An Atypical NF-κB-Regulated Pathway Mediates Phorbol Ester-Dependent Heme Oxygenase-1 Gene Activation in Monocytes

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An Atypical NF-κB-Regulated Pathway Mediates Phorbol Ester-Dependent Heme Oxygenase-1 Gene Activation in Monocytes

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Heme oxygenase (HO)-1 catalyzes the rate-limiting step of heme degradation and plays an important anti-inflammatory role via its enzymatic products carbon monoxide and biliverdin. In this study it is reported that the HO-1 gene is transcriptionally induced by the phorbol ester PMA in cell cultures of monocytic cells with a regulatory pattern that is different from that of LPS-dependent HO-1 induction in these cells. Activation of HO-1 by PMA was mediated via a newly identified κB element of the proximal rat HO-1 gene promoter region (−284 to −275). This HO-κB element was a nuclear target for the NF-κB subunit p65/RelA as determined by nuclear binding assays and transfection experiments with luciferase reporter gene constructs in RAW264.7 monocytes. Moreover, PMA-dependent induction of endogenous HO-1 gene expression and promoter activity was abrogated in embryonic fibroblasts from p65−/− mice. PMA-dependent HO-1 gene activation was reduced by an overexpressed dominant negative mutant of IκBα, but not by dominant negative IκB kinase-2, suggesting that the classical NF-κB pathway was not involved in this regulation. The antioxidant N-acetylcysteine and inhibitors of p38 MAPK or serine/threonine kinase CK2 blocked PMA-dependent HO-1 gene activation. Finally, it is demonstrated by luciferase assays with a Gal4-CHOP fusion protein that the activation of p38 MAPK by PMA was independent of CK2. Taken together, induction of HO-1 gene expression by PMA is regulated via an IκB kinase-independent, atypical NF-κB pathway that is mediated via the activation of p38 MAPK and CK2. The Journal of Immunology, 2008, 181: 4113–4123.

Heme oxygenase (HO)-1 is the first and the rate-limiting enzyme of heme degradation (1). The catalytic cleavage of the prooxidant heme by HO produces iron, biliverdin, and carbon monoxide (2). Biliverdin is converted into the potent antioxidant bilirubin (3) via biliverdin reductase (4), and HO-derived carbon monoxide plays an important physiological role as a signaling gas (5, 6). HO-1 is highly inducible by a variety of oxidative stress stimuli and has been known for many years to provide antioxidant cellular protection (6). More recently, HO-1 knockout mice and a human case of genetic HO-1 deficiency have been shown to exhibit phenotypical alterations of chronic inflammation (7, 8). Furthermore, HO-1−/− mice were highly susceptible to the toxicity of the proinflammatory mediator LPS (7, 9), and induction of HO-1 expression, either by gene transfer or by pharmacological stimulation, has emerged to be of potential therapeutic use for the treatment of inflammatory diseases in animal models (10–15).

HO-1 is regulated primarily at the level of transcription (6, 16). An array of cis-acting regulatory elements (RE), which are targeted by transcription factors (TF) such as NF-E2-related factor 2 (Nrf2), AP-1, or USF-2, have been identified in the promoter regions of avian and mammalian HO-1 genes and are involved in HO-1 regulation (6, 17). Although the TF NF-κB, which provides cytoprotection against oxidative stress (18), has been shown to be activated by various stimuli that are also known to up-regulate HO-1 gene expression such as curcumin (19), LPS (20), or dietary polyphenols (21), the regulatory role of NF-κB for HO-1 gene regulation is discussed controversially (17, 22). Moreover, a functional κB site of the HO-1 promoter, which is the direct target of this TF, has not been identified to date. Thus, the goal of the present study was to investigate the regulation of HO-1 by the phorbol ester PMA, which is a prototypical activator of NF-κB in monocytic cells and a potent inducer of protein kinase C (PKC) (23).

In this article it is reported that PMA induces HO-1 gene expression in monocytes. This up-regulation is mediated via a newly identified κB element of the rat HO-1 proximal promoter that is a target of the NF-κB subunit p65/RelA. An atypical IκB kinase (IKK)-independent NF-κB pathway, which requires the activation of p38 MAPK and CK2, is involved in PMA-dependent induction of HO-1 gene expression in monocytes.

Materials and Methods

Materials

DMEM, RPMI 1640, and MEM were obtained from PAA Laboratories, FBS was from Biochrom, Ficoll-Paque was from Pharmacia, CD14-
immunomagnetic microbeads were from Miltenyi Biotec, and polyvinylidi- 

dene difluoride membranes were from Millipore. All other chemicals were 
purchased from Sigma-Aldrich and Roche Applied Science unless other-
wise indicated.

Cell isolation and culture

Liver tissue macrophages (LTM), peritoneal macrophages (24), rat hepa-
tocytes (25) and human PBMC were isolated and cell culture was main-
tained in culture as described previously (26). RAW264.7 cells were from 
American Type Culture Collection, mouse embryonic fibroblasts (MEF) 
from p65−/− mice were from Dr. H. Nakano (Department of Immunology, 
Jutendo University School of Medicine, Tokyo, Japan) (27) and were 
grown in DME supplemented with 10% FBS, 100 U/ml penicillin, and 
100 μg/ml streptomycin. All cell cultures were kept under air/CO2 (19:1) 
at 100% humidity. Treatment of cells with PMA (0.5 μM) and LPS (Esher-
ichia coli 0111:B4; 1 μg/ml) was performed with serum-free medium. 
Specific inhibitors of CK2, apigenin, 5,6-dichloro-1-β-D-ribofuranosylben-
zimidazole (RFD) (Calbiochem), rabbit IgG HRP and anti-mouse IgG HRP (DPC Biermann) and were recommended by the manufacturers. Secondary Abs were goat anti-

Abs for the detection of phosphorylated IκBα (Cell Signaling) and 5,6-
dichloro-1-β-D-ribosylbenzimidazole (RFD) (Calbiochem), and N-acetylcysteine (NAC) were added to the culture medium 30 min to 1 h before treatment 
with PMA, as indicated.

RNA isolation, Northern blot analysis, and hybridization

Total RNA isolation and Northern blot analysis were essentially per-
duced as described previously and, as a probe for hybridization, the 
cDNA of rat HO-1 and a 28S ribosomal RNA oligonucleotide was applied 
(28).

Western blot analysis

Cells were washed with 0.9% NaCl and thereafter lysis was performed 
as described (29). The lysate was centrifuged for 5 min at 13,000 × g 
at 4°C and the protein concentration in the supernatant was determined 
by the BCA (bicinchoninic acid) protein assay kit (Pierce). Fifty mi-
gals of protein were applied (28).

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gals of protein were applied (28).

Preparation of nuclear extracts (NE) and EMSA

NE were prepared as described previously (29). The sequences of the 
biont-labeled oligonucleotides (MWG-Biotech) used for the EMSA are 
as follows: HO-κB-B, (5’-CTTAGTTCGAGACCATTCCAGATTTCT 
GA-A3’), HO-κB-Bmut (5’-CTTAGTTCGAGACATCTCCAGATTTCT 
GA-A3’), HO-κB-Bmut (5’-CTTAGTTCGAGACATCTCCAGATTTCT 
GA-A3’), and NF-κB consensus oligonucleotide with sequence 
(5’-AGTGGGAGGGATCTCCAGACC-G3’) with respective 
oligonucleotides of the noncoding strand. For competition assays, 
excess of unlabelled oligonucleotide was added as indicated. After pre-
incubation for 10 min at room temperature, the biotin-labeled probe was 
added and incubation was continued for another 20 min. For supershift 
analysis, 3 μl of an Ab directed against the NF-
κB p65 subunit (Cell Signaling) was added to the EMSA reaction. The reaction mixture was 
loaded on a 6% native polyacrylamide gel in 0.5% Tris-borate-EDTA 
and blotted onto nylon membranes (Pierce). After UV-cross-linking, the 
LightShift chemiluminescent EMSA kit (Pierce) was used to detect in-
teraction between the biotin end-labeled DNA and the protein with a 
 streptavidin-HRP conjugate and the chemiluminescent substrate.

Results

PMA induces endogenous HO-1 gene expression in 
mononuclear phagocytes

To investigate the regulation of HO-1 gene expression by the 
phorbol ester PMA in cell cultures of the monocytic cell line 
RAW264.7, we determined mRNA levels of HO-1 after exposure 
to PMA. For a comparison, the effect of PMA on HO-1 

mRNA expression was also determined in primary rat LTM, 
peritoneal macrophages, and hepatocytes. PMA induced HO-1 
mRNA levels in RAW264.7 cells and to a similar extent also in 
primary LTM (Fig. 1A) and peritoneal macrophages (data not shown), 
but not in hepatocytes (Fig. 1A). Subsequently, we ex-

amine the effect of PMA on the regulation of endogenous 
HO-1 protein expression in cell cultures of RAW264.7 cells and 
LTM. Similarly as for the regulation of HO-1 mRNA levels 
in response to PMA, HO-1 protein expression was markedly in-
duced by this treatment (Fig. 1B). It is also remarkable, that 
PMA-dependent induction of HO-1 gene expression was ob-
served in human PBMC (Fig. 1B). The data indicate that PMA 
induced HO-1 gene expression in the monocytic cell line 
RAW264.7 and in various primary monocytic cells, but not in 

hepatocytes.

The time course of HO-1 gene induction by PMA is 
regulated in part by LPS in RAW264.7 cells

The proinflammatory mediator LPS has previously been shown 
to be a potent inducer of HO-1 (32). To further investigate the 
regulatory mechanisms(s) of PMA-dependent induction of HO-1 
in monocytic cells, we compared the time course of HO-1 protein 
expression by PMA with that elicited by LPS. Treatment 
with LPS activated HO-1 gene expression in a time-
dependent manner, whereas in the presence of PMA, 
the time course of HO-1 gene induction was 

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dependent manner, whereas in the presence of PMA, 
the time course of HO-1 gene induction was 

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with PMA induced HO-1 gene expression in RAW264.7 cells in a time-dependent manner with a maximum after 6 h that persisted up to 18 h, whereas LPS-dependent induction of HO-1 was retarded (Fig. 1C). Moreover, we have also determined the combined effect of PMA and LPS on HO-1 gene expression. Simultaneous treatment with PMA and LPS induced HO-1 gene expression in an additive manner (Fig. 1D). The data suggest that various mechanisms regulate HO-1 gene expression in response to PMA and LPS in RAW264.7 cells.

Identification of a functional proximal κB-site of the rat HO-1 promoter that mediates PMA-dependent induction of HO-1 gene transcription

PMA is not only known to induce HO-1 gene expression (33), but it also activates the NF-κB pathway in monocytes (34). To further study whether HO-1 induction by PMA could be mediated via NF-κB, we searched for potential κB elements in the proximal promoter region (positions −1338 to +1) of the rat HO-1 gene. Two putative κB elements, HO-κB-A (−1002 to −993) and HO-κB-B (−284 to −275), were identified. Both elements matched the consensus sequence of the prototypical κB element in eight or nine of 10 nucleotides, respectively (Fig. 2A). In addition, a macrophage-specific 12-O-tetradecanoylphorbol-13-acetate-responsive element (MTE), which has previously been shown to mediate PMA-dependent induction of the human HO-1 gene (33), was identified in the proximal 5′-flanking sequence of the rat HO-1 gene promoter (−140 to −131) (Fig. 2A).

To further characterize the HO-1 gene activation in response to PMA, reporter gene constructs with serially 5′-deleted HO-1 promoter sequences were transiently transfected into...
RAW264.7 cells. Loss of the distal HO-κB-A element in pHO-754 and pHO-347 did not cause a major reduction of the PMA-induced luciferase activity. By contrast, deletion of the proximal HO-κB-B site and the MTE in pHO-20 abrogated the PMA-dependent induction, indicating a possible regulatory function of these two elements (Fig. 2B).

To assess the regulatory capacity of the HO-κB-B element and the MTE, pHO-347 reporter gene constructs, either with or without mutations of the HO-κB-B site and the MTE, were transfected into RAW264.7 cells. Targeted mutations of the HO-κB-B site led to a marked reduction of PMA-dependent induction. By contrast, point mutations of the HO-κB-B sequence did not have a major effect on PMA-induced luciferase activity when compared with the wild-type construct (Fig. 2C). It is also important to note that targeted mutations within the HO-κB-A site of pHO-1338 did not affect PMA-dependent up-regulation of luciferase activity (data not shown). Taken together, the data suggest that the HO-κB-B site, but not the MTE, mediate PMA-dependent induction of rat HO-1 gene expression.

The HO-κB-B site is a target of NF-κB

In EMSA studies we examined the binding activity of nuclear proteins from RAW264.7 cells that were treated either with PMA or LPS to an oligonucleotide with the HO-κB-B site. NE from PMA-treated cells showed markedly stronger DNA-binding activity to the HO-κB-B oligonucleotide when compared with NE from control cells. By contrast, NE from cells that were treated with LPS only exhibited minor inducible DNA binding to the HO-κB-B site (Fig. 3A). The intensity of the band formed by the DNA-protein complex of HO-κB-B with NE from PMA-treated cells was decreased by an excess of unlabeled HO-κB-B oligonucleotide in a dose-dependent manner (Fig. 3B). Binding of NE to the HO-κB-B site was abolished by an excess of unlabeled oligonucleotides for HO-κB-B and NF-κB, respectively, but not by an excess of an oligonucleotide with a targeted mutation in the HO-κB-B site (Fig. 3C). Moreover, incubation of the binding reaction with an Ab against the NF-κB subunit p65 caused a reduction of DNA-protein complex formation (Fig. 3C), suggesting that the HO-κB-B site is a nuclear target for p65.

Overexpressed p65 induces HO-1 promoter activity

The NF-κB subunit p65, which is also termed RelA, is a member of the Rel family of proteins and is activated in response to a variety of stimuli (18). To investigate the functional regulatory role of p65 on HO-1 promoter activity, RAW264.7 cells were cotransfected with HO-1 reporter gene constructs and an expression vector for p65. Basal luciferase activity of the reporter gene constructs pHO-1338 and pHO-347, but not that of pHO-347κBmut with a targeted mutation of the HO-κB-B site, was markedly augmented by overexpressed p65. As a control, luciferase activity of a reporter gene plasmid with three copies of the prototypical κB site (pNF-κB) was induced by cotransfected p65 to a similar extent when compared with pHO-347 (Fig. 4). The data indicate that p65-dependent HO-1 activation is mediated via the proximal HO-κB-B site of the rat HO-1 gene promoter.

HO-1 is not induced by PMA in p65−/− MEF

To substantiate the involvement of NF-κB and its subunit p65 in PMA-dependent HO-1 induction, we examined the HO-1 gene expression in p65−/− and p65+/+ MEF. We found that pMA was not
PMA-induced HO-1 expression in wild-type p65

The classical activation pathway of NF-κB by proinflammatory stimuli such as LPS and TNF-α is mediated via IKK-dependent phosphorylation of IκBα at serine 32 and serine 36. Phosphorylation of these regulatory serine residues leads to proteolysis of cytosolic IκBα via the proteasome, after which p65/RelA is translocated into the nucleus (18, 35). To investigate whether IκBα may be involved in PMA-dependent induction of HO-1, we determined the effect of overexpressed dominant negative IκBα on the level of PMA-dependent HO-1 promoter induction. As demonstrated in Fig. 6A, dominant negative IκBα markedly inhibited up-regulation of HO-1 promoter activity by PMA. Moreover, PMA-dependent induction of the control plasmid pTNF-585, which is known to be regulated via functional κB elements, was inhibited by dominant negative IκBα to a similar extent.

To investigate the potential role of IKK2 for PMA-dependent induction of HO-1 gene expression, we also determined the effect of an overexpressed dominant negative mutant of IKK2 on HO-1 promoter activity. As shown in Fig. 6B, dominant negative IKK2 did not have an inhibitory effect on PMA-dependent up-regulation of luciferase activity of pHO-1338, but markedly reduced PMA-dependent induction of the control reporter gene construct pTNF-585. No regulatory effect on PMA-dependent HO-1 promoter regulation was observed for the specific pharmacological IKK2 inhibitor SC-514 (data not shown).

To determine the PMA-dependent activation of IκBα, RAW264.7 cells were cotransfected with luciferase reporter gene constructs pHO-1338, pHO-347, pHO-347 xBmut, pNF-κB, and an expression vector for wild-type p65 or empty control expression vector (ev). Twenty-four hours after transfection, luciferase assay and quantitation were performed as described in Fig. 2. Values are means ± SEM from at least three or four independent experiments with duplicates of each point. Student’s t test for paired values: *, significant differences p65 vs empty vector; **, p65 plus pHO-347 xBmut vs p65 plus pHO-347, p < 0.05.

Figure 4. Effect of overexpressed p65 on HO-1 promoter activity. RAW264.7 cells were cotransfected with luciferase reporter gene constructs pHO-1338, pHO-347, pHO-347 xBmut, pNF-κB, and an expression vector for wild-type p65 or empty control expression vector (ev). Twenty-four hours after transfection, luciferase assay and quantitation were performed as described in Fig. 2. Values are means ± SEM from at least three or four independent experiments with duplicates of each point. Student’s t test for paired values: *, significant differences p65 vs empty vector; **, p65 plus pHO-347 xBmut vs p65 plus pHO-347, p < 0.05.

Figure 3. Binding of nuclear proteins to the HO-κB-B site. A, A biotin-labeled oligonucleotide with the HO-κB-B element was incubated with 7 μg of NE from control cells or from cells treated with PMA (0.5 μM), LPS (1 μg/ml), or without NE as a free probe. B, For competition analyses the biotin-labeled HO-κB-B oligonucleotide was preincubated with 7 μg of NE from PMA-treated cells along with a 10-, 50- and 100-fold molar excess of unlabeled HO-κB-B oligonucleotide, as indicated. For supershift analysis, 3 or 5 μl of Ab directed against the NF-κB subunit p65 was preincubated with NE from PMA-treated cells along with a 50-fold molar excess of unlabeled HO-κB-B or HO-κB-Bmut or an oligonucleotide with the prototypical NF-κB site, as indicated. For supershift analysis, 3 or 5 μl of Ab directed against the NF-κB subunit p65 was preincubated with NE from PMA-treated cells along with a 50-fold molar excess of unlabeled HO-κB-B or HO-κB-Bmut or an oligonucleotide with the prototypical NF-κB site, as indicated. For supershift analysis, 3 or 5 μl of Ab directed against the NF-κB subunit p65 was preincubated with NE from PMA-treated cells along with a 50-fold molar excess of unlabeled HO-κB-B or HO-κB-Bmut or an oligonucleotide with the prototypical NF-κB site, as indicated.

PMA-dependent HO-1 gene activation requires IκBα, but not IKK2

The classical activation pathway of NF-κB by proinflammatory stimuli such as LPS and TNF-α is mediated via IKK-dependent phosphorylation of IκBα at serine 32 for up to 8 h. In contrast, PMA induced IκBα phosphorylation at serine 32 only to a minor extent (Fig. 6C, upper panel). Because it has previously been shown that IκBα can also be phosphorylated at tyrosine 42 by oxidative stress (36, 37), we also determined the phosphorylation of IκBα at this regulatory residue in response to PMA and LPS. Treatment with PMA caused a rapid and transient IκBα phosphorylation at tyrosine 42 after 15 min (second panel from top). In contrast, treatment with LPS caused a stronger and more persistent IκBα phosphorylation at tyrosine 42 as compared with PMA (Fig. 6C, second panel from top).
Taken together, the data suggest that PMA-dependent activation of HO-1 is mediated via a nonclassical NF-κB pathway that is independent of IKK2 activity. NAC attenuates HO-1 induction by PMA

PMA has previously been shown to up-regulate the generation of reactive oxygen species (ROS) in monocytes (38). To determine whether ROS as potential secondary messengers would be involved in HO-1 gene induction in our cell culture model of RAW264.7 cells, we examined the effect of the antioxidant NAC on PMA-dependent induction of HO-1. Pretreatment with NAC decreased PMA-dependent up-regulation of HO-1 in a dose-dependent manner (Fig. 7A). Moreover, we also determined the effect of NAC on the regulation of HO-1 promoter activity by PMA in RAW264.7 cells. Pretreatment with NAC significantly lowered PMA-induced promoter activity of the pH0-1338 reporter gene construct (Fig. 7B), suggesting that the induction of HO-1 gene expression by PMA is mediated via ROS.

p38 MAPK mediates PMA-dependent HO-1 gene induction

A major target of ROS in monocytes is p38 MAPK (39). Accordingly, phosphorylation of p38 was markedly induced by PMA in our model of RAW264.7 cells (Ref. 40 and data not shown). To investigate the potential role of p38 MAPK for PMA-dependent
up-regulation of HO-1 gene expression, we determined the effect of the pharmacological p38 inhibitor SB202190 on PMA-dependent induction of endogenous HO-1 gene expression and promoter activity. Pretreatment with SB202190 markedly decreased PMA-dependent up-regulation of HO-1 gene expression (Fig. 8A). We also examined the influence of this inhibitor on PMA-dependent HO-1 promoter activity. Pretreatment with SB202190 significantly attenuated the PMA-induced activity of the pHO-1338 reporter gene construct (Fig. 8B). Taken together, the data suggest that p38 MAPK is involved in PMA-dependent induction of HO-1 gene expression.

**CK2 is involved in PMA-dependent HO-1 gene induction**

CK2 is a stress-activated serine/threonine protein kinase (41, 42) that has previously been shown to be involved in IKK2-independent activation of NF-κB (43). To investigate the potential regulatory role of CK2 for PMA-dependent up-regulation of HO-1 gene expression, we determined the effect of two specific CK2 inhibitors, RFBD and apigenin, on PMA-dependent induction of endogenous HO-1 and promoter activity. Pretreatment with RFBD and apigenin markedly reduced the PMA-dependent up-regulation of HO-1 gene expression in a dose-dependent manner (Fig. 9, A and B). Similar to the observations for endogenous HO-1 gene regulation, pretreatment with RFBD or apigenin attenuated PMA-dependent induction of HO-1 promoter activity (Fig. 9C). Finally, we also evaluated the effect of cotransfection of an expression vector for CK2α on basal HO-1 promoter activity. Overexpressed CK2α markedly augmented the activity of the HO-1 promoter construct pHO-1338 and that of the control reporter gene plasmid pNF-κB (Fig. 9D). Thus, the data indicate that CK2 is involved in the regulation of HO-1 gene induction by PMA.

**CK2 is a downstream target of PMA-dependent p38 activation**

Activation of CK2 by various stress stimuli such as UV light has previously been shown to be regulated via p38 MAPK (43). To determine whether p38 MAPK is required for PMA-dependent CK2 activation, p38 MAPK activity was determined with a fusion plasmid containing the transactivation domain of the transcription factor CHOP and the DNA-binding domain of yeast Gal4 (pFA-CHOP). Transactivation via pFA-CHOP is specifically controlled...
by p38-dependent phosphorylation of two adjacent regulatory serine residues of the CHOP transactivation domain (44). Treatment with PMA strongly induced pFA-CHOP activity, and pretreatment with the p38 MAPK inhibitor SB202190 lowered PMA-dependent pFA-CHOP-mediated luciferase activity. By contrast, pretreatment with CK2 inhibitors had no effect on PMA-dependent, pFA-CHOP-mediated luciferase activity (Fig. 10), suggesting that p38 MAPK is an upstream kinase of PMA-dependent CK2 activation.

**Discussion**

Expression of HO-1 is up-regulated by multiple stress stimuli, and the enzymatic products of this reaction not only have antioxidant cytoprotective effects but also anti-inflammatory functions in various animal models (10–15). The present study demonstrates the following: 1) HO-1 is induced by PMA in monocytic cells with a regulatory pattern different from that by LPS; 2) HO-1 induction by PMA occurs on the transcriptional level and is mediated via a proximal NF-kB site of the rat HO-1 promoter that is a nuclear target of p65/RelA; and 3) an IKK-independent, atypical NF-kB pathway mediates PMA-dependent induction of HO-1 via activation of p38 MAPK and CK2.

**Transcriptional induction of HO-1 gene expression by PMA**

In the present report it is shown that HO-1 gene expression is induced by PMA in monocytic cells, but not in hepatocytes (Fig. 1A). These findings correspond with a previous report demonstrating that PMA induced HO-1 gene expression in a monocyte-specific manner in human myelomonocytic cells (33). The regulatory mechanism(s) that mediate(s) HO-1 gene induction by PMA appear(s) to be different from that by LPS, because HO-1 gene expression was up-regulated with a different time course by these two compounds (Fig. 1C). This assumption is also supported by the observation that simultaneous treatment with PMA and LPS induced HO-1 gene expression in an additive manner (Fig. 1D). Distinct kinetics of gene induction by PMA and LPS were also reported for cyclooxygenase-2 gene expression in monocyes (26, 45).
LPS may also correspond with the distinct binding of nuclear proteins to the HO-κB-B site (Fig. 3).

Identification of a functional κB site in the rat HO-1 gene promoter

Activation of the TF NF-κB is a major pathway for mediating cell survival during oxidative stress (18). Because multiple stress stimuli that are known to induce HO-1 gene expression also activate NF-κB (19–21), we hypothesized that the HO-1 promoter may be targeted by this TF. Two potential κB sites within the proximal rat HO-1 gene promoter have been identified that share high sequence identity with the prototypical NF-κB consensus sequence (5'-GGGRNNYYCC-3') (Fig. 24). Although two potential candidate κB elements were found, we demonstrate that PMA-dependent induction of HO-1 promoter activity was only mediated via the proximal HO-κB-B site. In addition, it is shown that this HO-κB-B element was a nuclear target for the NF-κB subunit p65/RelA (Figs. 2–4). Although NF-κB has been implicated in the transcriptional regulation of HO-1 gene expression (17), to our knowledge the rat HO-κB-B site is the first functional RE of the HO-1 gene that is directly targeted by NF-κB. In an earlier report on the human HO-1 gene promoter, a putative κB site has been shown to exhibit in vitro DNA-binding with the recombinant NF-κB subunit p50, but the functionality of this RE has not been examined (46). In other reports, the in vitro binding activity of nuclear proteins to synthetic NF-κB oligonucleotides, which were not necessarily found in the HO-1 gene promoter, have been correlated with the induction of HO-1 gene expression by various identical stimuli (17, 21, 47). It is conceivable that a functional HO-1 κB element may have been overlooked in earlier studies, because human and mouse HO-1 gene promoter regions, which have previously been studied in more detail, exhibit significant sequence differences when compared with the rat HO-1 gene promoter. Sequence alignment of the first 1338 bp of the promoter 5'-flanking region of the rat, mouse, and human HO-1 genes revealed only 47% (rat vs human), 49% (mouse vs human), and 69% (rat vs mouse) sequence similarity, respectively. Remarkably, the murine sequence corresponding to the functional rat HO-κB-B element did not contain a homologous κB sequence, which may suggest species-specific functionality of the HO-κB-B site. Independently, the rat sequence that corresponds with a previously identified PMA-responsive MTE of the human HO-1 gene promoter (33) was not functional in the context of the rat HO-1 gene promoter (Fig. 2). In conclusion, discrepancies of the promoter structure may explain species-specific differences of HO-1 gene regulation that have also been observed for HO-1 induction by hypoxia or heat shock (17, 48). The present study, however, does not exclude the possibility that PMA-dependent induction of HO-1 is regulated by TF other than NF-κB. In fact, the TF AP-1 and Nrf2 have also been shown to mediate PMA-dependent induction of the mouse HO-1 gene (49, 50).

Signaling pathway of PMA-dependent HO-1 gene induction

The classical NF-κB pathway is regulated via IKK-dependent phosphorylation of serine 32 and 36 in the N-terminal region of IκBα in response to a variety of stimuli such as LPS and TNF-α (18, 35). Accordingly, inhibition of PMA-dependent HO-1 promoter activation by overexpressed dominant negative IκBα, but not by dominant negative IKK2 (Fig. 6), indicated that the classical NF-κB pathway does not play a major role for this regulation. This conclusion is consistent with the observation that IκBα is phosphorylated to a minor extent at serine 32 in response to PMA rather than in response to LPS (Fig. 6C).

Furthermore, it has been proposed that ROS could be involved in the activation of atypical NF-κB regulatory pathways, because PMA could increase intracellular levels of ROS in mononuclear phagocytes (38) and affect the phosphorylation of IκBα at tyrosine 42 (36, 37). In line with this proposal, we showed in the present study that the action of PMA on HO-1 gene expression was abolished by treatment with the antioxidant NAC (Fig. 7), which indicates the involvement of ROS. We also examined whether PMA would affect phosphorylation of IκBα at tyrosine 42 and found a minor and transient level of IκBα phosphorylation at tyrosine 42 after treatment with PMA (Fig. 6C). This minor up-regulation of IκBα phosphorylation at tyrosine 42 did not correlate with the marked PMA-dependent induction of HO-1 gene expression (Fig. 1) and the inducible DNA binding of nuclear extracts to the HO-κB-B site in response to PMA (Fig. 3). Therefore, IκBα phosphorylation at tyrosine 42 does not seem to play a major role for PMA-dependent induction of HO-1 gene expression. These latter observations may correspond with a report, in which H2O2 has been demonstrated to stimulate NF-κB, but phosphorylation of IκBα at tyrosine 42 per se was not sufficient for NF-κB activation (36). Moreover, PMA as a prototypical activator of PKC, which mimics the intracellular effects of the endogenous mediator diacylglycerol (23), has recently been found to activate PKC in a p38 MAPK-dependent manner (51). This assumption would partially correspond with our data on PMA-dependent induction of HO-1 gene expression by NF-κB via ROS and a p38 MAPK/CK2-dependent pathway (Figs. 7–10). Thus, the present study strongly suggests that this signaling cascade is mediated via an atypical NF-κB pathway that involves phosphorylation of regulatory sites in the C-terminal domain of IκBα. A similar regulatory signaling cascade has previously been shown for the activation of NF-κB by UV light in HeLa cells (43).

Physiological significance of PMA-dependent HO-1 gene induction in monocytes

The findings of our present study, along with those of a previous report (33) that demonstrate a monocyte-specific induction of HO-1 gene expression by PMA, suggest an important physiological role of PKC-dependent HO-1 up-regulation in monocyte differentiation and/or activation. This assumption would correspond with a recent study in which it has been shown that the activation of PKC is essential for the differentiation of CD14+ monocytes into macrophages or dendritic cells (52). Moreover, the proinflammatory mediator LPS has recently been shown to exert its cellular effect via activation of PKC and NF-κB in macrophages, which may also involve the induction of HO-1 gene expression (53).

Inflammatory processes play a major role in the pathogenesis of cancer and cardiovascular disease. Evidence has accumulated that HO-1 has potent anti-inflammatory functions, because genetic HO-1 deficiency causes a chronic inflammatory phenotype and high vulnerability to LPS (7, 9). Anti-inflammatory protection via the induction of HO-1 has initially been described in a model of acute complement-dependent pleurisy (54). More recently, the potential clinical relevance of HO-1 has also been shown in various animal models of inflammatory diseases and organ transplantation, in which targeted overexpression of HO-1 provided efficient protection (10–15, 55). Finally, it is remarkable that HO-1 gene expression is not only regulated via NF-κB as demonstrated in the present report, but that HO-1 can modulate the activity of NF-κB in various cell types (56–58).
In conclusion, the present study defines a new regulatory mechanism of HO-1 gene expression by PMA in monocytic cells and may help to further understand the complexity of the gene regulation of this protective gene.

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Disclosures

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References