ANTIOXIDANTS & REDOX SIGNALING
Volume 6, Number 5, 2004

Forum Original Research Communication

Role of NF-κB and p38 MAP Kinase Signaling Pathways in the Lipopolysaccharide-Dependent Activation of Heme Oxygenase-1 Gene Expression

NASTITI WIJAYANTI,[1] SEBASTIAN HUBER,[1] ANATOLY SAMOYLENKO,[2] THOMAS KIETZMANN,[2] and STEPHAN IMMENSCHUH[1]

ABSTRACT

Heme oxygenase (HO)-1 is the inducible isoform of the rate-limiting enzyme of heme degradation, which is up-regulated by a host of stress stimuli. The bacterial cell membrane component lipopolysaccharide (LPS) is a prototypical activator of monocytic cells. Here, it is shown that LPS induced the endogenous HO-1 gene expression in RAW264.7 monocytic cells. To investigate the molecular mechanisms of HO-1 gene induction by LPS, we performed transfection experiments with reporter gene constructs containing sequences of the proximal rat HO-1 gene promoter. Deletion and mutation analysis indicated that a cyclic AMP response element/activator protein-1 site (−664/−657), but not an E-box motif (−47/−42), played a major role for LPS-dependent HO-1 gene induction. Up-regulation of HO-1 promoter activity by LPS was decreased by pharmacological nuclear factor-κB (NF-κB) inhibitors and by cotransfected expression vectors with dominant negative isoforms of NF-κB-inducing kinase, inhibitor of NF-κB (IκB) kinase β, and IκBα. Moreover, the p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580 and overexpressed dominant negative p38β decreased, whereas dominant negative p38δ increased, LPS-dependent induction of HO-1 gene expression. The results suggest that the NF-κB and p38 MAPK signaling pathways mediate the LPS-dependent induction of HO-1 gene expression via DNA sequences of the proximal promoter region. *Antioxid. Redox Signal.* 6, 802–810.

INTRODUCTION

Heme oxygenase (HO) catalyzes the first and rate-limiting step of heme degradation (29). HO not only controls the cellular heme availability, but also produces carbon monoxide, iron, and biliverdin. At least two genetically distinct isozymes have been identified of which the inducible form, HO-1, is up-regulated by various stress stimuli, such as heme, heat shock, heavy metals, ultraviolet light, and hyperoxia (for reviews, see 19, 22). Although the functional role of HO-1 gene induction in response to oxidative stress is not understood in detail, it has been demonstrated that overexpression of HO-1 provides protection against oxidative stress in various cell culture and *in vivo* models (1, 5, 10). The physiological role of the HO-1 gene has also been demonstrated in HO-1-deficient mice (23, 24) and in a case of human genetic HO-1 deficiency (31). In both mouse and human HO-1 deficiency, organ manifestations such as iron overload of the liver with signs of a chronic hepatitis, as well as an iron deficiency anemia with paradoxical increased levels of ferritin, were observed (23, 24, 31).

Lipopolysaccharide (LPS) is a component of the cell wall of gram-negative bacteria and is the major mediator of sepsis, septic shock, and systemic inflammatory response syndrome (28). Treatment of monocytic cells with LPS induces the production of reactive oxygen species and nitric oxide,

[1]Institut für Klinische Chemie und Pathobiochemie, Justus-Liebig-Universität Giessen, D-35392 Giessen, Germany.
[2]Institut für Biochemie und Molekulare Zellbiologie, Georg-August-Universität Göttingen, D-37073 Göttingen, Germany.

the secretion of cytokines such as tumor necrosis factor α (TNFα) or interleukin-1, and the up-regulation of intracellular autoprotective proteins such as heat shock protein 70 and HO-1 (8, 30). LPS-dependent induction of HO-1 gene expression in monocytic cells has previously been demonstrated to be mainly mediated via antioxidant response elements (AREs), also termed stress response elements (StREs), of the far distal mouse HO-1 gene promoter (3), which are activated via the transcription factor (TF) Nrf2 (2). The involvement of the nuclear factor-κB (NF-κB) signaling pathway for HO-1 gene regulation, however, which is the major signaling pathway activated by LPS in monocytes (8, 15), is not well understood. Therefore, the goal of the present study was to identify the regulatory elements (REs) and signal cascades that are involved in the LPS-dependent induction of HO-1 gene activity in monocytic cells.

It is shown that the LPS-dependent induction of HO-1 gene expression is mediated via a previously characterized cyclic AMP response element (CRE)/activator protein-1 (AP-1) element of the proximal promoter region of the rat HO-1 gene. Moreover, it is demonstrated that the NF-κB and p38 mitogen-activated protein kinase (MAPK) signaling pathways play important regulatory roles in the LPS-dependent induction of HO-1 gene expression.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle medium was obtained from GibcoBRL/Invitrogen (Karlsruhe, Germany), fetal bovine serum from Biochrom KG (Berlin, Germany), polyvinylidene difluoride membranes from Millipore (Bedford, MA, U.S.A.), the Lumi-light chemiluminescent detection system for western blot from Roche Applied Science (Mannheim, Germany), and restriction endonucleases from New England Biolabs (Cambridge, MA, U.S.A.). Falcon tissue culture dishes were from BD Bioscience (Heidelberg, Germany). The primary polyclonal rabbit anti-HO-1 antibody was obtained from Stressgen (Victoria, BC, Canada). All other chemicals were purchased from Sigma (Munich, Germany) and Roche Molecular Biochemicals (Basel, Switzerland) unless otherwise indicated.

Cell culture

RAW264.7 cells were from American Type Culture Collection (Manassas, VA, U.S.A.) and were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 100 U of penicillin/ml, and 100 μg of streptomycin/ml. Cell cultures were kept under air/CO_2 (19:1). Treatment of cells with LPS (1 μg/ml) was performed with serum-free medium.

Western blot analysis

Cell cultures were washed twice with 0.9% NaCl. After addition of 300 μl of 1× Laemmli buffer (2% sodium dodecyl sulfate, 10% glycerol, bromophenol blue, 0.4 mol/L dithiothreitol, 4% protease inhibitor), cells were scraped from culture dishes and then homogenized by passing through a 25-gauge needle. The homogenate was incubated for 3 min at 95°C, and the protein content was determined in the supernatant by the Bradford method. Total protein (60 μg) was loaded onto a 12% sodium dodecyl sulfate–polyacrylamide gel and was blotted onto polyvinylidene difluoride membranes by electroblotting. Membranes were blocked with Tris-buffered saline containing 5% dry milk, 50 m*M* Tris-HCl (pH 7.5), 150 m*M* NaCl, and 0.1% Tween, for 1 h at room temperature. The primary polyclonal antibody against rat HO-1 was added at 1:1,000 dilution, and the blot was incubated for 1 h at room temperature. The secondary anti-rabbit IgG was diluted 1:10,000 and the chemiluminescent detection system was used for detection according to the manufacturer's instructions. Monoclonal mouse anti-rabbit glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (HyTest Ltd., Turku, Finland) was used as a loading control. The primary antibodies for the detection of phosphorylated and total MAPKs were from Cell Signaling (Beverly, MA, U.S.A.) and were applied at the concentrations recommended by the manufacturer.

Plasmid constructs

The luciferase reporter gene constructs pHO-1338, pHO-754, and pGL3basic have been described previously (13). The luciferase reporter gene constructs pHO-347, pHO-754Em and pHO-754Am/Em, were generated with the Quick change™ XL site-directed mutagenesis kit (Stratagene) as described previously (16). The pNF-κB reporter gene plasmid with four tandem copies of the NF-κB consensus sequence was obtained from Clontech (Palo Alto, CA, U.S.A.), and plasmid p3xStRE with three copies of the HO-1 SRE was a gift from Dr. Jawed Alam (New Orleans, LA, U.S.A.). Expression vectors for dominant negative forms of NF-κB-inducing kinase (NIK), inhibitor of NF-κB (IκB) kinase β (IKKβ), and IκBα were from Dr. Richard Gaynor (Houston, TX, U.S.A.), and dominant negative mutants (AF) of p38α, β, γ and δ isoforms were from Dr. Jiahuai Han (La Jolla, CA, U.S.A.).

Transfection and luciferase assay

After growth for 24 h, transfection of plasmid DNA into RAW264.7 cells was performed by the liposome method using Fugene® (Roche Molecular Biochemicals) as described previously (9). Unless otherwise stated, cells were transfected with 0.5 μg of reporter plasmid and in cotransfection experiments with 0.01–1 μg of the indicated expression vectors. Luciferase activity was determined with a commercial luciferase assay system (Promega; Madison, WI, U.S.A.) as described previously (9). Cells were either harvested 24 h after transfection or treated for another 18 h with LPS, as indicated. Relative light units of luciferase activity were normalized with sample protein.

RESULTS

LPS-dependent induction of HO-1 gene expression in RAW264.7 cells

It has previously been demonstrated that HO-1 gene expression is induced by LPS in macrophages and monocytic

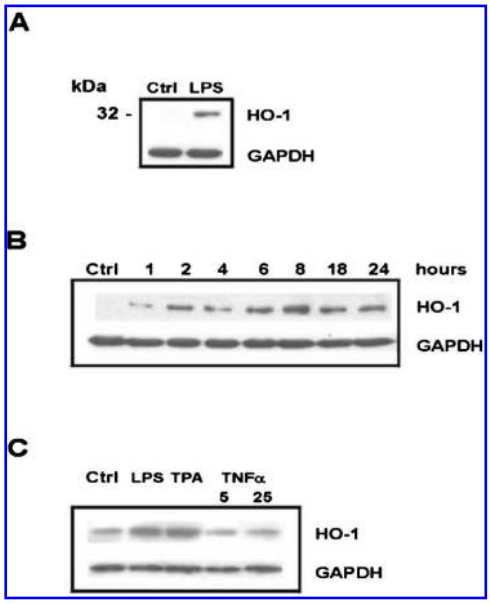

FIG. 1. LPS-dependent induction of endogenous HO-1 gene expression in RAW264.7 cells. RAW264.7 cells were cultured as described in Materials and Methods. Confluent cells were cultured (**A**) for 18 h with or without LPS (1 μg/ml) in serum-free medium, (**B**) in the presence of LPS for the times indicated, or (**C**) with LPS (1 μg/ml), TPA (0,5 μ*M*), TNFα (5 μg/ml and 25 μg/ml), or control medium as indicated. Total protein (60 μg) was subjected to western blot analysis with a polyclonal antibody against rat HO-1 or GAPDH as a loading control (A–C). Similar results were obtained in at least three independent experiments, and a representative autoradiogram is shown. Ctrl, control.

cells (11, 12). To investigate the regulatory mechanisms of LPS-dependent HO-1 gene induction in these cells in more detail, we applied the monocytic cell line RAW264.7 for the present study. Treatment with LPS induced HO-1 protein expression in RAW264.7 cells as determined by Western blot analysis (Fig. 1A). Up-regulation of HO-1 protein expression was time-dependent with a maximum at 8 h (Fig. 1B). HO-1 gene expression was also induced by treatment with LPS on the mRNA level (data not shown).

As LPS-dependent induction of HO-1 gene expression has previously been shown to be mediated by the proinflammatory cytokine TNFα, we compared the regulatory effect of LPS with that of TNFα and the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA). As demonstrated in Fig. 1C, treatment with TNFα had no effect on HO-1 gene expression, whereas TPA strongly up-regulated HO-1 gene expression. LPS-dependent induction of HO-1 gene promoter activity appeared to be cell-specific because no induction was observed in cell cultures of LLC-PK1 (porcine kidney epithelial cells), H35T718 (hepatoma cells), HeLa (human cervix epithelial cells), and human umbilical cord endothelial cells (ECV304) (data not shown). The data suggest that LPS induces the endogenous HO-1 gene expression in RAW264.7 cells.

Identification of regulatory DNA sequences of the proximal rat HO-1 gene promoter region that mediate the LPS-dependent induction of HO-1 gene expression

The up-regulation of HO-1 gene expression by most stimuli occurs on the transcriptional level. Accordingly, pretreatment of cell cultures with the transcription inhibitor actinomycin D prevented the LPS-dependent HO-1 mRNA induction in our model of RAW264.7 cells (data not shown). To investigate the molecular mechanisms of LPS-dependent HO-1 gene induction, we determined the effect of LPS on the activity of luciferase reporter gene constructs containing either the proximal 1,338 bp of the rat HO-1 gene promoter (pHO-1338), four copies of the NF-κB consensus sequences (pNF-κB), or three copies of the HO-1 ARE [p3xStRE (2)] in transiently transfected RAW264.7 cells (Fig. 2). A marked LPS-dependent induction of luciferase activity was determined for the pHO-1338 reporter gene construct, and a lower level of induction was observed for the pNF-κB and the p3xStRE constructs.

To identify potential regulatory DNA sequences that may mediate the LPS-dependent induction of HO-1 gene expression, we performed transfection experiments with reporter gene constructs containing serially 5′-deleted sequences of the proximal HO-1 promoter region. Deletion of a putative NF-κB site (−1,002/−994) did not alter the LPS-dependent induction of luciferase reporter gene activity. The inducibility by LPS of HO-1 reporter gene constructs, however, was markedly reduced after deletion of DNA sequences from −754 to −347 and −347 to −50. Suprisingly, even a construct with 50 bp of the proximal HO-1 promoter gene region containing an E-box motif (−47/−42) was still up-regulated by LPS (Fig. 3A). To assess further the functional role of this

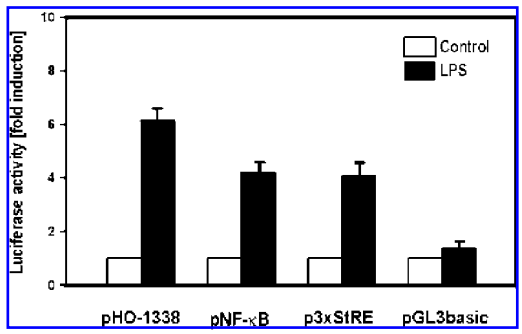

FIG. 2. LPS-dependent induction of rat HO-1 promoter activity. RAW264.7 cells were transiently transfected with reporter gene constructs containing either the proximal 1,338 bp of the HO-1 promoter 5′-flanking region (pHO-1338), four copies of the consensus sequence of NF-κB (pNF-κB), three copies of the HO-1 StRE (p3xStRE), or the control plasmid pGL3basic. Twenty-four hours after transfection, cells were cultured in the absence or presence of LPS (1 μg/ml) for another 18 h. Cell extracts were assayed for luciferase activity as described in Materials and Methods, and the rate of induction was determined relative to the control, which was set equal to 1. Values are means ± SEM from at least three independent experiments.

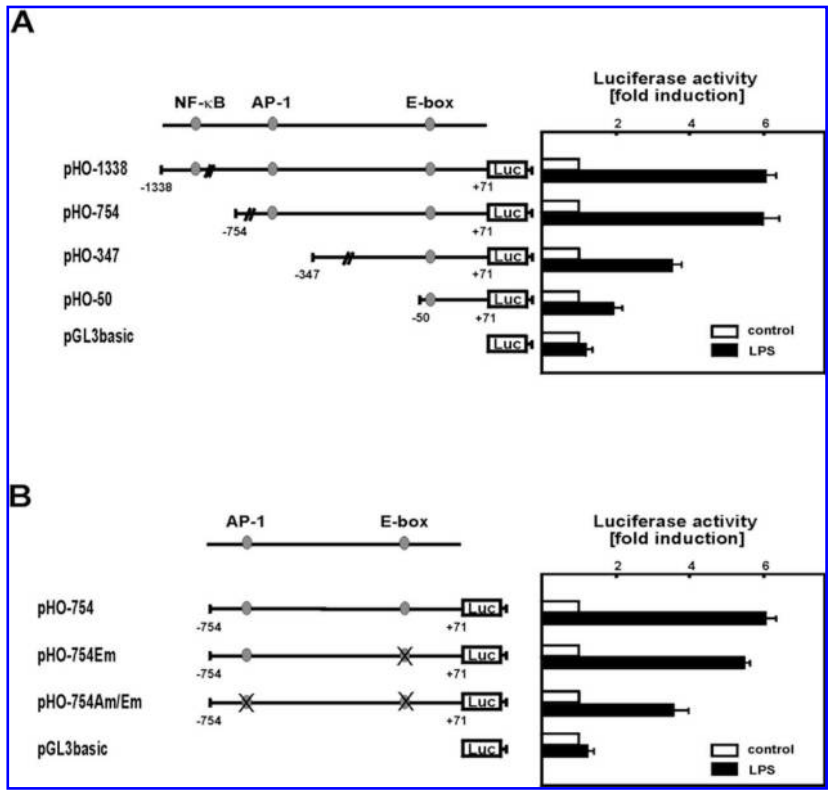

FIG. 3. Regulation of rat HO-1 gene promoter sequences by LPS in transiently transfected RAW264.7 cells. (**A**) The indicated serially deleted rat HO-1 reporter gene constructs were transiently transfected into RAW264.7 cells. Twenty-four hours after transfection, cells were treated for 18 h with or without LPS (1 μg/ml). The localization of putative NF-κB, AP-1, and E-box motifs is indicated. Cell extracts were assayed for luciferase activity as described in Materials and Methods, and the rate of induction was determined relative to the control, which was set equal to 1. (**B**) HO-1 reporter gene constructs with mutations in the AP-1 and E-box sites of pHO-754 were generated as described under Materials and Methods. Cell extracts were assayed for luciferase activity, and the rate of induction was determined relative to the control, which was set equal to 1. Values are means ± SEM from at least three independent experiments.

proximal E-box motif, which could be a potential target for the TF upstream stimulatory factor (USF) that mediates LPS-dependent gene regulation (7, 25, 26) and a previously identified HO-1 CRE/AP-1 site (−664/−657) (13, 16), we examined the LPS-dependent regulation of HO-1 reporter gene constructs with targeted mutations of these sites. As demonstrated in Fig. 3B, LPS-dependent induction of luciferase activity of pHO-754Em with a mutated E-box site was not reduced as compared with wild-type pHO-754. By contrast, up-regulation of reporter gene activity of construct pHO-754Am/Em by LPS was markedly decreased. Treatment with TNFα had no effect on reporter gene activity of pHO-1338 (data not shown).

The data suggest that activation of HO-1 gene expression by LPS in RAW264.7 cells is mediated via regulatory DNA sequences of the proximal HO-1 promoter region.

Role of the NF-κB signaling pathway in LPS-dependent activation of HO-1 promoter activity

The NF-κB signaling pathway has previously been shown to play a major role in LPS-dependent gene regulation (15). Therefore, the effects of the pharmacological NF-κB inhibitors caffeic acid phenethyl ester (CAPE) and Nap-tosyl-L-lysine chloromethyl ketone (TLCK) on LPS-dependent induction of HO-1 promoter activity was determined in RAW264.7 cells. The up-regulation of HO-1 promoter activity by LPS was inhibited by pretreatment with CAPE and TLCK in a dose-dependent manner (Fig. 4). For a comparison, the reporter gene construct pNF-κB showed a similar regulatory pattern.

To examine further the potential role of the NF-κB signaling pathway in LPS-dependent induction of HO-1 gene expression, we performed cotransfection experiments with expression vectors of wild-type and dominant negative forms of kinases of the NF-κB pathway. Overexpression of wild-type NIK, which is an upstream kinase of the NF-κB signaling cascade, led to a dose-dependent increase of luciferase activity of the pHO-1338 construct (Fig. 5). In addition, LPS-dependent induction of HO-1 promoter activity was inhibited by dominant negative NIK (Fig. 6, left panel), but not by dominant negative transforming growth factor-β-activated kinase-1 or MAPK/extracellular signal-regulated kinase (ERK) kinase kinase (MEKK)-1 (data not shown).

The regulatory role of the downstream IKKβ and IκBα was examined by cotransfection of RAW264.7 cells with construct pHO-1338 and expression vectors of dominant negative isoforms of IKKβ and IκBα. LPS-dependent induction of HO-1 promoter activity was markedly decreased by overexpressed dominant negative IKKβ and IκBα (Fig. 6, left panel). Taken together, the data demonstrate that activation of the NF-κB signaling pathway plays an important role in LPS-dependent induction of HO-1 gene expression.

Activation of MAPKs by LPS in RAW264.7 cells

To determine the potential role of MAPKs in LPS-dependent induction of HO-1 gene expression, RAW264.7 cells were treated with LPS for various lengths of time and cell extracts were analyzed for phosphorylated and total MAPKs. An increase in the level of phosphorylated ERK1 and ERK2 was observed in LPS-treated cells, reaching a

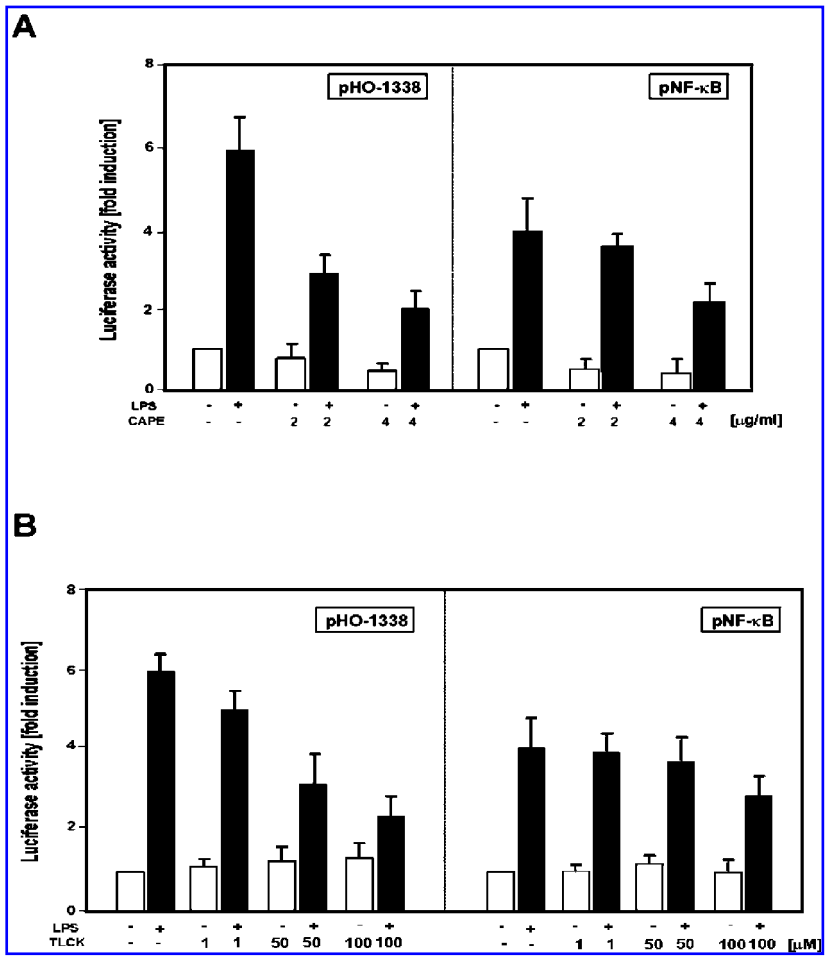

FIG. 4. Effect of pharmacological NF-κB inhibitors on LPS-dependent induction of HO-1 promoter activity in RAW264.7 cells. RAW264.7 cells were transiently transfected with the reporter gene constructs pHO-1338 or pNF-κB. Twenty-four hours after transfection, cells were treated for 30 min with the NF-κB inhibitors (**A**) CAPE and (**B**) TLCK at the indicated concentrations before incubation for another 18 h with or without LPS (1 μg/ml). Cell extracts were assayed for luciferase activity, and the rate of induction relative to the control was determined. Values are means ± SEM from at least three independent experiments.

maximum after 15 min (Fig. 7). Moreover, phosphorylation of c-Jun N-terminal kinase (JNK) and p38 was observed in LPS-treated cells (Fig. 7), suggesting that the three major MAPK pathways are activated by LPS in RAW264.7 cells.

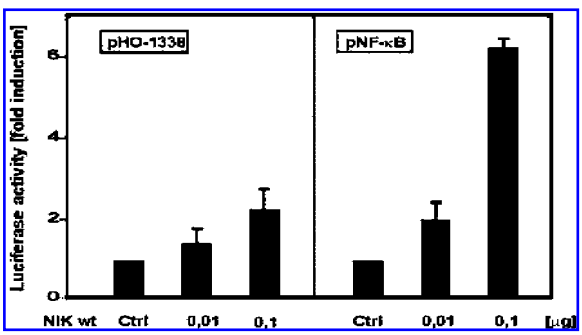

FIG. 5. Effect of overexpressed NIK on HO-1 promoter activity. RAW264.7 cells were cotransfected with pHO-1338 or pNF-κB and an expression vector for NIK at the indicated concentrations. Twenty-four hours after transfection, cell extracts were assayed for luciferase activity, and the rate of induction relative to the control was determined. Values are means ± SEM from at least three independent experiments.

Opposite regulatory roles of p38β and p38δ in the LPS-dependent regulation of HO-1 gene expression

To investigate the regulatory role of MAPKs in LPS-dependent regulation of HO-1 gene expression, we applied pharmacological MAPK inhibitors. Pretreatment for 30 min with the p38 MAPK inhibitor SB203580 strongly reduced LPS-dependent induction of HO-1 promoter activity (Fig. 8A). By contrast, pretreatment with the ERK inhibitor PD98059 and the JNK inhibitor SP600125 did not affect LPS-dependent induction of HO-1 gene expression (data not shown). To elucidate further the role of p38 for LPS-dependent HO-1 gene induction, we examined the effect of overexpression of dominant negative p38 α, β, γ, and δ isoforms. Overexpressed dominant negative p38β inhibited, whereas dominant negative p38δ increased, LPS-dependent induction of HO-1 promoter activity (Fig. 8B). The effects of overexpressed dominant negative p38β and p38δ were confirmed for the endogenous HO-1 gene expression in RAW264.7 cells (Fig. 8C), indicating that p38β and p38δ have opposite regulatory roles in the LPS-dependent induction of HO-1 gene expression.

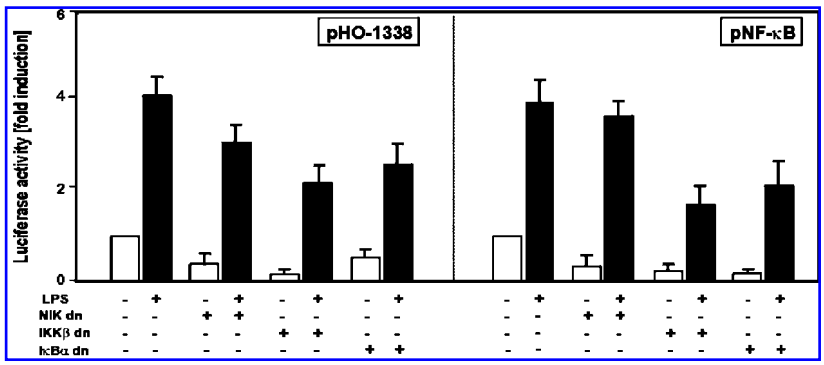

FIG. 6. Inhibition of HO-1 promoter activity by overexpressed dominant negative NIK, IKKβ, and IκBα. Cells were cotransfected with reporter gene constructs pHO-1338 or pNF-κB and expression vectors with dominant negative (dn) forms of NIK, IKKβ, and IκBα as indicated. Twenty-four hours after transfection, cells were treated with LPS (1 μg/ml) or control medium for another 18 h. Cell extracts were assayed for luciferase activity, and the rate of induction relative to the control was determined. Values are means ± SEM from at least three independent experiments.

DISCUSSION

In the present study, we demonstrate that the LPS-dependent induction of HO-1 gene expression is mediated via a CRE/AP-1 element of the proximal promoter region of the rat HO-1 gene in monocytic cells. Moreover, it is shown that the NF-κB and p38 signaling pathways play a major regulatory role in HO-1 gene induction in response to LPS.

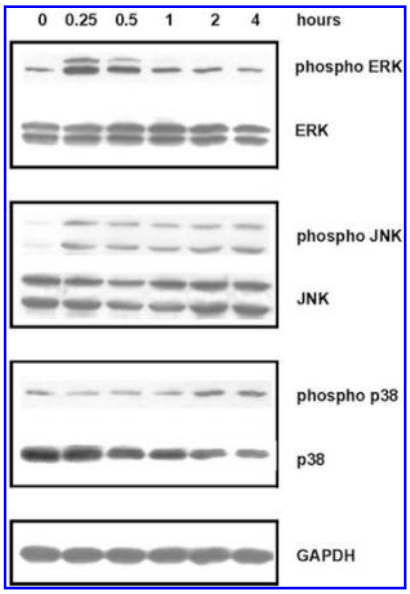

FIG. 7. Activation of MAPKs by LPS in RAW264.7 cells. RAW264.7 cells were cultured as described in Materials and Methods and were treated with LPS (1 μg/ml) for the times indicated. Total protein (60 μg) was analyzed by western blot analysis with polyclonal antibodies for the indicated MAPKs. Filters were used to detect phosphorylated MAPKs and then stripped and probed with antibodies against total MAPKs and GAPDH as a control. Similar results were obtained in three independent experiments. Autoradiograms of a representative experiment are shown.

Transcriptional induction of HO-1 gene expression by LPS in monocytic cells

The present study demonstrates that treatment with LPS leads to an induction of the endogenous HO-1 gene expression in RAW264.7 cell cultures (Fig. 1), which agrees with observations of previous reports (3, 11, 12). Two distal enhancer regions localized at −4.0 kb and −10.0 kb of the mouse HO-1 gene promoter 5′-flanking region have been demonstrated to mediate the LPS-dependent induction in RAW264.7 cells (3). These distal enhancer regions of the mouse gene promoter contain AREs, which are the nuclear target sequences of the TF Nrf2 (2). By contrast, in the present report, we have identified functional DNA sequences of the proximal rat HO-1 gene promoter that are different from AREs and confer LPS responsiveness to luciferase reporter gene constructs. By deletion and mutation analysis, it is demonstrated that a previously characterized CRE/AP-1 element (−668/−654) (13, 16) appears to play a major role in the LPS-dependent up-regulation of HO-1 gene expression. For a comparison, a reporter gene construct with three copies of the HO-1 StRE was up-regulated to a minor degree by LPS (Fig. 2). A putative NF-κB site (−1,002/−994) was not involved in the transcriptional regulation of HO-1 gene expression as judged by transfection experiments, because deletion of this element did not alter the LPS responsiveness of luciferase constructs (Fig. 3A). Interestingly, it has previously been demonstrated that an NF-κB site could be involved in the heme-dependent transcriptional regulation of the human HO-1 gene in an erythroid cell line (17) and that age-dependent induction of HO-1 gene expression in liver was mediated by NF-κB (18).

As the TF USF has been shown to mediate the up-regulation of the alcohol dehydrogenase gene expression by LPS in liver (25, 26) and LPS induces the occupation of an E-box motif of the murine inducible nitric oxide synthase gene (7), we hypothesized that the proximal E-box motif of the HO-1 gene promoter could be involved in the LPS-dependent induction of HO-1 gene expression. The present data, however, suggest that the E-box of the rat HO-1 gene promoter does not play a major role in the transcriptional induction of HO-1 gene expression

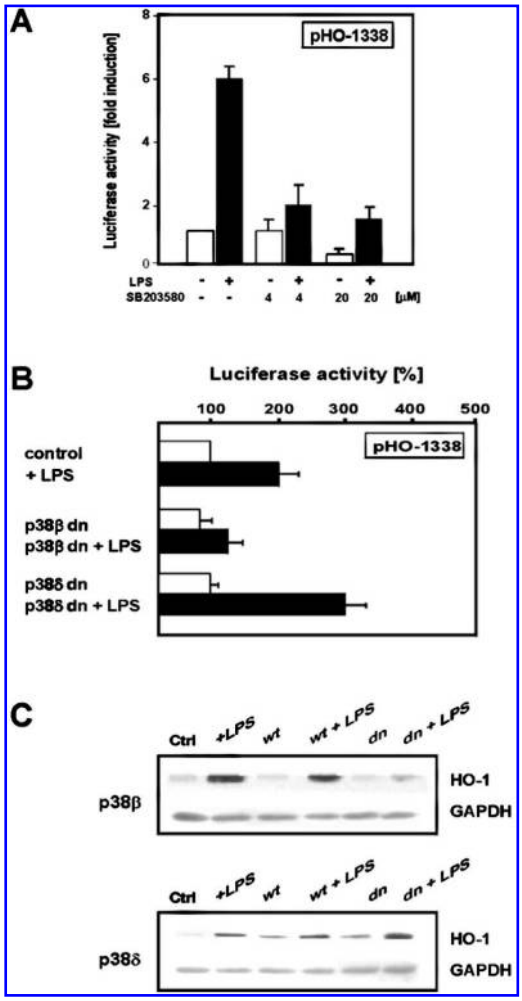

FIG. 8. Involvement of p38 MAPK in the regulation of HO-1 promoter activity by LPS. (**A**) RAW264.7 cells were transiently transfected with reporter gene construct pHO-1338. Twenty-four hours after transfection, cells were pretreated with the p38 MAPK inhibitor SB203580 (4 and 20 μ*M*) for 30 min, after which cells were incubated for another 18 h in the absence or presence of LPS (1 μg/ml) as indicated. Cell extracts were assayed for luciferase activity, and the rate of induction relative to the control was determined. Values are means ± SEM from at least three independent experiments. (**B**) RAW264.7 cells were cotransfected with expression vectors for dominant negative (dn) p38β, p38δ, or empty expression vector and reporter gene construct pHO-1338. Twenty-four hours after transfection, cells were incubated for 18 h in the absence or presence of LPS (1 μg/ml), cell extracts were assayed for luciferase activity, and the rate of induction relative to the control was determined. Values are means ± SEM from at least three independent experiments. (**C**) Endogenous HO-1 protein levels were detected in RAW264.7 cells that were transfected with expression vectors of wild-type (wt) and dominant negative (dn) p38β or p38δ. Twenty-four hours after transfection, cells were treated with LPS (1 μg/ml) as indicated. Total protein (60 μg) was subjected to western blot analysis and detected with polyclonal antibodies against rat HO-1 or GAPDH as a loading control. Similar results were obtained in three independent experiments, and autoradiograms of a representative experiment are shown.

in monocytic cells. Interestingly, the group of Shibahara has previously shown that USF is involved in the TPA-dependent induction of the human HO-1 gene in the myelomonocytic cell line THP-1 (20). Deletion experiments also suggested that additional DNA sequences of the proximal 347 bp of the rat HO-1 gene promoter are involved in LPS-dependent regulation of HO-1 gene expression (Fig. 3A).

Signal transduction pathways that mediate LPS-dependent induction of HO-1 gene expression

The cytokine TNFα has previously been shown to be a principal mediator of the LPS-dependent induction of HO-1 gene expression in liver possibly via an autocrine mechanism (8, 21). Our results, however, indicate that TNFα is not involved in the LPS-dependent induction of HO-1 in RAW264.7 monocytic cells (Fig. 1C). The data rather suggest that induction of HO-1 gene expression is directly mediated via activation of LPS-dependent signaling cascades. At least two distinct pathways are involved in the regulation of HO-1 gene expression by LPS: the NIK/IKKβ/NF-κB signaling pathway and the p38 MAPK pathway. The inhibition of LPS-dependent induction of HO-1 promoter activity by pharmacological NF-κB inhibitors (Fig. 4) and dominant negative forms of kinases of the NF-κB signaling pathway (Fig. 6) strongly suggest a major regulatory role for this signaling cascade. It is highly likely, however, that additional pathways are involved in LPS-dependent induction of HO-1 gene expression (for a review, see 8). For example, it has been shown that LPS-dependent induction of NF-κB can be mediated via transforming growth factor-β activated kinase-1 (14). The present data on the effect of the NF-κB inhibitor CAPE (Fig. 4A) contradict a previous report in which CAPE has been demonstrated to induce the expression of the endogenous HO-1 gene in an astrocyte cell line (27). These conflicting observations may be explained by cell-specific differences between astrocytes and RAW264.7 macrophages. Our data also indicate that activation of the p38 MAPK signaling pathway is involved in the regulation of HO-1 gene expression in RAW264.7 cells (Fig. 8). Surprisingly, overexpression of dominant negative forms of p38β and p38δ had opposite effects on the LPS-dependent induction of HO-1 gene expression in RAW264.7 cells (Fig. 8). Whether the NF-κB and p38 signaling pathways interact in the regulation of HO-1 gene expression cannot be concluded from our data. Interestingly, Carter and colleagues have previously demonstrated that p38-dependent phosphorylation of the TF TATA-binding protein by LPS is necessary for the activation of NF-κB-dependent gene regulation (4). Moreover, a complex interplay of upstream signaling cascades has been demonstrated to be involved in the NF-κB-dependent gene regulation of the monocyte chemoattractant protein-1 gene (6). Clearly, further studies are necessary to elucidate the interaction of these signaling cascades in the gene regulation of HO-1 gene expression.

As LPS-dependent activation of monocytic cells plays a crucial role in the pathogenesis of sepsis, the present data have important physiological and therapeutic implications. The modification of HO-1 gene expression in monocytic cells via

targeted regulation of signaling cascades may ultimately lead to the development of novel therapeutic strategies.

ACKNOWLEDGMENTS

We wish to thank Evelyn Welzel and Dennis Brusius for excellent technical assistance. This study was supported by the Deutsche Forschungsgemeinschaft SFB 402 A1 and GRK335 (to T.K.) and SFB 402 A8 and GRK335 (to S.I.).

ABBREVIATIONS

AP-1, activator protein-1; ARE, antioxidant response element; CAPE, caffeic acid phenethyl ester; CRE, cyclic AMP response element; ERK, extracellular signal-regulated kinase; GAPDH; glyceraldehyde-3-phosphate dehydrogenase; HO, heme oxygenase; IκB, inhibitor of NF-κB; IKKβ, IκB kinase β; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; NIK, NF-κB-inducing kinase; RE, regulatory element; StRE, stress RE; TF, transcription factor; TLCK, Nap-tosyl-L-lysine chloromethyl ketone; TNFα, tumor necrosis factor α; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; USF, upstream stimulatory factor.

REFERENCES

1. Abraham NG, Lavrovsky Y, Schwartzman ML, Stoltz RA, Levere RD, Gerritsen ME, Shibahara S, and Kappas A. Transfection of the human heme oxygenase gene into rabbit coronary microvessel endothelial cells: protective effect against heme and hemoglobin toxicity. *Proc Natl Acad Sci U S A* 92: 6798–6802, 1995.
2. Alam J, Stewart D, Touchard C, Boinapally S, Choi AM, and Cook JL. Nrf2, a cap'n'collar transcription factor, regulates induction of the heme oxygenase-1 gene. *J Biol Chem* 274: 26071–26078, 1999.
3. Camhi SL, Alam J, Wiegand GW, Chin BY, and Choi AM. Transcriptional activation of the HO-1 gene by lipopolysaccharide is mediated by 5′-distal enhancers–role of reactive oxygen intermediates and AP-1. *Am J Resp Cell Mol Biol* 18: 226–234, 1998.
4. Carter AB, Knudtson KL, Monick MM, and Hunninghake GW. The p38 mitogen-activated protein kinase is required for NF-kappaB-dependent gene expression. The role of TATA-binding protein (TBP). *J Biol Chem* 274: 30858–30863, 1999.
5. Foresti R and Motterlini R. The heme oxygenase pathway and its interaction with nitric oxide in the control of cellular homeostasis. *Free Radic Res* 31: 459–475, 1999.
6. Goebeler M, Gillitzer R, Kilian K, Utzel K, Brocker EB, Rapp UR, and Ludwig S. Multiple signaling pathways regulate NF-kappaB-dependent transcription of the monocyte chemoattractant protein-1 gene in primary endothelial cells. *Blood* 97: 46–55, 2001.
7. Goldring CE, Reveneau S, Chantome A, Pance A, Fleury C, Hume DA, Sester D, Mignotte B, and Jeannin JF. Heat shock enhances transcriptional activation of the murine-inducible nitric oxide synthase gene. *FASEB J* 14: 2393–2395, 2000.
8. Guha M and Mackman N. LPS induction of gene expression in human monocytes. *Cell Signal* 13: 85–94, 2001.
9. Hess A, Wijayanti N, Neuschafer-Rube AP, Katz N, Kietzmann T, and Immenschuh S. Phorbol ester-dependent activation of peroxiredoxin I gene expression via a protein kinase C, Ras, p38 mitogen-activated protein kinase signaling pathway. *J Biol Chem* 278: 45419–45434, 2003.
10. Immenschuh S and Ramadori G. Gene regulation of heme oxygenase-1 as a therapeutic target. *Biochem Pharmacol* 60: 1121–1128, 2000.
11. Immenschuh S, Stritzke J, Iwahara S, and Ramadori G. Up-regulation of heme-binding protein 23 (HBP23) gene expression by lipopolysaccharide is mediated via a nitric oxide-dependent signaling pathway in rat Kupffer cells. *Hepatology* 30: 118–127, 1999.
12. Immenschuh S, Tan M, and Ramadori G. Nitric oxide mediates the lipopolysaccharide dependent upregulation of the heme oxygenase-1 gene expression in cultured rat Kupffer cells. *J Hepatol* 30: 61–69, 1999.
13. Immenschuh S, Hinke V, Katz N, and Kietzmann T. Transcriptional induction of the heme oxygenase-1 gene expression by okadaic acid in primary rat hepatocyte cultures. *Mol Pharmacol* 57: 610–618, 2000.
14. Irie T, Muta T, and Takeshige K. TAK1 mediates an activation signal from toll-like receptor(s) to nuclear factor-kappaB in lipopolysaccharide-stimulated macrophages. *FEBS Lett* 467: 160–164, 2000.
15. Karin M and Ben-Neriah Y. Phosphorylation meets ubiquitination: the control of NF-κB activity. *Annu Rev Immunol* 18: 621–663, 2000.
16. Kietzmann T, Samoylenko A, and Immenschuh S. Transcriptional regulation of heme oxygenase-1 gene expression by MAP kinases of the JNK and p38 pathways in primary cultures of rat hepatocytes. *J Biol Chem* 278: 17927–17936, 2003.
17. Lavrovsky Y, Schwartzman ML, Levere RD, Kappas A, and Abraham NG. Identification of binding sites for transcription factors NF-κB and AP-2 in the promoter region of the human heme oxygenase 1 gene. *Proc Natl Acad Sci U S A* 91: 5987–5991, 1994.
18. Lavrovsky Y, Song CS, Chatterjee B, and Roy AK. Age-dependent increase of heme oxygenase-1 gene expression in the liver mediated by NF-κB. *Mech Ageing Dev* 114: 49–60, 2000.
19. Maines MD. The heme oxygenase system: a regulator of second messenger gases. *Annu Rev Pharmacol Toxicol* 37: 517–554, 1997.
20. Muraosa Y and Shibahara S. Identification of a cis-regulatory element and putative trans-acting factors responsible for 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-mediated induction of heme oxygenase expression in myelomonocytic cell lines. *Mol Cell Biol* 13:7881–7991, 1993.
21. Oguro T, Takahashi Y, Ashino T, Takaki A, Shioda S, Horai R, Asano M, Sekikawa K, Iwakura Y, and Yoshida T. Involvement of tumor necrosis factor alpha, rather than

interleukin-1alpha/beta or nitric oxides in the heme oxygenase-1 gene expression by lipopolysaccharide in the mouse liver. *FEBS Lett* 516: 63–66, 2002.
22. Otterbein LE and Choi AM. Heme oxygenase: colors of defense against cellular stress. *Am J Physiol Lung Cell Mol Physiol* 279: L1029–L1037, 2000.
23. Poss KD and Tonegawa S. Heme oxygenase is required for mammalian iron reutilization. *Proc Natl Acad Sci U S A* 94: 10919–10924, 1997.
24. Poss KD and Tonegawa S. Reduced stress defense in heme oxygenase 1-deficient cells. *Proc Natl Acad Sci U S A* 94: 10925–10930, 1997.
25. Potter JJ, Cheneval D, Dang CV, Resar LM, Mezey E, and Yang VW. The upstream stimulatory factor binds to and activates the promoter of the rat class I alcohol dehydrogenase gene. *J Biol Chem* 266: 15457–15463, 1991.
26. Potter JJ, Rennie-Tankersley L, and Mezey E. Endotoxin enhances liver alcohol dehydrogenase by action through upstream stimulatory factor but not by nuclear factor-kappa B. *J Biol Chem* 278: 4353–4357, 2003.
27. Scapagnini G, Foresti R, Calabrese V, Giuffrida Stella AM, Green CJ, and Motterlini R. Caffeic acid phenethyl ester and curcumin: a novel class of heme oxygenase-1 inducers. *Mol Pharmacol* 61: 554–561, 2002.
28. Sweet MJ and Hume DA. Endotoxin signal transduction in macrophages. *J Leukoc Biol* 60: 8–26, 1996.
29. Tenhunen R, Marver HS, and Schmid R. The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase. *Proc Natl Acad Sci U S A* 61: 748–755, 1968.
30. Ulevitch RJ and Tobias PS. Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. *Annu Rev Immunol* 13: 437–457, 1995.
31. Yachie A, Niida Y, Wada T, Igarashi N, Kaneda H, Toma T, Ohta K, Kasahara Y, and Koizumi S. Oxidative stress causes enhanced endothelial cell injury in human heme oxygenase-1 deficiency. *J Clin Invest* 103: 129–135, 1999.

Address reprint requests to:
Stephan Immenschuh
Institut für Klinische Chemie und Pathobiochemie
Justus-Liebig-Universität Giessen
Gaffkystrasse 11
35392 Giessen, Germany

E-mail: Stephan.Immenschuh@klinchemie.med.uni-giessen.de

Received for publication May 20, 2004; accepted June 13, 2004.

This article has been cited by:

1. Hwa Jin Jang , Young Min Kim , Konstantin Tsoyi , Eun Jung Park , Young Soo Lee , Hye Jung Kim , Jae Heun Lee , Yeonsoo Joe , Hun Taeg Chung , Ki Churl Chang . 2012. Ethyl Pyruvate Induces Heme Oxygenase-1 Through p38 Mitogen-Activated Protein Kinase Activation by Depletion of Glutathione in RAW 264.7 Cells and Improves Survival in Septic Animals. *Antioxidants & Redox Signaling* **17**:6, 878-889. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links] [Supplemental material]
2. Jeremy L. Herrmann , Troy A. Markel , Aaron M. Abarbanell , Brent R. Weil , Meijing Wang , Yue Wang , Jiangning Tan , Daniel R. Meldrum . 2009. Proinflammatory Stem Cell Signaling in Cardiac Ischemia. *Antioxidants & Redox Signaling* **11**:8, 1883-1896. [Abstract] [Full Text PDF] [Full Text PDF with Links]
3. Prof. Mahin D. Maines . 2005. The Heme Oxygenase System: Update 2005. *Antioxidants & Redox Signaling* **7**:11-12, 1761-1766. [Citation] [Full Text PDF] [Full Text PDF with Links]
4. Auemduan Prawan , Joydeb K. Kundu , Young-Joon Surh . 2005. Molecular Basis of Heme Oxygenase-1 Induction: Implications for Chemoprevention and Chemoprotection. *Antioxidants & Redox Signaling* **7**:11-12, 1688-1703. [Abstract] [Full Text PDF] [Full Text PDF with Links]