus suggested that LPS induces MKK3 activation in astrocytes, which in turn stimulates a C/EBPβ and -δ binding element to mediate transcriptional activation of *CYP2E1*.

CYP2E1 is a member of the cytochrome P450 (*CYP*) gene superfamily and is active in the oxidation of fatty acids and ketones. CYP2E1 has a key role in gluconeogenesis as the pathway of formation of lactate or glucose from the ketone body acetone is initiated by CYP2E1, and it has been shown that the pathway contributes 5–10% of gluconeogenetic demands (1, 2). The pathway is physiologically relevant as acetone levels are highly elevated in *Cyp2E1* null mice compared with wild type, during fasting (3). In addition to acetone, CYP2E1 accepts numerous pharmaceuticals as substrates including neuroactive agents such as chlorzoxazone, a centrally acting muscle relaxant, and general anesthetics. In the absence of substrate, CYP2E1 can still be reduced and produce superoxide anions that may in turn contribute to oxidative stress in the cell (4, 5). The property of oxygen radical generation is likely also the background to the contribution of CYP2E1 to apoptotic cell injury in hepatic and glial cell lines (6, 7).

Although expression levels of CYP2E1 are highest in the liver, CYP2E1 is expressed also in the lung and kidney as well as in the central nervous system of rats and humans. In the brain CYP2E1 is constitutively expressed, *e.g.* in hippocampal pyramid neurons, cortical astrocytes, and endothelial cells (8), and the enzyme has been found to be inducible and catalytically active in the brain (9–11).

Several cytochrome P450 members of families 1 to 4 are highly responsive to inflammatory mediators. Hepatic expression of CYP1A, CYP2B, CYP2C, CYP2E, and CYP3A members is significantly repressed by, *e.g.* lipopolysaccharide, interleukin-1β, interleukin-6, tumor necrosis factor α, and interferons α and β, often between 40 and 95% (12–14). The mechanisms of down-regulation of hepatic P450s in response to inflammatory stimuli are not completely clear, but may be specific for each P450, and for different stimuli. It has, *e.g.* been found that sphingomyelin hydrolysis may mediate CYP2C11 down-regulation by interleukin-1β (15), that the C/EBPβ mRNA-derived liver-enriched inhibitory protein down-regulates CYP3A4 in response to interleukin-6 (16), and that the decreased availability of the transcription factor HNF-1α is likely important for the down-regulation of CYP2E1 in response to interleukin-1β (17).

In contrast to the down-regulation of hepatic CYP2E1 by inflammatory factors, CYP2E1 has been shown to be highly inducible during inflammation both *in vivo* and *in vitro* in astrocytes. CYP2E1 is readily inducible in astrocytes *in vivo* following mechanical injury to the rat central nervous system (8), or following ischemic injury in the gerbil or in the rat (18). *In vitro*, CYP2E1 was found to be highly responsive to lipopolysaccharide and interleukin-1β in cultured astrocytes, and to a lesser extent to interleukin-6 (18). Interestingly, both *in vivo* and in culture, CYP2E1 is induced in and restricted to a subset of glial fibrillary acidic protein positive ($GFAP^+$) astrocytes, corresponding to ~8% of the cells (8, 18, 19). A few other P450s are now known to be induced, rather than down-regulated, during inflammation, as exemplified by the induction of CYP4A3 in hepatocytes from certain rat strains (20). Interestingly, it was recently found that CYP4F4 and 4F5 were induced in the hippocampus and frontal cortex, in the inflammatory phase following traumatic brain injury (21). The mechanisms remain, however, unknown.

The previously described induction of CYP2E1 by lipopolysaccharide in astrocytes (18) involved an increase in CYP2E1 mRNA, an unusual mode of induction for CYP2E1, and was sensitive to transcriptional inhibitors such as actinomycin D. We have now studied the mechanism of induction and present evidence for the rapid, transcriptional induction of CYP2E1 through a pathway involving MKK3-dependent stimulation of a C/EBPβ and C/EBPδ binding element at −486/−474.

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EXPERIMENTAL PROCEDURES

Primary Cortical Glial Cultures—Rat primary cortical glial cultures were established from cortices of newborn rats (6–12 h) as previously described (18). The cells were grown in minimum essential medium supplemented with 20% fetal calf serum, amino acids, vitamins, penicillin (250,000 IU/liter), and streptomycin (250 mg/liter) (all medias were from Invitrogen). The cells were stimulated with lipopolysaccharide at 90–95% confluence. Protein kinase inhibitors were added 30 min before stimulation with lipopolysaccharide (LPS).[1] Cell cultures were analyzed with microscopy for the composition of cells, and the expression of CYP2E1 and luciferase protein. The cells were verified to consist of ~85% astrocytes and ~3% microglial cells, as previously described (18, 22). In a similar manner CYP2E1 was verified to be induced in a subset of cells, as previously described (18). pGL3-reporter transfected cells, using DMRIE-C, were found to express luciferase protein in $GFAP^+$ cells. For microscopy, cells were fixed with 2% paraformaldehyde and subsequently incubated with polyclonal antisera prepared from rabbits immunized with purified rat liver CYP2E1 (18), or with luciferase antisera or preimmune sera. In other experiments, cells were incubated with antibodies reactive toward glial fibrillary acidic protein (GFAP, Dakopatts A/S, Denmark) or CD11b (BIOSOURCE Intl.). Secondary fluorescein isothiocyanate-labeled antibodies (Sigma) or TRITC-labeled antibodies (Roche Applied Science) were used. All experiments involving the establishment of primary cortical glial cultures were approved by the ethical committee at Karolinska Institute.

Preparation of Microsomal Fractions—After harvest of the rat primary cortical glial cultures, the cells were washed twice in phosphate-buffered saline, pH 7.4, and subsequently sonicated in 5 volumes of ice-cold 10 mM Tris-HCl buffer, pH 7.4, with 20% glycerol, 1.14% (w/v) KCl, 0.2 mM EDTA, 0.1 mM dithiothreitol, and, freshly dissolved, 0.1 mM phenylmethylsulfonyl fluoride and 20 μM butylated hydroxytoluene. Centrifugation for 10 min at 12,000 × *g* gave a nuclear and mitochondrial pellet, which was washed once. The supernatants were combined and centrifuged (100,000 × *g*) for 60 min to give a microsomal pellet. The pellets were dissolved in 50 mM potassium phosphate buffer, pH 7.4, and were stored at −70 °C.

Chlorzoxazone 6-Hydroxylation Assay—The 6-hydroxylation of chlorzoxazone (CZN) was monitored to detect CYP2E1-dependent catalytic activity. One mg of microsomal protein was incubated with 500 μM CZN and 0.5 mM NADPH in 50 mM potassium phosphate buffer, for 120 min. NADPH was added every 30 min, and the reaction was linear up to at least 150 min. After termination of the incubations with 43% *o*-phosphoric acid, internal standard (1 μg of paracetamol/acetaminophen or 5 μg of 5-fluorbenzoxazoline-2-one) was added to the incubations. After extraction twice with dichloromethane and evaporation to dryness under N_2, the residue was dissolved in 50 μl of mobile phase. The products of CZN oxidation were analyzed on a Millipore/Waters 510 HPLC system, on a Hibar prepacked column RT 250-4 (Merck, Germany). The mobile phase consisted of acetonitrile, 0.5% phosphoric acid (22:78), at a flow rate of 1.5 ml/min. The effluent was detected by a Millipore/Waters 480 LC spectrophotometer (at 250 nm for paracetamol/acetaminophen detection) and simultaneously by a LC-4A amperometric detector (Bioanalytical Systems Inc.), for sensitive analysis of the product 6-OH-chlorzoxazone. There was a potential of 1.3 volts over the electrochemical detector cell. The sensitivity for detection of 6-OH-CZN was determined to 0.5 pmol.

Electrophoretic Mobility Shift Assays—Nuclear extracts were isolated and electrophoretic mobility shift assay (EMSA) was carried out as previously described (22). In the binding reaction, 5–50 fmol of DNA labeled with [γ-^{32}P]dATP (3000 Ci/mmol, Amersham) was incubated in 10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 14% glycerol, and 150 mM NaCl with 5–10 μg of nuclear extract. Antibody interference assay was carried out by incubation with 2 or 4 μg of antibody, specifically directed to individual C/EBP factors or nuclear factor κB (NFκB) factors (Santa Cruz Biotechnology, Santa Cruz, CA). *CYP2E1* sequences of oligonucleotides used in EMSA were taken from Umeno *et al.* (23). The −492/−466 oligonucleotide has the following sequence: 5′-TGCTCTCATTT-TCCAAACAGGCCATT-3′. The same −492/−466 oligo, but with introduced mutations, has the following sequence: 5′-TGCTCTCATGT-TCAGAGCAGGCCATT-3′, and the consensus C/EBP binding oligonucleotide has the following sequence: 5′-TGCAGATTGCGCAATCTGCA-3′. The consensus NFκB oligonucleotide has the following sequence: 5′-AGTTGAGGGGACTTTCCCAGGC-3′.

Construction of Plasmids—The reporter gene constructs were prepared by amplifying the targeted 5′ regulatory fragments with PCR (23, 24) and cloning into pGL3-Basic plasmid (Promega, Madison, WI) in front of a luciferase reporter gene using BglII and HindIII sites. The mutated pGL3-CYP2E1(−489-+28) plasmid carried the same four mutations as the −492/−466 *CYP2E1* oligonucleotide described above. The correct identity of the constructs was verified by DNA sequencing. Expression plasmids for constitutively active MAP kinase kinases MEKK1 (pFC-MEKK1), MKK1 (pFC-MKK1), and MKK3 (pFC-MKK3), the GAL4-CHOP (C/EBP homology protein) plasmid, as well as the GAL4-UAS luciferase reporter were obtained from Stratagene (La Jolla, CA). The pCMV-C/EBPα, -β, and -δ expression plasmids and the dominant negative C/EBPβ expression plasmid have been previously described (25–27).

Transient Transfection Assay—Primary cortical glial cultures were transiently transfected as previously described (22). Briefly, cells were grown to 70–80% confluence and were then transfected with the cationic lipid transfection reagent DMRIE-C (Invitrogen). If no other quantity is given in the figure legends, 0.5 μg of pGL3 plasmid, 0.5 μg of pCMV-C/EBP plasmid, and/or 25 ng of pFC-MEKK1/pFC-MKK1/pFC-MKK3 was transfected to each well in 12-well plates (φ 22 mm). Except for the pGL3 firefly luciferase plasmid, a *Renilla* luciferase control plasmid was co-transfected. Medium was changed after 6–12 h, to normal growth medium, and cells were then harvested at around 48 h following transfection. Cell stimulation with 100 ng/ml lipopolysaccharide took place in growth medium. Using an antibody to the Firefly luciferase protein in microscopy, it was confirmed that $GFAP^+$ astrocytes were transfected with this procedure. For analysis of 6-OH-CZN formation, larger quantities of cells were needed. Large plates (φ 139 mm) with cortical glial cells 85% confluent were transfected with 4–17 μg of pFC-MEKK1, pFC-MKK3, or empty plasmid DNA, using DMRIE-C (35 μl) and 35 ml of Opti-MEM medium. The cells were incubated with the cationic lipid:DNA mixtures for 6 h, then kept for 48 h in normal growth medium and harvested. Microsomal fractions and chlorzoxazone assay were carried out as described above. GAL4-CHOP and GAL4-luciferase plasmid transfections were carried out as previously described (28).

Western Blotting—30 μg of cell extract protein from primary cortical glial cultures was subjected to 8.5% SDS-PAGE and transferred to nitrocellulose. The filters were, after transfer of proteins, washed in phosphate-buffered saline, pH 7.4, dried, and blocked in 5% dry milk in Tris-buffered saline, pH 7.4, containing 0.2% Tween 20 (TBS-Tween) overnight at +4 °C. The filters were then incubated with MKK3 or phospho-MKK3/6 antisera (Santa Cruz Biotechnology), diluted 1/1000 in TBS-Tween with 5% milk, washed three times, and incubated for 120 min with a horseradish peroxidase secondary antibody (Dakopatts A/S, Denmark) diluted 1/1000 in TBS-Tween with 5% milk. After thorough washing in TBS-Tween (5 × 30 min in large volumes), proteins were visualized with enhanced luminescence (Amersham Biosciences). Molecular weight standards used for molecular weight estimations were from Bio-Rad.

Data Analysis—Results are presented as mean ± S.D., and statistically analyzed by one-way ANOVA with Tukey-Kramer's multiple comparisons post-test. The GraphPad Instat program version 3.0 for Windows 95 (GraphPad software, San Diego, CA) was used for calculations.

Materials—SB203580, lavendustin A, genistein, and daidzein were obtained from Calbiochem. Water soluble SB203580 was used as organic solvents may affect CYP2E1 activity. Lipopolysaccharide, wortmannin, and paracetamol/acetaminophen reference standards were from Sigma, and were dissolved and used according to instructions with the products. Chlorzoxazone was purchased from Aldrich, and 5-chloro-6-hydroxy-2-benzoxazoline (6-OH-CZN) was obtained from McNeil Chemical Co., PA. 5-Fluorbenzoxazoline-2-one was a kind gift from Dr. Raimund Peter, University of Erlangen, Germany. Antibodies to C/EBP and NFκB proteins were from Santa Cruz Biotechnology.

RESULTS

Lipopolysaccharide-dependent Induction of CYP2E1 in Cortical Glial Cells—We have previously found the 6-hydroxylation of CZN in LPS-treated cortical glial cells to be catalyzed by CYP2E1 to a very large extent (11, 18, 19), and therefore to be a good and sensitive measure of functional CYP2E1 expression. A detailed analysis of the time course of induction of 6-hydroxylation of CZN in cortical glial cells in response to LPS revealed

[1] The abbreviations used are: LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; GFAP, glial fibrillary acidic protein; TRITC, tetramethylrhodamine isothiocyanate; CZN, chlorzoxazone; EMSA, electrophoretic mobility shift assay; ANOVA, analysis of variance; MKK, mitogen-activated protein kinase kinase; C/EBP, CCAAT enhancer-binding protein.

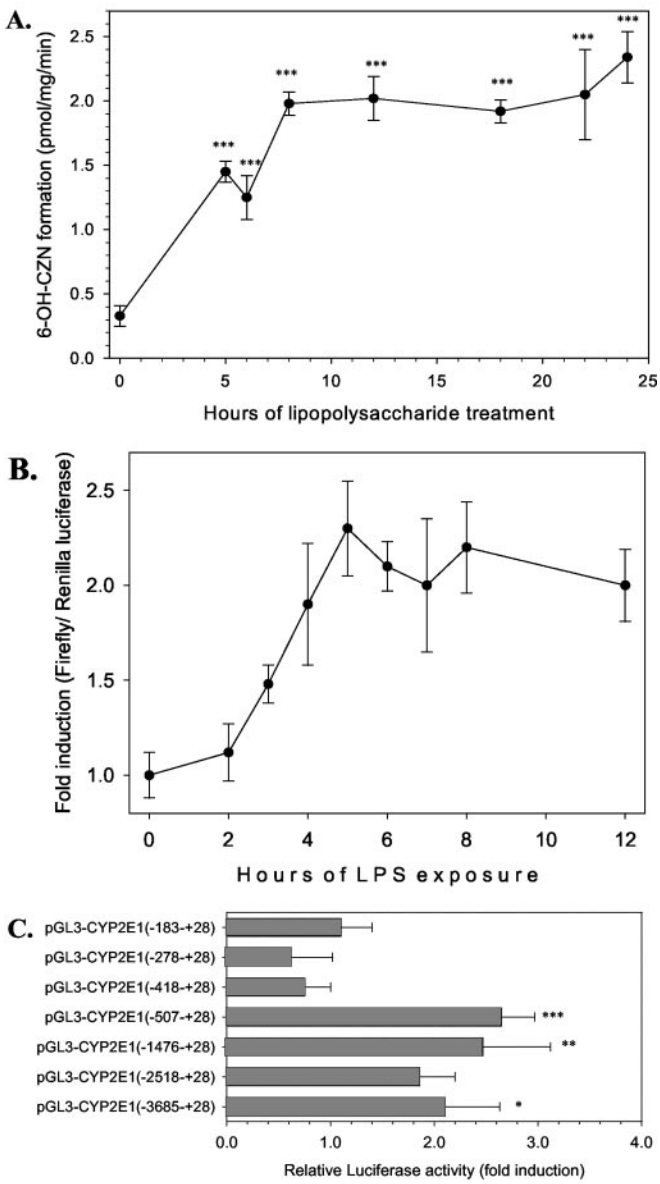

FIG. 1. **LPS induces CYP2E1 in cortical glial cultures.** *A*, effect of 100 ng/ml LPS on the rate of 6-hydroxylation of CZN, in microsomal preparations from rat cortical glial cultures. *B*, effect of 100 ng/ml LPS on luciferase activity in cortical glial cells transfected with pGL3-CYP2E1(−3685-+28). The peak of induction in individual experiments varied somewhat in different experiments, between 4 and 7 h, but the activity always remained elevated at 12 h. *C*, effect of 100 ng/ml LPS on luciferase activity in cortical glial cells transfected with several different pGL3-CYP2E1 plasmids. The pGL3-CYP2E1 plasmids include the *CYP2E1* 5′ flank sequence of decreasing length as indicated, all starting at +28 downstream of initiation. Statistical significance was analyzed with one-way ANOVA with Tukey-Kramer's post-test, where all plasmids were compared with pGL3-CYP2E1(−183-+28). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

that formation of 6-OH-CZN was increased more than 4-fold within 5 h, and that a 6–7-fold induction was seen at 8–24 h of LPS exposure (Fig. 1*A*). The 6-hydroxylation of CZN was inhibited by CYP2E1 antisera, but not preimmune sera, to ~85% (not shown), as we have described in previous reports (11, 18, 19). Thus, LPS appears to cause a rapid and potent induction of functional CYP2E1.

Inducible expression of CYP2E1 in cells of the central nervous system *in vivo* and *in vitro*, in response to injury, inflammatory factors, or phorbol esters, appears restricted to GFAP$^+$ astrocytes (8, 11, 18, 19). Therefore, a plasmid transfectional protocol was developed whereby GFAP$^+$ astrocytes, in cortical glial cultures, were transfected by cationic lipids. With this protocol, cortical glial cultures were transfected with a pGL3-CYP2E1 plasmid, containing ~3.7 kb of *CYP2E1* 5′ flank in front of the luciferase reporter. Stimulation with LPS gave 2–2.5-fold induction of luciferase activity, evident from 3 h of LPS treatment and more pronounced from 4 h on (Fig. 1, *B* and *C*). Using a number of pGL3-CYP2E1 deletion constructs (25), the LPS effect was found to depend on the *CYP2E1* 5′ flank sequence upstream of −418 (Fig. 1*C*).

Induction of 6-Hydroxylation of CZN by LPS Is Inhibited by Tyrosine Kinase Inhibitors—Because LPS signaling may involve tyrosine phosphorylation events, a number of protein-tyrosine kinase inhibitors were tested. The general tyrosine kinase inhibitor genistein gave a dose-dependent inhibition of the induction of 6-OH-CZN formation by LPS (Fig. 2*A*). Whereas 100 μM genistein caused a nearly complete inhibition of the induction by LPS (Fig. 2*A*), the inactive analogue daidzein was at 100 μM nearly without inhibitory effect (Table I). In contrast to genistein, herbimycin A displays a more restricted pattern of tyrosine kinase inhibition, which includes inhibition of the phosphorylation of MKK3 and p38 MAP kinase (29, 30). Herbimycin A inhibited the LPS-dependent induction of the formation of 6-OH-CZN to a very large extent, in a dose-dependent manner (Fig. 2*B*). A third protein-tyrosine kinase inhibitor, tyrphostin AG126, gave a partial inhibition, whereas a fourth inhibitor, lavendustin A, was without inhibitory effect (Table I).

Effect of p38 MAP Kinase Inhibitors on the Induction of 6-Hydroxylation of CZN by LPS—We have previously found that LPS stimulates p38 MAP kinase phosphorylation and activity in the cortical glial cells (22). We have also characterized the neuroprotective agent chlomethiazole as an inhibitor of p38 MAP kinase activity (22, 31), in the cortical glial cells. Addition of chlomethiazole to the cultures did inhibit the LPS-dependent induction of the rate of 6-OH-CZN formation, in a dose-dependent manner (Fig. 2*C*), as we have previously shown (18). SB203580 is a p38 MAP kinase inhibitor specific to p38 MAP kinase α and β, which is structurally distinct from chlomethiazole, and that has a different mechanism of action (22, 32). Addition of low concentrations of SB203580 to the cultures was found to significantly increase the induction of the rate of 6-hydroxylation of CZN in response to LPS, in a dose-dependent manner (Fig. 2*D*).

MAP Kinase Kinase-3 Is Phosphorylated by LPS and Activates pGL3-CYP2E1—As the inducibility of 6-hydroxylation of CZN was modulated by inhibitors of p38 MAP kinase, the pGL3-CYP2E1 containing 3.6 kb of 5′ flank was cotransfected to cortical glial cells with small amounts of plasmids for expression of constitutively active kinases stimulating MAP kinases. MEKK-1, stimulating the c-Jun N-terminal kinase pathway, and MKK1, stimulating the extracellular signal-regulated MAP kinase pathways had no or negligible effect on pGL3-CYP2E1 luciferase activity (Fig. 3*A*). In contrast, expression of constitutively active MKK3 stimulated pGL3-CYP2E1 luciferase activity at low concentrations, and with higher potency at increasing concentrations (Fig. 3, *A* and *B*). As was found for the LPS effect on pGL3-CYP2E1 deletion plasmids (Fig. 1*C*), MKK3 expression only resulted in increased luciferase activity if the pGL3-CYP2E1 plasmid contained the *CYP2E1* sequence to at least −489 base pairs upstream of the initiation site (Fig. 3*C*).

Analysis of the *CYP2E1* sequence revealed a putative C/EBP binding site at −486 to −474 (Transfac software; Ref. 33). Several new pGL3-CYP2E1 plasmids were thus constructed, and it was found that mutation of this site, or deletion of the

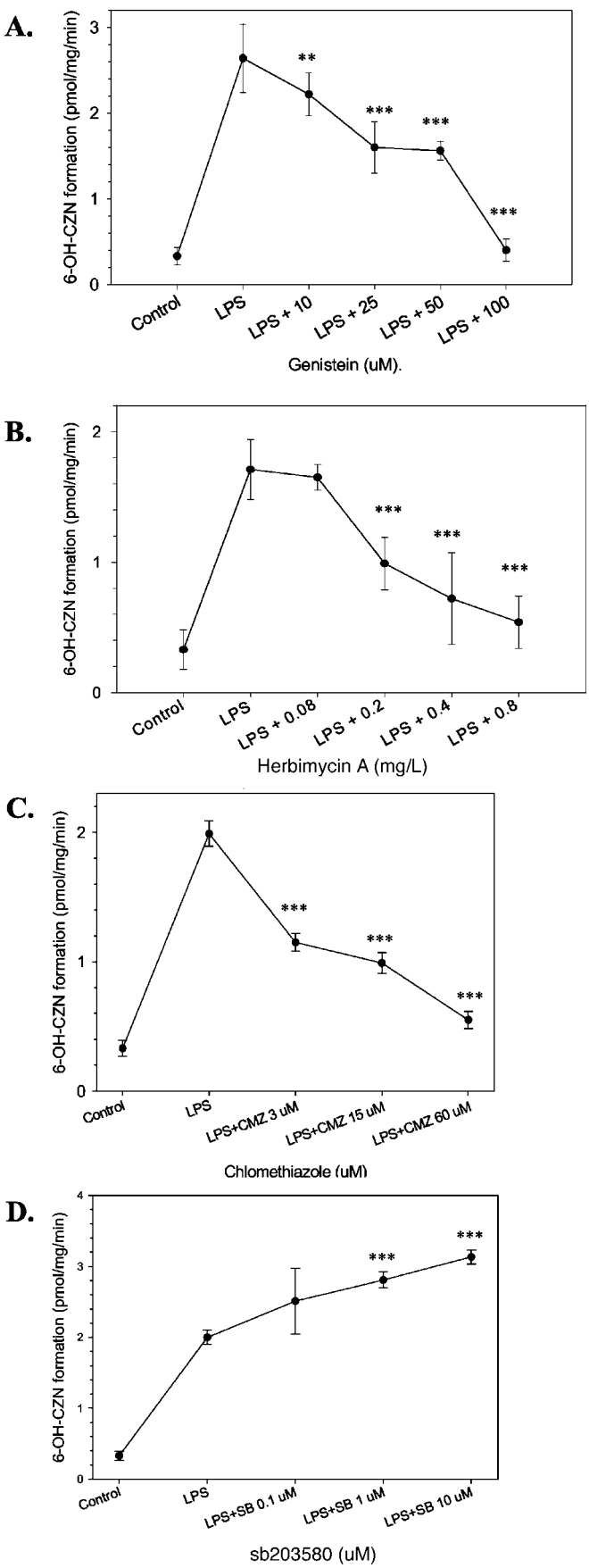

FIG. 2. **Effect of several kinase inhibitors on the inducibility of 6-hydroxylation of CZN by LPS, in cortical glial cells.** The effects of increasing concentrations of: *A*, genistein; *B*, herbimycin A; *C*, chlomethiazole; and *D*, SB203580, on the inducibility of 6-hydroxylation of CZN by LPS are shown. In all cases the inhibitor was added 45 min prior to 100 ng/ml LPS, and the cells were incubated with the inhibitors for 24 h before harvest. The effects of the inhibitors were statistically analyzed by one-way ANOVA with Tukey-Kramer's post-test; the statistical significance between samples was treated with only LPS ("LPS") or together with the indicated kinase inhibitor. **, $p < 0.01$; ***, $p < 0.001$.

TABLE I
Effect of kinase inhibitors on the inducibility of 6-hydroxylation of CZN, by LPS, in cortical glial cultures

Inhibitors were added at the indicated concentrations 30 min before stimulation with 100 ng/mL LPS. Stimulation then took place for 24 hs.

Compound	Maximal inhibition	Concentration
Genistein	97%	100 μM
Daidzein	4%	100 μM
Herbimycin A	85%	0.8 μg/ml
Tyrphostin AG126	34%	50 μM
Lavendustin A	0%	10 μM
Wortmannin	5%	100 nM
Dexametason	0%	1 μM

CYP2E1 sequence 3′ of the putative element (at −466) abolished the stimulatory effect of MKK3 expression (Fig. 3*C*). Constitutively active MKK3, encoded by pFC-MKK3, was also verified to be functionally active in the cortical glial cultures. The activation of the transcription factor CHOP, an indirect MKK3 target through the p38 MAP kinase (34) was assayed through the activation of a GAL4/CHOP construct, and a dose-dependent activation of CHOP was noted in response to MKK3 (Fig. 4*C*).

MKK3 was originally isolated from the central nervous system (35), and by using a highly specific antisera to MKK3, we detected a band with the expected mobility in cell extracts from cortical glial cells (Fig. 4*A*, *left panel*). Stimulation of cortical glial cells with LPS, for 30 min, induced a band reacting with highly specific anti-phosphoantibodies to MKK3/6, with the expected mobility (Fig. 4*A*, *right panel*). Because expression of MKK3 was found to stimulate transcription from the *CYP2E1* −486/−474 element, we analyzed the ability of expressed MKK3 and MEKK1 to stimulate CYP2E1-dependent catalytic activity: 6-hydroxylation of chlorzoxazone. It was found that MKK3, but not MEKK1, transfected to the cortical glial cells induced an increase in the rate of formation of 6-OH-CZN, as shown in Fig. 4*B*.

The Element at −486/−474 Binds C/EBPβ and -δ—To analyze the element at −486/−474 found to be sensitive to LPS and MKK3 expression, EMSA with antibody interference analysis was used. Protein complexes were found to bind to the oligonucleotide containing the element in a specific manner, as they were readily competed by an excess of the same cold oligonucleotide (Fig. 5) or by an oligonucleotide containing a consensus C/EBP binding element, but not by unrelated DNA (not shown). Antibody interference analysis with highly specific antibodies to four isoforms of C/EBP showed that antibodies directed to C/EBPβ or C/EBPδ produced supershifts (Fig. 5), whereas antibodies to C/EBPα or C/EBPε did not. Although no supershift occurred, antibodies to C/EBPε did, however, interfere with complex binding to some extent (Fig. 5).

In line with the antibody interference analysis, it was found in cotransfection experiments that careful overexpression of C/EBPβ or C/EBPδ, but not C/EBPα, stimulated a pGL3-CYP2E1 plasmid containing *CYP2E1* 5′ flank up to −489 (Fig. 6*A*). When C/EBPα, C/EBPβ, or C/EBPδ were instead cotransfected with a pGL3-CYP2E1 plasmid containing *CYP2E1* 5′ flank up to −489, but with a mutated C/EBP element, no stimulatory effect was seen (not shown).

In another series of cotransfection experiments, constitutively active MKK3 was cotransfected with pGL3-

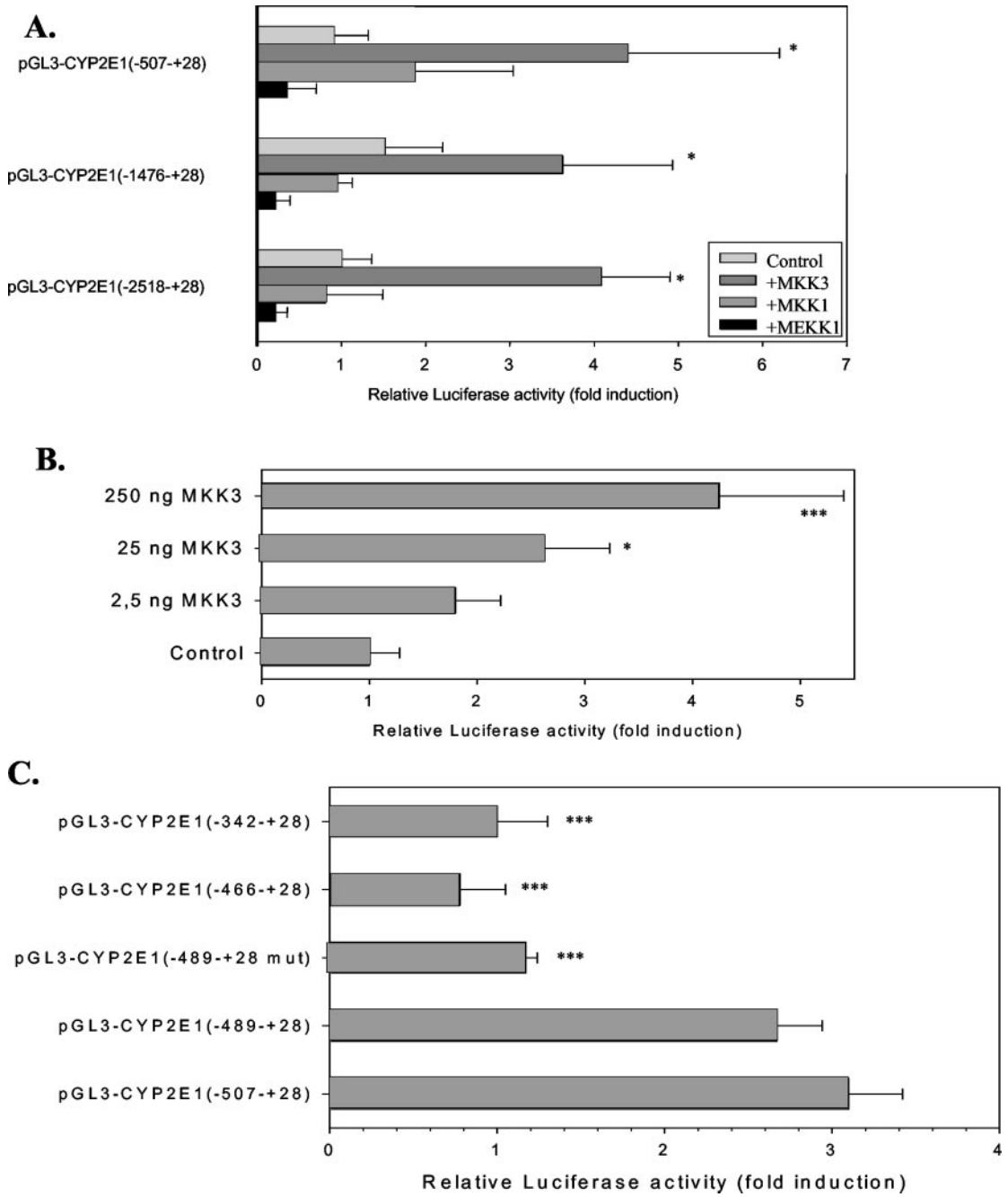

FIG. 3. **The effects of expression of constitutively active MKK3 on pGL3-CYP2E1 transfected cortical glial cells.** *A*, cortical glial cultures were transfected with the indicated pGL3-CYP2E1 plasmids and cotransfected with 25 ng/ml pFC-MKK3, pFC-MKK1, or pFC-MEKK1 plasmid expressing constitutively active MKK3, MKK1, and MEKK1, respectively. *B*, cortical glial cultures were transfected with pGL3-CYP2E1(−3685-+28) and cotransfected with the indicated amounts of pFC-MKK3, expressing a constitutively active MKK3 kinase. *C*, pGL3-CYP2E1 plasmids of different lengths were transfected by themselves, or cotransfected with pFC-MKK3 (25 ng of plasmid) in cortical glial cells. The induction with pFC-MKK-3 compared with control is shown. In the pGL3-CYP2E1(−489-+28 mut), 4 mutated residues were introduced in the putative response element (see "Experimental Procedures"). *, $p < 0.05$; ***, $p < 0.001$.

CYP2E1(−489-+28), in addition to C/EBPα, C/EBPβ, or C/EBPδ. Stimulation with MKK3 induced luciferase activity 3-fold, as expected, and co-transfection with C/EBPβ or C/EBPδ gave an additive effect on luciferase activity (Fig. 6*B*). The stimulatory effect of MKK3, C/EBPβ, or C/EBPδ was lost when plasmids instead were cotransfected with the mutated −489 pGL3-CYP2E1 plasmid (not shown). These experiments indicated that both the concentration of available C/EBPβ and C/EBPδ molecules at the element, as well as their degree of activation, appeared to be potentially of importance.

Increased Binding at the −486/−474 C/EBP Element by LPS at Later Time Points, but Not Initially—The results presented above showed that the CYP2E1 catalytic activity, and pGL3-CYP2E1 reporters, were potently induced within 3–5 h of LPS exposure. Therefore, EMSA was carried out with nuclear extracts treated with LPS for 2 h. Although the extracts were verified to be stimulated by LPS through the increased binding of NFκB, to an oligonucleotide with a consensus NFκB element, no increased binding was seen to the CYP2E1 −486/−474 C/EBP element-containing oligonucleotide (Fig. 7, gels to the *left*). At 12 h, however, the extent of protein binding to the CYP2E1 −486/−474 C/EBP element-containing oligonucleotide had increased (Fig. 7, gels to the *right*), and remained increased at 24 h (not shown). Protein complexes in nuclear

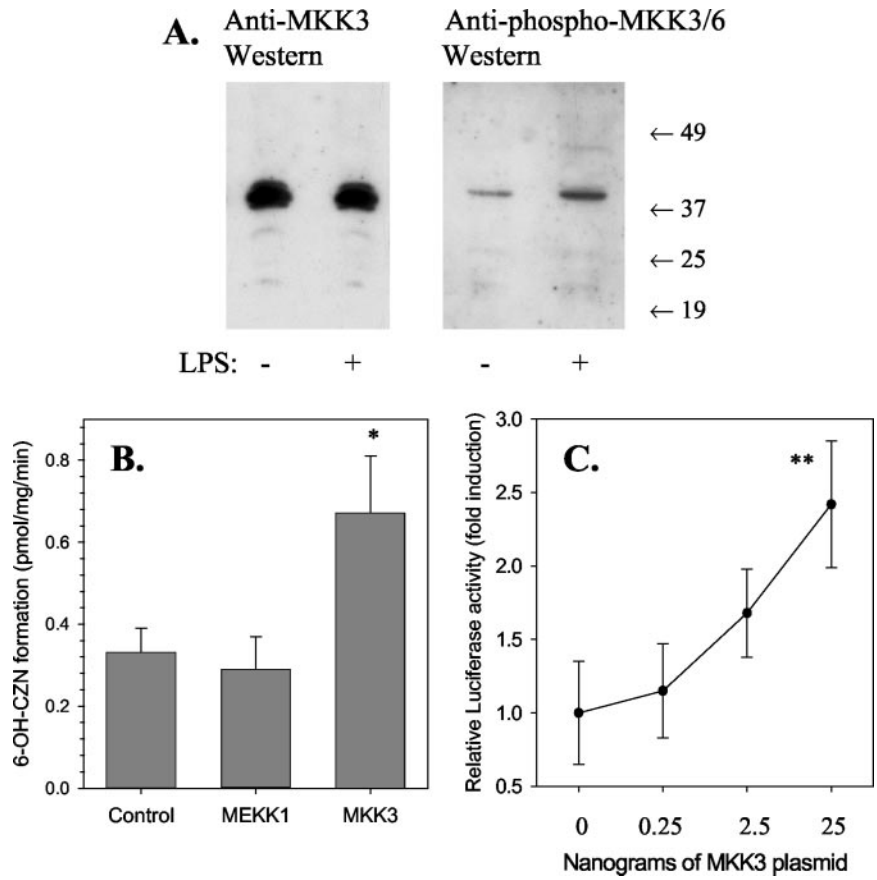

FIG. 4. **The effects of LPS stimulation on MKK3 phosphorylation, and expression of constitutively active MKK3 on 6-OH-CZN formation.** *A*, Western blot analysis of cell extracts from cortical glial cultures, using highly specific antisera to MKK3 (*left panel*) or antisera directed specifically against phosphorylated MKK3/6 (*right panel*). The indicated samples originate from cultures treated with 100 ng/ml LPS, for 30 min. *B*, effect of MKK3 or MEKK1 expression on the rate of 6-hydroxylation of CZN, in cortical glial cultures. Control cells were transfected with empty plasmid DNA. *C*, analysis of the effect of pFC-MKK3 transfection on a CHOP/GAL4 construct for activation of a GAL4-luciferase reporter plasmid, in cortical glial cultures. *, $p < 0.05$; **, $p < 0.01$.

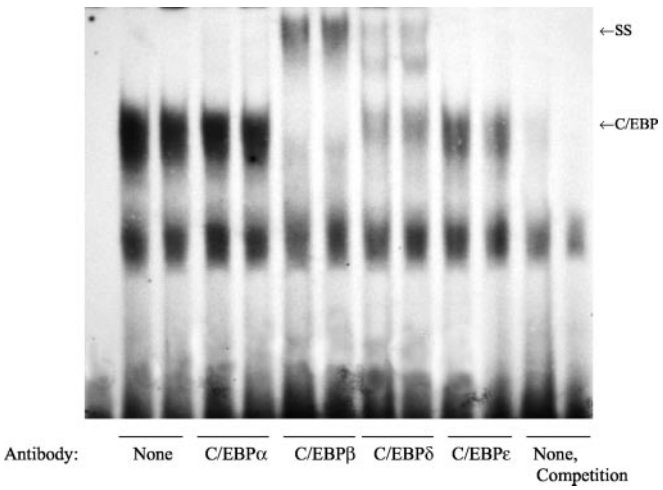

FIG. 5. **EMSA of the LPS/MKK3 responsive element at −486/−474 in *CYP2E1*.** Nuclear extracts from cortical glial cultures were incubated with a labeled *CYP2E1* oligonucleotide (−492/−466) and analyzed by PAGE on a 4% gel. Prior to addition of the labeled oligonucleotide, specific antibodies directed against C/EBP proteins were added; 2 μg in the *left lane* and 4 μg in the *right lane*, for all antibodies. In the last two lanes (to the *right*), instead of antibodies a 10- and 100-fold excess of unlabeled oligonucleotide, respectively, was added.

extracts stimulated with LPS for 12 h, bound to the CYP2E1 −486/−474 C/EBP element-containing oligonucleotide, were found to consist of C/EBPβ and C/EBPδ to a very large extent (EMSA antibody interference analysis, Fig. 8*A*). Interestingly, it was found also that antibodies to C/EBPα produced a small shift, at 12 h of LPS treatment (Fig. 8*A*). Complexes bound to the NFκB consensus oligonucleotide were supershifted with antibodies to p50/NFκB and p65/RelA (Fig. 8*B*), as expected.

Dominant Negative C/EBPβ Inhibits the Response of CYP2E1 to Both LPS and MKK3—The above experiments showed that although LPS treatment increased the binding of protein complexes to DNA, it did not change the composition of the complexes; both in the control situation and in the induced state, protein complexes were bound to DNA almost completely supershifted with C/EBPβ antibodies (Figs. 5 and 8). C/EBPβ has been shown to induce transcriptional activation both through post-translational modification of existing C/EBP, and by increased binding to DNA (48). We therefore transfected the cortical glial cells with an expression plasmid encoding dominant negative C/EBPβ and tested the response to LPS, or MKK3 cotransfection. It was found that expression of the dominant-negative C/EBPβ to a large extent inhibited the response to MKK3, and also to LPS (Fig. 9). As LPS stimulation took place for only 5 h, also an early response of the *CYP2E1* reporter plasmid appeared dependent on C/EBPβ.

DISCUSSION

MKK3 was originally isolated as a MAP kinase kinase from human brain mRNA, specifically phosphorylating p38 MAPK (35). Studies have shown also that MKK4 and MKK6 phosphorylate p38 MAP kinases. It appears, however, that MKK4 does not appreciably activate p38 MAPK *in vivo* (36), and that MKK6 is not expressed at very high levels in the brain (37, 38). Thus, MKK3 seems to be the major p38 MAPK activator in the central nervous system. In addition, MKK3 has been identified as the major MKK for activation of p38 MAP kinase in response to lipopolysaccharide (39). Previous studies from our laboratory have shown that chlomethiazole inhibited lipopolysaccharide-induced CYP2E1-dependent catalytic activity (18) and subsequently that chlomethiazole acts as a p38 MAPK inhibitor (22), in astrocytes. For all these reasons, MKK3 appeared to us to be a good first choice for studies of the involvement of the MKK3/4/6-p38 MAPK pathway in the regulation of *CYP2E1* in response to lipopolysaccharide in astrocytes.

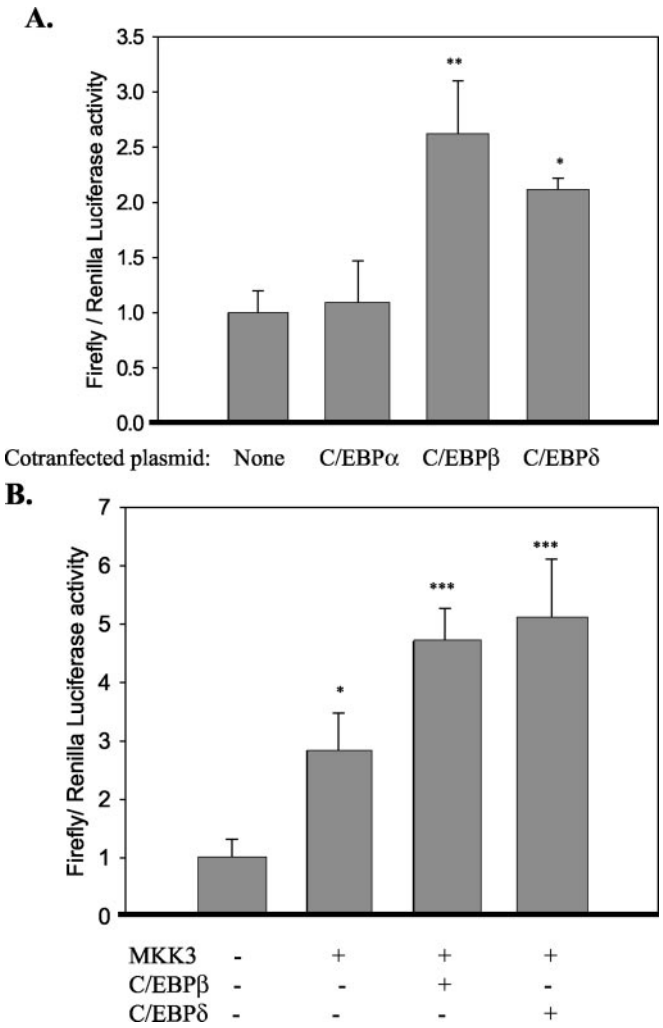

FIG. 6. **Overexpression of C/EBPβ or -δ stimulates pGL3-CYP2E1 transfected cortical glial cells.** Statistical analysis (one-way ANOVA with Tukey-Kramer's post-hoc test) gave the indicated results, and further showed that also the additive effect of either C/EBPβ or C/EBPδ on MKK3 induced luciferase activity was statistically significant *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

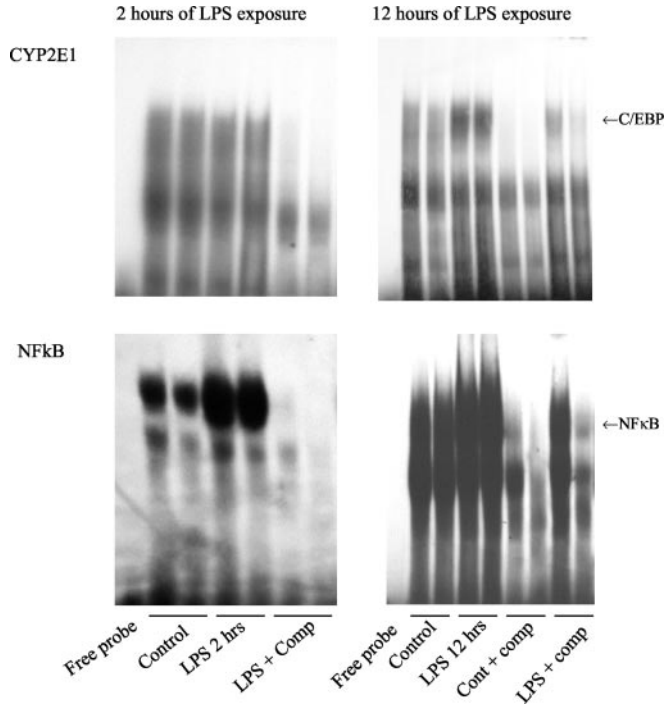

FIG. 7. **Increased binding to −486/−474 in *CYP2E1* after LPS treatment for 12 h.** Nuclear extracts were isolated from cortical glial cultures stimulated with LPS for 2 or 12 h. These extracts were incubated with the labeled *CYP2E1* oligonucleotide (−492/−466) (the *two gels* on *top*) or with a consensus NFκB oligonucleotide (the *two gels* at the *bottom*). 2 h of LPS exposure is shown to the *left*, and 12 h of LPS exposure to the *right*.

We found that the LPS-dependent induction of the rate of 6-OH-CZN formation was highly sensitive to the protein-tyrosine kinase inhibitors genistein and herbimycin A (Fig. 2). It was also found that tyrphostin AG126 had some inhibitory effect (Table I), whereas several other inhibitors were without effect. Genistein, herbimycin A, and tyrphostin AG126 have previously been found to inhibit a number of cellular responses to lipopolysaccharide exposure (29, 40). Although few details of the exact nature of the pathways leading from CD14 and the Toll-like receptors 2/4 (TLR2/TLR4), transducing LPS signals, to MKK3 are known, several reports have specifically shown that herbimycin A inhibits the phosphorylation and activation of MKK3 and p38 MAPK (29, 30, 41). Indeed, it was found that related adhesion focal tyrosine kinase may be involved in the cellular response to hyperosmolarity, leading to MKK3, but not MKK6, phosphorylation and activation (30). Except for tyrosine kinase inhibitors, we also tested the two structurally unrelated p38 MAPK inhibitors, SB203580 and chlomethiazole. It was found that chlomethiazole potently inhibited the induction of 6-OH-CZN formation in response to LPS, but that SB203580 showed no inhibition. This may be interpreted to indicate that p38 MAPK α or β isoforms are not involved in the response, but that other p38 MAPK isoforms or related kinases may be involved. The results must, however, be interpreted with caution as chlomethiazole binds to other molecules such as the $GABA_A$ receptor (although at much higher K_d) in addition to inhibiting p38 MAP kinase activity (22). Interestingly, SB203580 significantly induced the already increased rates of 6-OH-CZN formation (Fig. 2*D*). Up-regulation of activity in signaling pathways in response to SB203580 has previously been reported (31, 42, 43) and may be related to positive feedback loops upstream of p38 MAPK α and β, as exemplified by MKK6 (42). Thus, the positive effect of SB203580 on the LPS-induced increased rate of 6-OH-CZN formation indicated that enzymes upstream of p38 MAPK participates in the signaling pathway to CYP2E1. Subsequent experiments showed that expressed constitutively active MKK3 specifically stimulated *CYP2E1* luciferase reporters, and that transfection of the MKK3 plasmid to cortical glial cells also induced CYP2E1 catalytic activity, as the formation of 6-OH-CZN was increased. It was concluded that constitutively active MKK3 stimulated *CYP2E1* in its natural chromatin context, in astrocytes.

We identified a C/EBP binding element at −486/−474 in *CYP2E1* that was positively regulated by LPS and MKK3. EMSA and transfectional analysis indicated that especially C/EBPβ but also C/EBPδ have major roles for transcriptional activation of *CYP2E1* from the −486/−474 element (Figs. 5–9). The findings are in line with previous reports on C/EBP factors, where C/EBPβ and C/EBPδ have been found to be expressed in the nuclei of astrocytes (44), and to be induced in response to LPS (45). C/EBPβ and C/EBPδ, but not C/EBPα, were also shown to mediate the effect of lipopolysaccharide on interleukin-1 and interleukin-6 promoters, in monocytes (46), macrophages (47), and intestinal cells (48). Results presented in Figs. 6, 7, and 9 further suggested that transcriptional activation through the −486/−474 element appeared to involve both activation of prebound factors as well as increased binding of C/EBPβ and C/EBPδ to the site. Several post-translational

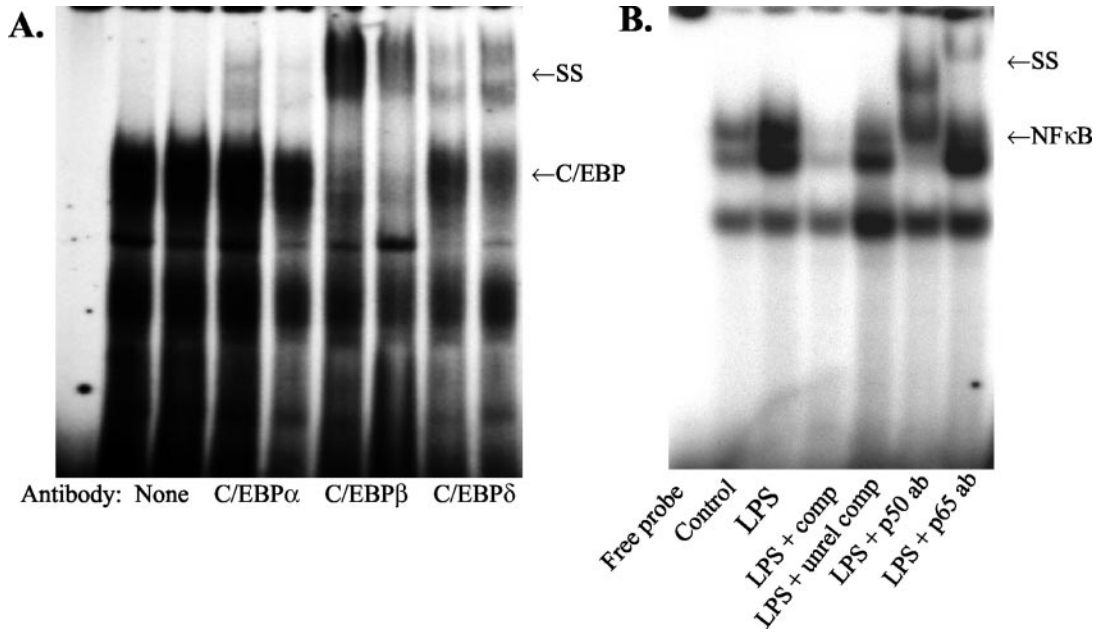

FIG. 8. **Antibody analysis of proteins bound to 486/−474 in *CYP2E1*, and NFκB oligo.** EMSA using the labeled *CYP2E1* oligonucleotide (−492/−466) (*A*) or the consensus NFκB oligonucleotide (*B*). The nuclear extracts were from cortical glial cells treated with 100 ng/ml LPS for 12 h. In *A*, 2 or 4 μg of specific antibodies to C/EBPα, C/EBPβ, or C/EBPδ were added to the incubation reaction. In *B*, 2 μg of antibodies to p50 or p65, as indicated, were added to the incubation reaction. Competition with a 50-fold excess of the consensus NFκB oligonucleotide, or unrelated, unlabeled DNA is shown.

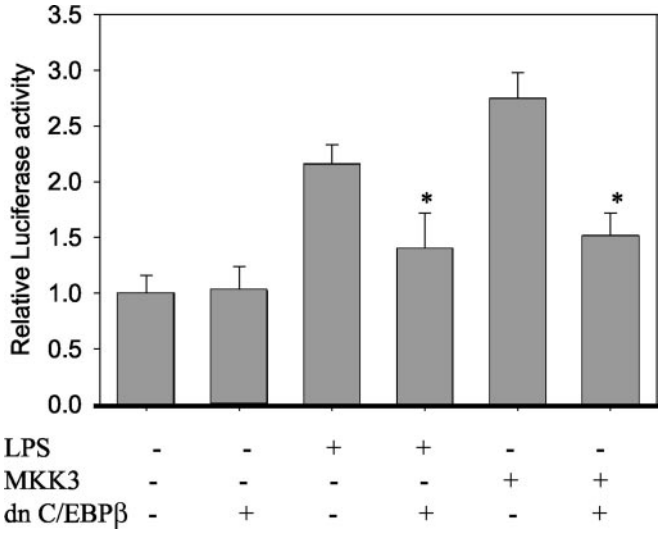

FIG. 9. **Effect of dominant negative C/EBPβ on *CYP2E1* reporter inducibility.** Cortical glial cultures were transfected with 0.5 μg of pGL3-CYP2E1(−1476-+28) and cotransfected with 25 ng of pFC-MKK3, expressing a constitutively active MKK3 kinase, or treated with LPS (100 ng/ml) for 5 h, in the presence or absence of cotransfected dominant negative C/EBPβ (1 μg), as indicated. Cells not transfected with dominant negative C/EBPβ were cotransfected with empty plasmid DNA (1 μg).

modifications that lead to activation of C/EBPβ have been described, including serine/threonine phosphorylation at different sites, in response to protein kinase C or extracellular signal-regulated kinase (see Ref. 49). Although C/EBPδ has not been described to be activated through post-translational modifications, lipopolysaccharide reportedly increases transcription of both C/EBPβ and C/EBPδ and subsequently increase binding to C/EBPβ and C/EBPδ elements (49). It may therefore be hypothesized that the early phase of *CYP2E1* transcriptional activation in response to lipopolysaccharide involves activation of prebound C/EBPβ, whereas the later phase may involve increased binding of both C/EBPβ and C/EBPδ. In support of this hypothesis are the findings that: 1) transfectional experiments showed that the −486/−474 site was the major response element to LPS and MKK3 (Figs. 1C and 3); 2) C/EBPβ was found to be the major protein bound to this site, both in the control situation and in the induced state (Figs. 5 and 8); 3) expression of dominant negative C/EBPβ inhibited both the LPS- and MKK3-dependent activation of a *CYP2E1* reporter gene (Fig. 9); and 4) overexpression of MKK3, C/EBPβ, or C/EBPδ caused by themselves activation of a *CYP2E1* reporter gene, whereas an additive effect of C/EBPβ or C/EBPδ was seen on MKK3 induced activation of a *CYP2E1* reporter gene (Fig. 6). Furthermore, the results from EMSA, with increased binding of C/EBPβ and C/EBPδ at 12 and 24 h, are also in agreement with the sustained induction of CYP2E1-dependent catalytic activity over 24 h (Fig. 1*A*).

The activation of C/EBP in response to extracellular stimuli have in several cases been indirectly linked to p38 MAP kinase. Activation of C/EBP in the fibroblast growth factor-binding protein promoter by serum, in squamous carcinoma cells (50), and lipopolysaccharide induced activation of a luciferase reporter gene, containing a C/EBP binding element from the interleukin-1β promoter, in monocytes (46), were both sensitive to SB203580. Similarly, Bhat *et al.* (51) recently reported that transcriptional activation of inducible nitric-oxide synthase in glial cells, in response to lipopolysaccharide, interferon γ, and tumor necrosis factor α, was inhibited by SB203580. In the same report, it was also shown that expression of constitutively active MKK3 induced inducible nitric-oxide synthase transcription, and this was to some extent inhibited through transfection of a dominant negative C/EBPβ. The direct binding of, or transcriptional contribution of C/EBPβ to inducible nitric-oxide synthase transcription was, however, not presented. It may be concluded that p38 MAP kinases α and β (which are inhibited by SB203580) likely are involved in the signaling from extracellular stimuli such as lipopolysaccharide, to C/EBPβ in the above referred studies. The exact mechanisms, including the direct C/EBP kinase, remain, however, unidentified.

The exact nature of the mediator of the MKK3 effect on the

CYP2E1 −486/−474 C/EBP binding site was not addressed in the presented study, and is currently unknown. At least four substrates of MKK3 have, however, been identified, including p38 MAPK isoforms α, γ, and δ (52). Of these, the activity of p38α is potently inhibited by SB203580 (32), and a contribution of p38α in the response of CYP2E1 to LPS is therefore not likely (Fig. 2*D*). Interestingly, in addition to p38 MAP kinases, Mirk is an arginine-directed Ser/Thr protein kinase that recently has been described as a MKK3 substrate. Transcriptional activation induced by Mirk was enhanced by interaction with and phosphorylation by MKK3 (53). The contribution of p38γ, p38δ, and Mirk to the LPS and MKK3 effects on *CYP2E1* transcription is currently under investigation. In conclusion, we present data indicating that lipopolysaccharide may signal through MKK3 to stimulate CYP2E1 expression through stimulation of C/EBPβ and C/EBPδ at a specific binding element at −486/−474.

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Lipopolysaccharide Induces *CYP2E1* in Astrocytes through MAP Kinase Kinase-3 and C/EBP β and -δ

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