Signaling Pathways and Transcriptional Regulation of Antioxidant Genes Peroxiredoxin I and Heme Oxygenase-1 Gene activation in RAW264.7 Monocytes

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Signaling Pathways and Transcriptional Regulation of Antioxidant Genes Peroxiredoxin I and Heme Oxygenase-1 Gene activation in RAW264.7 Monocytes

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Declarations

"I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation."

"This dissertation is submitted as a cumulative thesis according to the guidelines provided by the PhD-program of Faculty of Veterinary Medicine and Medicine of the Justus-Liebig University. The thesis includes three original papers addressing one topic, two of which comprise the majority of my experimental work during the course of PhD. The third paper was a collaborative effort with a colleague (Nastiti Wijayanti)."

Srivatsava Naidu

Dedication

To

Sri Harsha Naidu

i. Abbreviations

AP-1	activator protein-1
ARE	antioxidant response element
bZIP	basic leucine zipper
Btk	bruton's tyrosine kinase
CK2	casein kinase 2
Cox-2	cyclooxygenase-2
DHE	dihydroethidium
EMSA	electrophoretic mobility shift assay
ERK	extracellular signal-regulated kinase
FCS	fetal calf serum
HO	heme oxygenase
HSF	heat-shock factor
IKK	IκB kinase
IκB	inhibitor of NF-κB
JNK	c-jun N-terminal kinase
LPS	lipopolysaccharide
MAPK	mitogen activated protein kinase
MEF	mouse embryonic fibroblast(s)
NF-κB	nuclear factor-κB
Nrf2	nuclear factor-erythroid-2 related factor 2
PBS	phosphate buffered saline
PKC	protein kinase C
PMA	phorbol myristate acetate
Prx	peroxiredoxin
RE	regulatory element
ROS	reactive oxygen species
StRE	stress response element
TF	transcription factor
TLR4	toll-like receptor-4
TNF-α	tumor necrosis factor-α

ii. List of Papers

I. Wijayanti, N., **S. Naidu**, T. Kietzmann and S. Immenschuh. 2008. Inhibition of phorbol ester-dependent peroxiredoxin I gene activation by lipopoly-saccharide via phosphorylation of RelA/p65 at serine 276 in monocytes. *Free Radic. Biol. Med.* 44: 699-710.

II. **Naidu, S**., N. Wijayanti, S. Santoso, T. Kietzmann and S. Immenschuh. 2008. An atypical NF-kappaB-regulated pathway mediates phorbol ester-dependent heme oxygenase-1 gene activation in monocytes. *J. Immunol*. 181: 4113-4123.

III. **Naidu, S**., V. Vijayan, S. Santoso, T. Kietzmann and S. Immenschuh. Inhibition and genetic deficiency of p38 MAPK up-regulates heme oxygenase-1 gene expression via NF-E2-related factor-2 (Nrf2) (Submitted).

1. INTRODUCTION

1.1. Peroxiredoxins

Peroxiredoxins (Prxs) (EC 1.11.1.15) are multifunctional antioxidant thioredoxin-dependent peroxidases that have been ubiquitously identified in organisms ranging from bacteria to mammals. Prx proteins are abundantly expressed in mammalian cells and are primarily localized at the sites of peroxide production including cytosol, mitochondria and peroxisomes (Immenschuh and Baumgart-Vogt, 2005).

1.1.1. Classification of Prxs

Thus far, six isoforms of Prxs have been identified in mammals and are classified into three subgroups based on the content and usage of highly conserved cysteinyl residues in the catalytic site. Typical 2-cysteine Prxs contain N-terminal and C-terminal cysteine residues, and both are required for the catalytic function (Prx I – IV). Atypical 2-cysteine (Prx V) and 1-cysteine (Prx VI) Prxs contain only the N-terminal cysteine, but atypical 2-cysteine Prxs require an additional cysteine for catalytic activity (Figure 1).

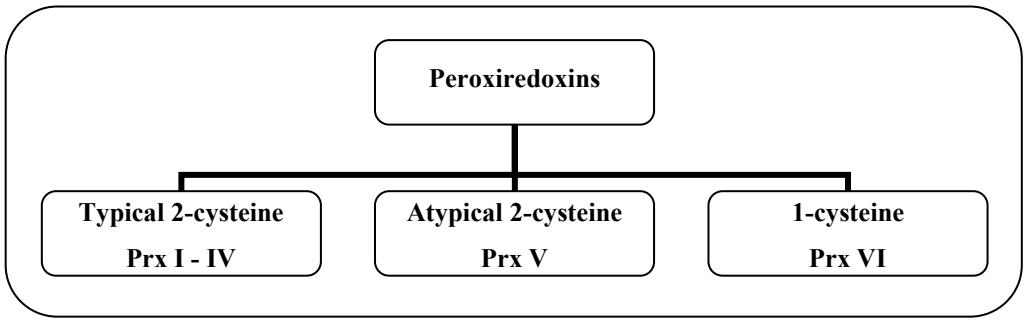

Figure 1. Classification of Prxs

1.1.2. Prxs - mechanism of action

Prx-mediated peroxidase reaction is a two step mechanism and is similar for all Prxs. The cysteinyl residues present in the active site are redox sensitive and undergo a cycle of peroxide-dependent oxidation and thiol-dependent reduction during the catalytic process (Rhee et al., 2005). In the first step, the catalytic cysteine residues attack the peroxide substrate and are oxidized. The attacked peroxides are converted into water and alcohol. In the second step, the oxidized Prxs are reduced and converted into the active form by electron donors such as thioredoxin and sulfiredoxin (Wood et al., 2003) (Figure 2).

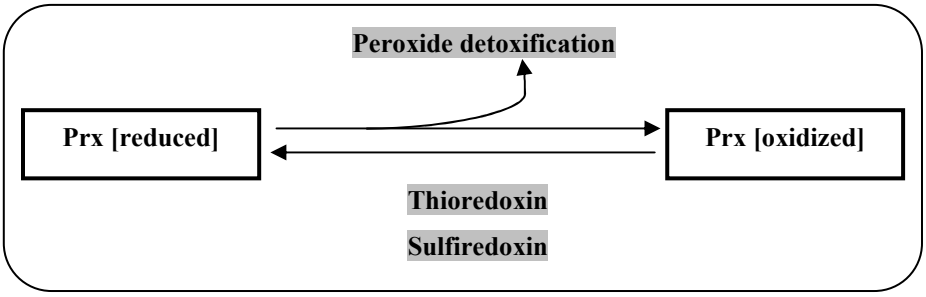

Figure 2. A simplified mechanism of the Prx-mediated enzyme reaction

1.1.3. Physiological role of Prxs

Reactive oxygen species (ROS) are chemical species that are formed upon incomplete reduction of oxygen. The major forms of ROS are oxygen ions, free radicals and peroxides. Prxs play a significant role in intracellular peroxide and peroxynitrite detoxification. The antioxidant properties of Prx proteins have been confirmed in studies demonstrating that mice with genetic deficiency of various Prxs are highly susceptible to oxidative stress-mediated injury. In addition, recent studies have shown that Prx activities are involved in various cellular functions such as peroxide-dependent signaling, regulation of cell cycle, proliferation, differentiation and apoptosis (Immenschuh and Baumgart-Vogt, 2005).

1.1.4. Peroxiredoxin I (Prx I)

Prx I is the best characterized isoform of the known mammalian Prxs. Prx I gene expression is up-regulated by various oxidative stress stimuli and may serve as an antioxidant cellular response against stress-related toxicity (Immenschuh et al., 1995, Ishii et al., 2000, Li et al., 2002). Mice with targeted disruption of the Prx I gene have been shown to progressively accumulate intracellular ROS and to exhibit an increased incidence of various malignancies (Neumann et al., 2003, Egler et al., 2005, Neumann and Fang, 2007). More recently, Prx I has also been shown to have anti-inflammatory functions in an experimental model of atherosclerosis (Kisucka et al., 2008). Therefore, it is conceivable that Prx I is not only involved in the regulation of cellular redox homeostasis, but may also have potent anti-tumorigenic and anti-inflammatory functions.

1.1.5. Regulation and signal transduction of Prx I gene expression

Regulation of Prx I gene expression by various oxidative stimuli is mediated via a highly conserved *cis*-acting antioxidant response element (ARE) (Ishii et al., 2000, Kim et al., 2007). The ARE, also known as stress response element (StRE) has been identified as a crucial regulatory element (RE) for the induction of various detoxifying enzymes and stress proteins. Redox-regulated transcription factors (TFs) such as nuclear factor-erythroid-2 related factor 2 (Nrf2) and activator protein-1 (AP-1) have previously been shown to target the StRE (Nguyen et al., 2003). In line with this notion, induction of Prx I gene expression by oxidative stress stimuli has previously been shown to be mediated via a StRE in a Nrf2- and AP-1-dependent manner (Immenschuh and Baumgart-Vogt, 2005). In addition, various studies suggested that the Prx I gene is also regulated via posttranslational mechanisms such as protein phosphorylation, redox-dependent oligomerization, proteolysis and modification of ligand binding (Wood et al., 2003). Activation of signaling pathways such as protein kinase C (PKC) and p38 mitogen-activated protein kinase (MAPK) has been shown to mediate Prx I gene activation (Hess et al., 2003).

1.2. Heme oxygenase

Heme oxygenase (HO) (EC 1.14.99.3) is a microsomal enzyme that catalyzes the first and rate-limiting step of oxidative heme (Fe-protoporphyrin-IX) degradation. HO cleaves the tetrapyrrole ring of heme into equimolar amounts of biliverdin-IXα, carbon monoxide (CO) and iron (Fe).

Biliverdin-IXα is subsequently converted into bilirubin-IX via a NAD(P)H-dependent reductase (Maines, 1997) (Figure 3).

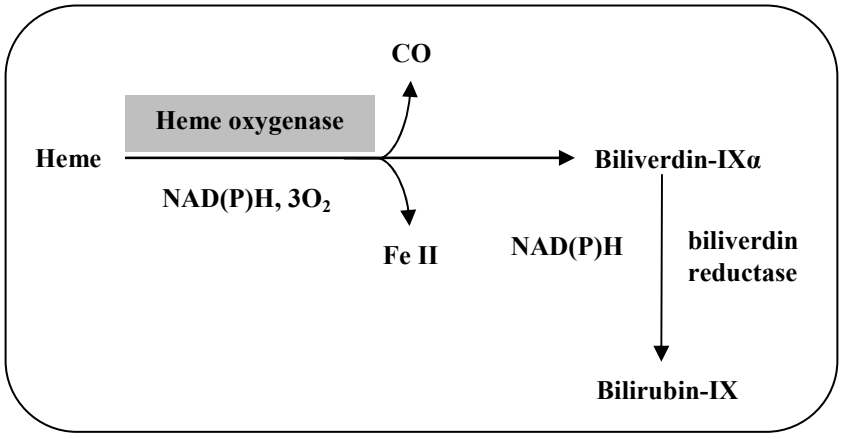

Figure 3. Catalytic degradation of heme by HO

1.2.1. Isoforms of HO

The HO system includes two genetically distinct isoforms: the inducible isoform HO-1 and the constitutively expressed isoform HO-2. Both isoforms share ~ 43 % amino acid homology and exhibit different gene regulatory patterns and tissue distribution. While induction of HO-1 is considered a cytoprotective response against oxidative stress, HO-2 may serve as a sensor of intracellular heme levels (Wagener et al., 1999).

1.2.2. Physiological role of HO

HO not only degrades the prooxidant heme, but also plays a crucial role in maintaining the cellular redox homeostasis. Besides its well-defined metabolic

functions, an increasing body of evidence suggests important biological functions to the products of the HO enzyme reaction. In particular, CO and biliverdin/bilirubin have been shown to exert unique antioxidant, signaling and immunomodulatory functions (Otterbein et al., 2003).

1.2.3. Heme oxygenase-1 (HO-1)

HO-1 is the inducible isoform of HO and was initially identified in 1968 (Tenhunen et al., 1968). HO-1, also known as 32-kDa heat shock protein, is barely expressed under basal conditions in most cells and tissues, but is highly up-regulated in response to a host of stimuli including both chemical and physical stress agents (Ferrandiz and Devesa, 2008). Up-regulation of HO-1 during oxidative insults has been shown to have cytoprotective effects, in particular in immune cells such as monocytes (Tracz et al., 2007). In addition, recent studies in HO-1 knockout mice (Poss and Tonegawa, 1997) and a human case of HO-1 genetic deficiency (Yachie et al., 1999) emphasized the antioxidant, anti-inflammatory and immunomodulatory functions of HO-1 (Table 1).

Table 1. Phenotypical alterations in human and mouse genetic HO-1 deficiency

Findings	Human	Mouse
Intrauterine death	Stillbirth, abortion	20% birth rate
Growth failure	+	+
Anemia	+	+
Fragmentation	+	?
Iron binding capacity	Increased	Increased
Ferritin	Elevated	Elevated
Iron deposition	+	+
Hepatomegaly	+	+
Splenomegaly	Asplenia	+
Lymph node swelling	+	+
Leukocytosis	+	+
Thrombocytosis	+	?
Coagulation abnormality	+	?
Endothelial injury	+	?
Hyperlipidemia	+	?

Adopted from Yachie et al., 1999

1.2.4. Gene regulation and signal transduction pathways of HO-1

HO-1 gene activation by most stimuli is predominantly regulated at the transcriptional level. A comprehensive analysis of various mammalian HO-1 gene promoters has revealed several REs, which serve as binding sites for a variety of TFs. In addition, two important enhancer sequences have been identified in the distal 5'-flanking region of the human and mouse HO-1 gene promoters at – 4 kb and –10 kb. These enhancer sequences contain multiple StREs and are essential for the basal and inducible HO-1 gene expression (Ryter et al., 2006). Considerable structural and spatial differences, however, have been found between rodent and human HO-1 gene promoters, which may explain the species-specific regulation of HO-1 gene expression (Exner et al., 2004, Naidu et al., 2008).

It is generally accepted that the basic leucine zipper **(**bZIP) TF Nrf2 is the major regulator of oxidative stress-dependent induction of HO-1 gene expression. Nevertheless, other TFs such as AP-1, heat-shock factor (HSF) and nuclear factor-κB (NF-κB) have also been shown to play a significant role for the regulation of HO-1 expression in response to various stimuli (Alam and Cook, 2007).

Accumulating evidence indicates that HO-1 induction is mediated via activation of multiple signaling pathways. In particular, a major regulatory role for HO-1 gene expression has been ascribed to the MAPK p38, extracellular signal-regulated kinase (ERK) and c-jun N-terminal kinase (JNK). Recent studies, however, have reported significant variations among the signaling cascades that regulate HO-1 expression in a cell-, tissue- or inducer-specific manner (Ryter et al., 2006).

1.3. Regulation of Prx I and HO-1 gene expression in monocytes – Objective of the study

Mononuclear phagocytes (monocytes, macrophages) play a critical role for the regulation of inflammation. Mononuclear phagocytes are activated in response to tissue injury or microorganisms and generate substantial amounts of intracellular ROS, which serve potent microbicidal and signaling functions (Beaman and Beaman, 1984).

The unique defensive properties of these cells would cause irreversible tissue damage, if they would not be controlled in a specific manner (Richard and Johnston, 1988). Therefore, endogenous antioxidant and anti-inflammatory protective mechanisms play a major physiological role in these cells.

Evidence has accumulated that Prx I and HO-1 gene expression is up-regulated by oxidative stress and proinflammatory stimuli in monocytic cells (Abbas et al., 2008, Wijayanti et al., 2004). Since Prx I and HO-1 have potent antioxidant and immunomodulatory functions, up-regulation of these two genes in monocytic cells may have beneficial effects during oxidative stress-related disorders (Figure 4).

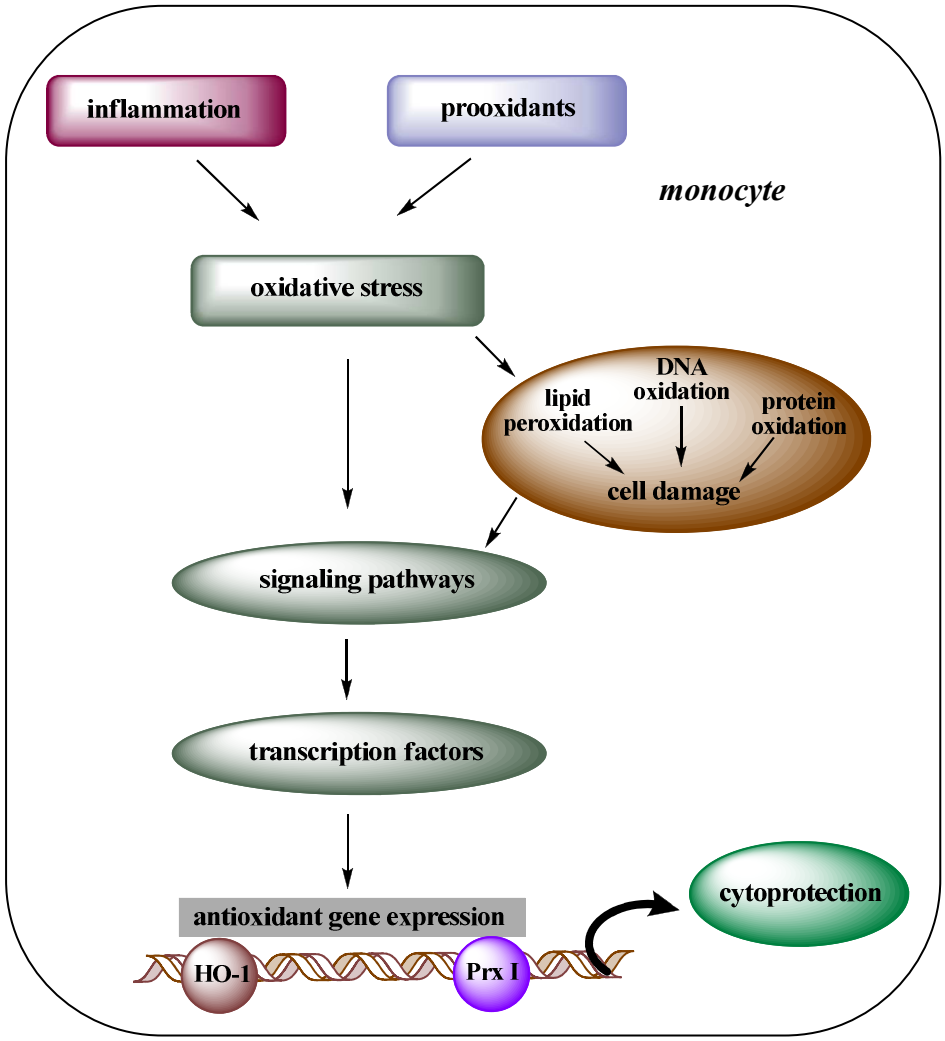

Figure 4. Rationale of the study

The proinflammatory mediator lipopolysaccharide (LPS) and the PKC activator phorbol myristate acetate (PMA), also termed 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), are prototypical activators of monocytic cells. Both, PMA and LPS, have previously been shown to increase the generation of ROS in a NAD(P)H oxidase-dependent manner in monocytic cells (Datta et al., 2000, Park et al., 2004). In addition, treatment with PMA and LPS has previously been shown to up-regulate HO-1 and Prx I gene expression in monocytic cells (Muraosa et al., 1993, Hess et al., 2003). Understanding the regulatory networks of these two genes in monocytes may help to prevent and treat chronic inflammatory disorders, which may ultimately lead to cancer (Karin et al., 2006). Therefore, the objective of the present studies was to elucidate the molecular mechanisms and signaling pathways that regulate gene expression of Prx I and HO-1 in response to PMA and LPS in monocytic cells. The primary objective of the studies was as follows:

1.3.1. Specific aim I: To elucidate the signaling pathway(s) that regulate(s) Prx I gene expression in response to PMA and LPS in monocytic cells.

It is shown that PMA and LPS induced Prx I gene expression in RAW264.7 monocytic cells. Treatment with LPS, however, inhibited PMA-dependent induction of Prx I gene expression indicating cross talk of signaling pathways that are activated by these stimuli. The LPS-dependent inhibition of Prx I gene activation by PMA is mediated via a transcriptional mechanism that involves a 'non-classical' activation of the TF NF-κB and the non-receptor tyrosine kinase Bruton's tyrosine kinase (Btk) (Paper I).

1.3.2. Specific aim II: To elucidate the signaling pathway(s) that induce HO-1 gene expression by PMA in monocytes, and the specific role of p38 MAPK for the regulation of HO-1 gene expression.

It is demonstrated that HO-1 gene expression was induced by PMA in RAW264.7 monocytic cells and was transcriptionally regulated 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 IκB kinase (IKK)-independent, atypical NF-κB pathway mediated PMA-dependent induction of HO-1 gene expression via activation of p38 MAPK and CK2 (Paper II).

HO-1 gene expression was up-regulated by genetic deficiency or pharmacological inhibition of p38 MAPK. Induction of HO-1 gene expression by p38 inhibition is transcriptionally regulated via the stress-inducible TF Nrf2 (Paper III).

2. MATERIALS and METHODS

A detailed description of the materials and methods are given in the respective papers of this thesis. All the materials that were used for the study are of reagent grade and were obtained from different suppliers as indicated. This chapter briefly summarizes the methodology applied in various parts of the experimental studies.

Plasmid preparations: Competent *Escherichia. coli* were transformed with plasmid DNA and cultured according to standard protocols. Plasmid preparations were performed using QIAGEN endotoxin-free kits. DNA concentrations were determined by the measurement of absorbance at 260 and 280 nm.

Generation of luciferase promoter constructs: Luciferase reporter gene constructs used in the study were generated using standard molecular cloning techniques or received as gifts from various sources cited accordingly. Mutated reporter gene constructs were generated using QuikChange XL site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. Insert orientation or mutations in the plasmids were verified by DNA sequencing in both directions.

Transient transfection and luciferase assay: Transfection of plasmid DNA into cells was performed using a liposome-based transfection reagent FuGENE® 6 (Roche). Plasmid DNA at amounts of 0.5 to 1 µg and for contransfection experiments 0.1 to 1.5 µg was added to 2 to 3 µl of transfection reagent in serum free medium. The DNA-lipid complex was added dropwise to the cell cultures in complete media. After 24 h of transfection or treatment with various stimuli before harvesting, cells were lysed using cell culture lysis reagent (Promega). The lysate was assayed for luciferase activity using the Dual luciferase assay kit (Promega). Relative light units of *Firefly* luciferase activity were normalized with *Renilla* luciferase activity.

Electrophoretic mobility shift assay (EMSA): The DNA-protein complexes were resolved on 6 % native polyacrylamide gels and blotted onto nylon membranes (Pierce). The interactions between biotin-labeled DNA and proteins were detected with a non-radioactive LightShift chemiluminescent EMSA kit (Pierce) according to the manufacturer's instructions.

Cell culture: All cell lines or primary cells used in the study were cultured in suitable growth media supplemented with 10 % FCS, 100 U/ml penicillin and 100-µg/ml streptomycin. Cell cultures were maintained under air/CO_2 (19:1) at 100 % humidity.

Western blotting: Protein concentration was determined with the BCA protein assay kit (Pierce). Proteins were separated on 12 % SDS-polyacrylamide gels and electro-blotted onto polyvinylidene difluoride membranes. Membranes were blocked either with 5 % fat-free milk buffer or 5 % bovine serum albumin, and probed with corresponding primary and secondary antibodies at the concentrations indicated by the manufacturers. The ECL chemiluminescent detection system was applied for detections and the signals were visualized either by exposure to X-ray films or with the Fluorchem FC2 gel documentation system.

Subcellular fractionation: Cells were washed twice in ice-cold PBS, and incubated in 200 µl of ice-cold homogenizing buffer. Thereafter, cells were scraped from the dishes, dounce-homogenized with 50 strokes and centrifuged at 2500 x *g* for 20 min at 4°C to separate nuclei from cytosol. The supernatant was collected as cytosolic fraction, and the pelleted nuclear fraction was washed twice in homogenizing buffer and incubated in 100 µl extraction buffer. Samples were rocked at 4°C for 20 min and centrifuged at 12,000 x *g* for 10 min. This fraction was collected as nuclear extract (NE). All fractions were brought to equal volumes and protein content was determined for the cytosol and nuclear extract fractions (Buffer compositions see Paper III).

Measurement of ROS: Intracellular generation of ROS was detected with dihydroethidium (DHE) dye (Invitrogen). After stimulation, cells were incubated with 5 µM DHE for 20 min, washed with PBS and the levels of ROS were determined with a FACScalibur flow cytometer. DHE-detectable superoxide anion (FL2-H) fluorescent signals were displayed as histograms.

Statistics: Quantitative data obtained either from Western blot analysis or luciferase assays were analyzed by Student's *t* test and presented as mean values ± S.E. from at least three independent experiments. A value of $p \leq 0.05$ was considered as statistically significant.

3. RESULTS and DISCUSSION

The two major aspects of the thesis are presented in three original articles. The first paper demonstrates the regulatory role of the TF NF-κB for Prx I gene expression by PMA and LPS. The second paper identified an atypical NF-κB pathway, which mediated the PMA-dependent induction of HO-1 gene expression via a newly identified functional κB element of the rat HO-1 promoter. The third paper deals with an unexpected up-regulation of HO-1 gene expression by inhibition of the p38 MAPK pathway, which was mediated via the TF Nrf2.

3.1. Regulation of Prx I gene expression by PMA and LPS in monocytic cells (Paper I)

The monocyte activators PMA or LPS alone up-regulated Prx I gene expression in RAW264.7 monocytic cells. Unexpectedly, simultaneous treatment of LPS inhibited Prx I gene activation by PMA. This inhibitory effect of LPS, which appears to be cell-specific for monocytes, was not observed for gene regulation of Cox-2 and TNF-α, both of which are also known to be induced by PMA and LPS (*Figure 1, paper I*). A similar inhibitory pattern of LPS has previously been reported for the expression of the epoxide hydrolase and glutathione *S*-transferase genes (Choi et al., 1998).

LPS-dependent inhibition of Prx I gene activation by PMA occurred on the transcriptional level and was regulated via the TF NF-κB, which binds to a newly identified Prx-κB site (*Figures 3 and 4, paper I*). Various oxidative stress stimuli and inflammatory mediators activate the classical NF-κB pathway, which includes stimulus-coupled phosphorylation of the IκB-kinase complex and phosphorylation of IκB. Phosphorylation of IκB leads to its degradation via the proteasome and, subsequently, p65 is translocated into the nucleus (Chen et al., 2004).

This pathway, however, does not seem to be involved in the regulation of LPS-dependent repression of Prx I gene activation by PMA, because overexpression of dominant negative IκB had no effect on the inhibitory effect of LPS. In addition, simultaneous treatment of LPS did not alter PMA-dependent phosphorylation of IκB (*Figure 5, paper I*).

These observations led to the conclusion that a 'non-classical' mode of NF-κB activation is involved in this regulatory mechanism. In line with this conclusion, an IκB-independent activation of NF-κB, including phosphorylation of p65 at regulatory serine 276, was involved in the regulation of inhibition of Prx I gene induction by LPS. Treatment with PMA induced phosphorylation of p65 at serine 276, which was markedly attenuated by simultaneous exposure to LPS. In addition, functional studies with Gal4-p65 fusion constructs indicated that phosphorylation of p65 at serine 276 mediates LPS-dependent repression of Prx I gene activation by PMA (*Figure 6, paper I*). Phosphorylation of p65 at serine 276 is a post-translational mechanism that controls NF-κB activity, and is essential for the interaction of this TF with the transcriptional coactivator p300/CREB-binding protein (Hayden and Ghosh, 2004). Accordingly, overexpression of p300 up-regulated Prx I promoter activity suggesting that p300 is involved in the NF-κB-dependent regulation of Prx I gene expression (*Figure 7, paper I*).

Btk, a non-receptor tyrosine kinase has previously been shown to mediate LPS-dependent toll-like receptor-4 (TLR4) signaling by modulating NF-κB activity in monocytic cells (Horwood et al., 2003 and Doyle et al., 2005). Therefore, the role of Btk for the regulation of Prx I gene expression was investigated. Treatment with the Btk inhibitor LFM-A13 not only attenuated LPS-dependent repression of Prx I gene activation by PMA, but also down-regulated this effect on phosphorylation of p65 at serine 276 (*Figures 8 and 9, paper I*).

In conclusion, LPS-dependent inhibition of Prx I gene activation by PMA is regulated via a pathway that involves phosphorylation of the NF-κB subunit p65 at serine 276 (Figure 5). In addition, the present findings suggest cross-talk between signaling mechanisms that regulate Prx I gene expression by PMA and LPS in monocytic cells.

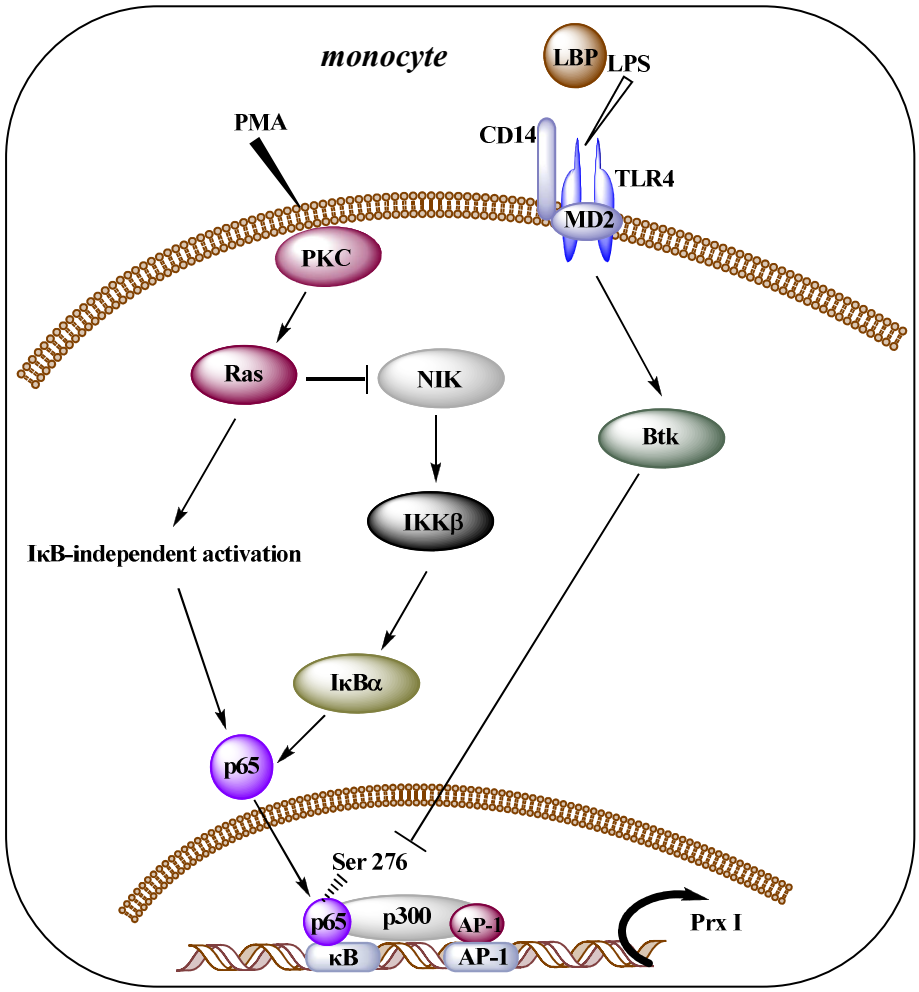

Figure 5: Signaling pathways of Prx I gene activation by PMA and LPS

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Original Contribution

Inhibition of phorbol ester-dependent peroxiredoxin I gene activation by lipopolysaccharide via phosphorylation of RelA/p65 at serine 276 in monocytes

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Abstract

Peroxiredoxin I (Prx I) is an antioxidant enzyme with thioredoxin-dependent peroxidase activity which is involved in various cellular processes such as regulation of cell proliferation. Here, it is shown that the proinflammatory mediator lipopolysaccharide (LPS) inhibits the induction of Prx I expression and promoter activity by the phorbol ester 12-*O*-tetradecanoylphorbol- 13-acetate (TPA) in RAW264.7 monocytes, but not that of cyclooxygenase-2. LPS-dependent repression of Prx I induction by TPA was mediated via a newly identified κB site in the Prx I promoter, but the "classical" NF-κB cascade was not involved in this regulatory pathway, because IκB did not affect LPS-mediated Prx I repression. By contrast, phosphorylation of p65 at serine 276, which enhances the transcriptional activity of NF-κB, was up-regulated by TPA and was reduced by simultaneous exposure to LPS. Functional studies with Gal4–p65 constructs revealed that serine 276 is crucial to confer LPS-dependent repression of TPA-mediated induction of p65 transactivation. Finally, repression of TPA-dependent Prx I induction by LPS was mediated via Bruton's tyrosine kinase as indicated by studies with the pharmacological inhibitor LFM-A13. In summary, LPS-dependent inhibition of Prx I gene activation by TPA in monocytes is regulated via a pathway that involves phosphorylation of the NF-κB subunit p65 at serine 276.

Keywords: Antioxidant; Peroxiredoxin; Gene expression; NF-κB; Oxidative stress; Monocytes; Inflammation; Signaling; Free radicals

Peroxiredoxin (Prx) I is a multifunctional protein with thioredoxin-dependent peroxidase activity and belongs to the Prx protein family [1–3]. Prx I was initially identified as stress-inducible macrophage 23-kDa stress protein in mouse peritoneal macrophages (MSP23) [4], as proliferation-associated gene (PAG) [5] in various human cell lines, and as heme-binding protein 23 (HBP23) [6] in rat liver. The physiological functions of Prx I comprise cytoprotection against oxidative stress, modulation of intracellular signaling cascades that apply hydrogen peroxide as a second-messenger molecule, and regulation of cell proliferation and differentiation [7]. Mice with a targeted deletion of the Prx I gene have been reported to develop hemolytic anemia with an increase in reactive oxygen species in erythrocytes and malignancies such as sarcomas, lymphomas, and carcinomas, suggesting that Prx I may function as a tumor suppressor [8,9]. In contrast, it has also been demonstrated by others that Prx I is highly expressed in a number of malignant tumors and may promote an aggressive survival type of tumor cells and resistance to tumor therapy [10,11]. A number of stimuli, among them the phorbol ester 12-*O*-tetradecanoylphorbol- 13-acetate (TPA), induce Prx I gene expression at the transcriptional level [12–15]. We have previously demonstrated that TPA-dependent Prx I gene expression is activated in monocytic cells via a protein kinase C (PKC)/Ras-dependent signaling pathway that targets the transcription factor (TF) activator protein-1 (AP-1) [16].

Abbreviations: AP-1, activator protein-1; Btk, Bruton's tyrosine kinase; COX-2, cyclooxygenase-2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IκB, inhibitor of nuclear factor-κB; LPS, lipopolysaccharide; NE, nuclear extract; PKC, protein kinase C; Prx, peroxiredoxin; TF, transcription factor; TLR4, toll-like receptor-4; TNF-α, tumor necrosis factor-α; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

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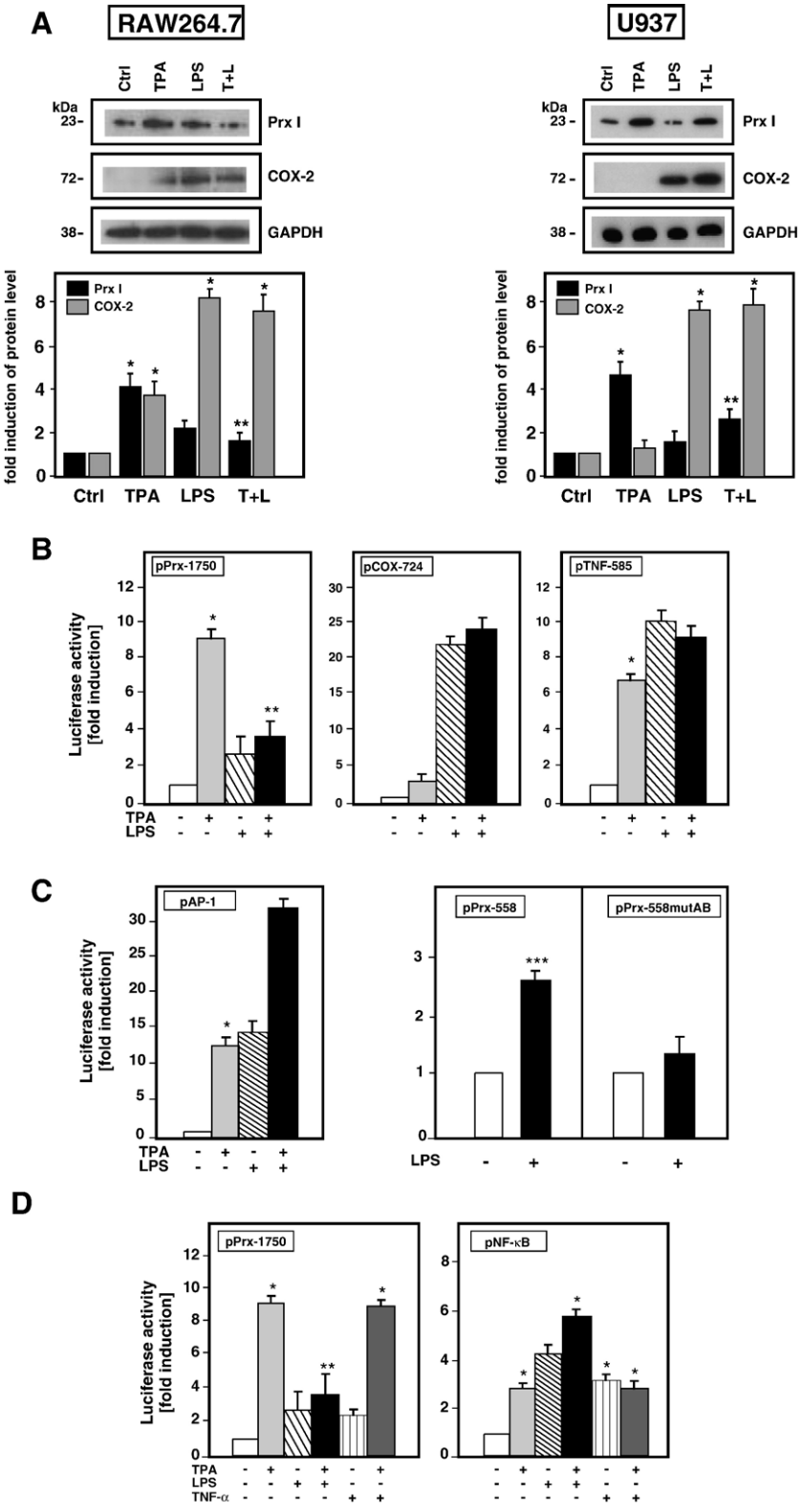
Because chronic inflammation is a major risk factor for cancer [17] and Prx I may be involved in the complex sequence of events that cause malignancy in inflammatory disease, we asked whether the prototypical proinflammatory mediator lipopolysaccharide (LPS), which activates toll-like receptor-4 (TLR4) signaling in immune cells [18], would affect Prx I gene expression in monocytic cells. Thus, the goal of the present study was to investigate the molecular mechanisms that regulate Prx I gene expression in response to TPA and LPS and the potential cross-talk of signaling pathways that are activated by these two compounds in mononuclear cells.



Here, it is reported that exposure to LPS markedly inhibits the TPA-dependent induction of Prx I gene expression in monocytic cells. This repression of Prx I induction by LPS did not involve the "classical" NF-κB signaling cascade, but was regulated via a Bruton's tyrosine kinase (Btk)-dependent pathway that was mediated by phosphorylation at serine 276 of NF-κB subunit p65.

Materials and methods

Materials

Dulbecco's modified Eagle medium, RPMI 1640, and MEM were obtained from PAA Laboratories (Pasching, Germany). Medium M199 was from Gibco (Grand Island, NY, USA), fetal bovine serum was from Biochrom KG (Berlin, Germany), polyvinylidene difluoride membranes were from Millipore (Bedford, MA, USA), radioisotopes were from Amersham Biosciences. All other chemicals were purchased from Sigma and Roche Applied Science, unless otherwise indicated.

Cell culture

All cell lines were from the American Type Culture Collection (Manassas, VA, USA). RAW264.7 cells were grown in Dulbecco's modified Eagle medium, U937 cells in RPMI 1640, Chinese hamster ovary cells in MEM, and ECV304 cells in M199. All media were supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cell cultures were kept under air/CO_2 (19/1) at 100% humidity. Treatment of cells with TPA (0.5 μM), LPS (1 μg/ml; *Escherichia coli* 0111:B4), or recombinant mouse tumor necrosis factor-α (TNF-α, 25 μg/ml) was performed with serum-free medium. The small-molecule inhibitor of Btk LFM-A13 (Calbiochem) was added to the culture medium at the indicated concentrations 1 h before treatment with TPA and/or LPS.

Western blot analysis

After cell cultures were washed twice with 0.9% NaCl, 300 μl of 1× Laemmli buffer (2% SDS, 10% glycerol, bromophenol blue, 0.4 mol/L dithiothreitol, 4% protease inhibitor) was added, and 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 (30–60 μg) was loaded onto a 12% SDS–polyacrylamide gel and was blotted onto polyvinylidene difluoride-membranes by electroblotting. Membranes were blocked with Tris-buffered saline containing 5% skim-milk or 5% bovine serum albumin, 50 mM Tris/HCl (pH 7.6), 150 mM NaCl, and 0.1% Tween 20, for 1 h at room temperature. The primary polyclonal rabbit antibody against rat Prx I [19] was added at 1:5000 dilution in blocking buffer and the membrane was incubated for 1 h at room temperature. The primary rabbit polyclonal antibody against cyclooxygenase (COX)-2 (Alexis Biochemicals; Grünberg, Germany) was used at 1:3000 and the mouse monoclonal antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (HyTest Ltd., Turku, Finland) was used at 1:1000 dilution. The primary antibodies for the detection of the phosphorylated inhibitor of nuclear factor-κB (IκB)-α (serine 32) and phosphorylated serine 276 of p65 were from Cell Signaling (Beverly, MA, USA) and were applied at the concentrations recommended by the manufacturer. The secondary antibodies were goat anti-rabbit IgG horseradish peroxidase and anti-mouse IgG horseradish peroxidase (DPC Biermann, Bad Nauheim, Germany) and used at 1:20,000 and 1:100,000, respectively. A commercial detection system (Lumi-Light; Roche Applied Science) was used for the detection of chemiluminescence signals according to the manufacturer's instructions. The obtained chemiluminescent autoradiographic signals were scanned by videodensitometry and quantitated with ImageQuant software [20].

Plasmid constructs

The luciferase reporter gene constructs pPrx-1750, pPrx-1209, pPrx-897, pPrx-558, and pPrx-558mutAB have previously been described [16]. The reporter gene construct pPrx-1750-κBmut, which contains targeted mutations of the Prx-κB site (−1024 to −1015) within the proximal Prx I promoter region (Fig. 3A), was generated with the QuickChange XL-site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions with the plasmid pPrx-1750 as a template. Reporter gene plasmid NF-κB with three copies of the NF-κB consensus sequence was obtained from Dr. Lienhard Schmitz (University of Giessen, Giessen, Germany), plasmid pAP-1 with six -AP-1 repeats in front of a minimal fos promoter was from Dr. Craig Hauser (Burnham Institute, La Jolla, CA, USA) [21], plasmid pCOX-724 with the −724 bp proximal promoter of the mouse COX-2 gene was from Dr. Harvey Herschman (UCLA, Los Angeles, CA, USA) [22], and plasmid pTNF-585 was from

Fig. 1. LPS inhibits TPA-dependent Prx I gene expression in monocytic cells. (A) Cell cultures were treated with TPA (0.5 μM), LPS (1 μg/ml), TPA plus LPS (T + L), or control medium (Ctrl) for 18 h, as indicated. Total protein (30 μg) was subjected to Western blot analysis and probed sequentially with antibodies against Prx I, COX-2, and GAPDH. A representative autoradiogram is shown and autoradiographic signals of three independent experiments were obtained by chemiluminescence and scanned by videodensitometry. Values ± SE represent the fold- induction of Prx I or COX-2 normalized to GAPDH from these three experiments. Student's *t* test for paired values: significant differences, treatment versus control, $*p \le 0.05$; TPA + LPS versus TPA, $**p \le 0.05$. (B) RAW264.7 cells were transiently transfected with reporter gene construct pPrx-1750, pCOX-724, or pTNF-585 or (C) pAP-1, pPrx-558, or pPrx-558mutAB. Transfected cells were treated with TPA (0.5 μM), LPS (1 μg/ml), TPA plus LPS, or control medium for 18 h, as indicated, after which cell extracts were assayed for luciferase activity. The -fold induction relative to the control was determined. Values are means ± SE from at least three independent experiments with duplicates of each point. Student's *t* test for paired values: significant differences, *TPA versus control, **TPA + LPS versus TPA, ***LPS versus control, $p \le 0.05$. (D) Cells were transfected with pPrx-1750 and pNF-κB. After transfection cells were treated with recombinant mouse TNF-α (25 μg/ml), LPS, and TPA for another 18 h, as indicated. Luciferase analysis was done in the same way as described for (B). Student's *t* test for paired values: significant differences, *treatment versus control, **TPA + LPS versus TPA, $p \le 0.05$.

Table 1
Regulation of Prx I promoter activity by TPA and LPS in chinese hamster ovary and human endothelial cells (ECV 304)

Treatment	-fold induction of luciferase activity	
	CHO	ECV304
Ctrl	1.0	1.0
TPA	1.5±0.3	3.5±0.3
LPS	1.5±0.1	1.0±0.1
TPA+ LPS	2.0±0.2	4.0±0.2

CHO and ECV304 cells were transfected with reporter gene construct pPrx-1750. After transfection cells were treated with TPA (0.5 μM), LPS (1 μg/ml), TPA plus LPS or control medium, as indicated. After 18 h cell extracts were assayed for luciferase activity and the - fold induction relative to the control was determined. Values are means±S.E from of at least three independent experiments with duplicates of each point. *CHO*, *chinese hamster ovary.*

Dr. Gordon Duff (University of Sheffield, Sheffield, UK) [23]. The expression vector with the constitutive active Ras has been described previously [16,24]. The expression vector with the dominant negative mutant of IκB was from Dr. Richard Gaynor (Southwestern Medical Center, Houston, TX, USA). Plasmids pGal4-p65, pGal4-p65 S276C, and pGal4-p65 S536A, which contain the transactivation domain of TF p65/RelA fused to the DNA-binding domain of yeast Gal4, were gifts from Dr. Guy Haegeman (University of Gent, Gent, Belgium) [25]. Empty control vector pFC2-dbd and the luciferase reporter vector pFR-luc with five copies of the yeast Gal4 binding element were from Stratagene. Expression plasmid pRSV-NF-κB, which expresses RelA/p65, was from Dr. Dieter Schmoll (Sanofi Aventis Pharma; Frankfurt/Main, Germany) [26].

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 Applied Science) [16]. Unless otherwise stated cells were transfected with 1 μg of luciferase reporter plasmid and in cotransfection experiments with 0.1 to 1.5 μg of the indicated expression vectors along with 0.25 μg *Renilla* luciferase expression vector (pRL-SV40) to control for transfection efficiency [20]. Cells were lysed with 1× luciferase lysis reagent (Promega) and luciferase activity was determined with the commercial Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Cells were either harvested 24 h after transfection or cultured for another 18 h under the indicated conditions. Relative light units of firefly luciferase activity were normalized with *Renilla* luciferase activity, as described previously [20].

Preparation of nuclear extracts (NE) and electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared as described [16]. The sequences of the oligonucleotides used for the EMSA are as follows: Prx-κB, 5′-CGAGACCCTTGGGACTCTCTGGGT-CTGGTT-3′; Prx-κB mutant (Prx-κBmut), 5′-CGAGACCCTT-GCTACAGTCTGGGTCTGGTT-3′; and NF-κB consensus oligonucleotide, 5′-AGTTGAGGGGACTTTCCCAGGC-3′, along with the respective oligonucleotides of the non-coding strand. Equal amounts of complementary oligonucleotides were annealed and labeled by 5′-end labeling with [γ-^{32}P]-dATP and T4 polynucleotide kinase and were purified with a nucleotide removal kit. Binding reactions were carried out in a total volume of 20 μl containing 50 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 5% glycerol, 10 μg of nuclear extract, 250 ng poly(dI–dC), and 5 mM dithioerythritol. For competition analyses a 100-fold molar excess of unlabeled oligonucleotides was added. After preincubation for 5 min at room temperature, 1 μl of the labeled probe (10^4 cpm) was added and the incubation was continued for an additional 10 min. For supershift analysis an antibody directed against the NF-κB p65 subunit (sc-8008; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit preimmune serum as a control were added to the EMSA reaction, which was incubated at 4 °C for 2 h. Electrophoresis was performed with a 4.5% non-denaturing polyacrylamide gel in TBE buffer (89 mM Tris, 89 mM boric acid, 5 mM EDTA) at 200 V. After electrophoresis the gels were dried and exposed to X-ray films.

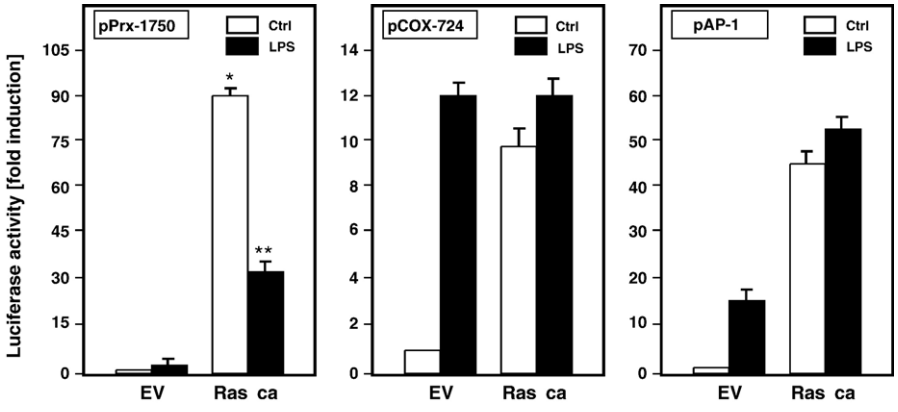


Fig. 2. Inhibitory effect of LPS on Ras-dependent induction of Prx I promoter activity. RAW264.7 cells were cotransfected with pPrx-1750 and an expression vector with a constitutively active mutant of Ras (Ras ca) or an empty control vector (EV). After transfection cells were treated with LPS (1 μg/ml) for another 18 h. Cell extracts were assayed for luciferase activity and the -fold induction relative to the control was determined. Values are means±SE from at least three independent experiments with duplicates of each point. Student's *t* test for paired values: significant differences, *control medium+ Ras ca versus control medium + EV, **LPS + Ras ca versus control medium + Ras ca; $p \leq 0.05$.

Results

LPS inhibits the TPA-dependent induction of Prx I gene expression

We examined the effects of the phorbol ester TPA and the proinflammatory mediator LPS on endogenous Prx I protein expression in the monocytic cell line RAW264.7. In line with a previous report [16] we found that TPA induced Prx I expression, whereas LPS caused only a minor enhancement of Prx I expression. Interestingly, simultaneous treatment of cells with TPA and LPS reduced TPA-dependent induction of Prx I expression (Fig. 1A). A similar Prx I gene expression pattern was also found in the human monocyte cell line U937 (Fig. 1A). To test the specificity of the inhibitory LPS effect on Prx I gene regulation, we also examined the gene expression of COX-2, which is known to be induced by these two stimuli [27]. Treatment of cells with TPA and LPS, either alone or in combination, up-regulated COX-2 expression (Fig. 1A), indicating that the inhibitory action of LPS on TPA-dependent induction is specific for Prx I.

To investigate whether LPS-mediated inhibition of TPA-dependent induction of Prx I gene expression occurs on the transcriptional level, we determined the regulation of Prx I promoter activity by these two compounds in transiently transfected RAW264.7 cells. Similar to the regulatory pattern of endogenous Prx I gene expression in RAW264.7 cells, TPA induced luciferase activity of a reporter gene construct with the proximal 1750 bp of the rat Prx I gene promoter (pPrx-1750) (Fig. 1B). LPS caused only a minor up-regulation of Prx I promoter activity and reduced the TPA-dependent induction of Prx I promoter activity when added in combination with TPA (Fig. 1B). To examine the specificity of this LPS-mediated repression of Prx I, we studied the effects of TPA and LPS on the activity of the COX-2 (pCOX-724) and TNF-α (pTNF-585) gene promoters, both of which are known to be prominently up-regulated by these two compounds [27,28]. In contrast to Prx I, combined treatment with TPA plus LPS did not significantly reduce COX-2 and TNF-α promoter activities (Fig. 1B). Because TPA-dependent Prx I gene induction has previously been shown to be mediated via AP-1 [16], we also examined the regulation of a reporter gene construct with six copies of the AP-1 consensus sequence [21]. Simultaneous treatment with TPA plus LPS caused an additive induction of AP-1 reporter gene activity (Fig. 1C). To further elucidate the weak induction of Prx I promoter activity by LPS, we also determined the regulation of a reporter gene construct with two functional AP-1 sites of the proximal Prx I promoter region (Fig. 1C; pPrx-558). Exposure to LPS up-regulated reporter gene activity of this construct to an extent similar to that observed for pPrx-1750 (Fig. 1B). In contrast, this up-regulation was not observed for a construct in which these two AP-1 sites were inactivated by site-directed mutagenesis (pPrx-558mutAB; Fig. 1C).

Because LPS and TNF-α activate overlapping signaling cascades in mononuclear cells [18,29,30], we also determined the effects of TNF-α on TPA-dependent Prx I promoter induction. In contrast to LPS, exposure to TNF-α did not affect induction of Prx I promoter activity by TPA (Fig. 1D). Regulation of a reporter gene construct with three copies of the κB enhancer consensus sequence by these compounds is shown as a control (Fig. 1D). Finally, a different regulation of Prx I promoter activity was observed in transfected Chinese hamster ovary cells and the endothelial cell line ECV304. Although in both cell lines Prx I promoter activity was induced by TPA and LPS (Table 1), the TPA-dependent induction of Prx I was not inhibited by simultaneous treatment with LPS.

Taken together, the data indicate that LPS inhibits the TPA-dependent induction of Prx I gene expression in a cell-specific manner in monocytes.

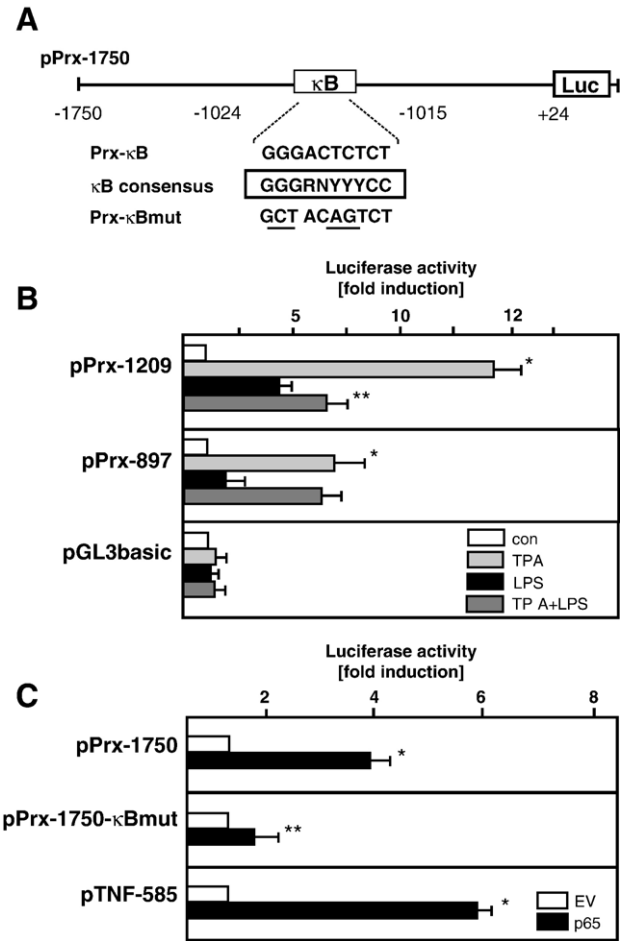


Fig. 3. Role of the Prx-κB site for regulation of Prx I promoter activity. (A) Localization of the Prx-κB site within the Prx I promoter and DNA sequence comparison with the κB enhancer consensus sequence (box). Mutated bases within the pPrx-1750-κBmut construct are underlined. (B) The indicated Prx I reporter gene constructs were transfected into RAW264.7 cells. Cells were treated with TPA (0.5 μM), LPS (1 μg/ml), TPA plus LPS, or control medium for 18 h and luciferase activity was determined as described for Fig. 1. Values are means ± SE from at least three independent experiments with duplicates of each point. Student's *t* test for paired values: significant differences, *TPA versus control, **pPrx-1209 + TPA + LPS versus pPrx-1209 + TPA, $p \leq 0.05$. (C) RAW264.7 cells were cotransfected with the constructs pPrx-1750, pPrx-1750-κBmut, or pTNF-585 and an expression vector for the NF-κB subunit p65 or empty control vector (EV), as indicated. 24 h after transfection cells were treated for another 18 h with TPA or control medium. Luciferase analysis was done in the same way as described for (B). Student's *t* test for paired values: significant differences, *pPrx-1750 + p65 versus pPrx-1750 + EV, pTNF-585 + p65 versus pTNF-585+ EV, **pPrx-1750-κBmut + p65 versus pPrx-1750 + p65, $p \leq 0.05$.

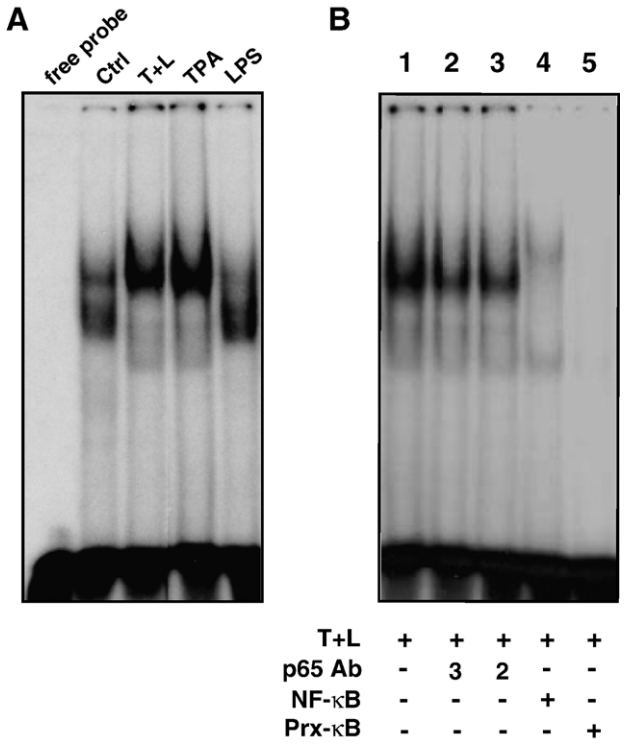


Fig. 4. Binding of nuclear protein to the Prx-κB site. (A) EMSA. A ^{32}P-labeled oligonucleotide with the Prx-κB element was incubated with 20 μg of NE from control (Ctrl) or TPA- (0.5 μM), LPS- (1 μg/ml), or TPA plus LPS- (T + L)-treated cells or without NE as a free probe, as indicated. (B) For supershift analysis NE from TPA-treated cells was preincubated with 3 and 2 μl of antibody against the NF-κB p65 subunit for 1 h at 4 °C before the radiolabeled Prx-κB probe was added (lanes 2 and 3). For competition analyses the radiolabeled Prx-κB oligonucleotide was preincubated with NE from TPA-treated cells along with a 50-fold molar excess of unlabeled NF-κB consensus or Prx-κB site oligonucleotides, as indicated (lanes 4 and 5). DNA–protein complexes were separated by electrophoresis on a 4.5% polyacrylamide gel.

LPS inhibits Ras-dependent activation of Prx I transcription

The small GTP-binding protein Ras has previously been shown to be involved in TPA-dependent induction of Prx I gene expression [16]. To evaluate the role of Ras in the LPS-dependent inhibition of Prx I gene induction by TPA, we examined the effect of LPS on the regulation of Prx I promoter activity by a cotransfected expression vector with a constitutively activated mutant of Ras. Treatment with LPS markedly decreased the Ras-dependent induction of Prx I promoter activity (Fig. 2). By contrast, LPS had an additive effect on Ras-dependent induction of reporter gene constructs with either the COX-2 promoter region or six copies of the AP-1 consensus site (Fig. 2, for a comparison see also Fig. 1).

The data indicate that LPS-dependent inhibition of TPA-dependent Prx I promoter induction occurs downstream of Ras.

Identification of a functional κB site in the proximal Prx I gene promoter region

A common intracellular target of TPA- and LPS-dependent signaling is the NF-κB pathway, which is a major regulator of numerous genes in mononuclear cells. NF-κB is able to bind promoter and enhancer regions of various genes that contain κB sites [31,32]. A potential κB element of the Prx I promoter was identified between positions −1024 and −1015 bp relative to the transcription initiation site, which matches the κB consensus sequence in 9 of 10 bp (Fig. 3A). To assess the regulatory capacity of this putative κB enhancer element, we generated two Prx I reporter gene constructs, either with (pPrx-1209) or without the Prx-κB site (pPrx-897). Transfection of these constructs into RAW264.7 cells revealed that up-regulation of Prx I promoter activity in response to TPA was reduced after deletion of the Prx-κB site (Fig. 3B). Importantly, the LPS-dependent inhibition of Prx I induction by TPA was not observed for pPrx-897 (Fig. 3B). In the following, we determined the functionality of the Prx-κB site by mutation studies. When cotransfected with an expression vector for the NF-κB subunit p65, which is also termed RelA, targeted mutation of the Prx-κB site (pPrx-1750-κBmut) reduced the extent of reporter gene induction compared to the wild-type construct (Fig. 3C). The magnitude of TPA-dependent induction was significantly lower for the Prx I reporter gene construct with the mutated κB site (data not shown). This seemed to be specific, because p65-dependent regulation of a TNF-α promoter construct (pTNF-585) with functional κB- sites was similar to that of pPrx-1750 when used as a control (Fig. 3C, bottom).

Taken together, the data suggest that the Prx-κB site is involved in LPS-dependent inhibition of Prx I gene transcription and serves as a functional target for the NF-κB subunit p65.

The Prx-κB site is a nuclear target of p65

DNA–protein interactions in NE from RAW264.7 cells were determined for an oligonucleotide with the Prx-κB site by

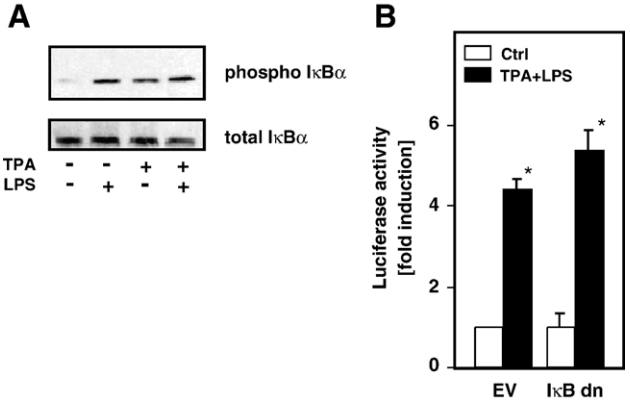


Fig. 5. Role of IκB in the regulation of Prx I promoter activity. (A) RAW264.7 cells were treated with TPA (0.5 μM), LPS (1 μg/ml), TPA plus LPS, or control medium for 3 h, as indicated. Total protein (60 μg) was subjected to Western blot analysis and probed sequentially with antibodies against phosphorylated IκBα (serine 32) or total IκBα. (B) Cells were cotransfected with pPrx-1750 and an expression vector for dominant negative IκB (IκB dn) or empty control vector (EV), as indicated. After transfection cells were treated with TPA plus LPS for another 18 h. Cell extracts were assayed for luciferase activity and the -fold induction relative to the control was determined. Values are means ± SE from at least three independent experiments with duplicates of each point. Student's *t* test for paired values: significant difference, TPA + LPS versus control, $*p \leq 0.05$.

EMSA. NE from cells which were treated with TPA alone showed a markedly stronger DNA-binding activity to the Prx-κB oligonucleotide than NE from control cells (Fig. 4A). By contrast, NE from cells that were treated with LPS alone did not show inducible DNA-binding to the Prx-κB site and, unexpectedly, NE from cells that were treated with a combination of TPA plus LPS exhibited a DNA–protein band similar to that observed for TPA alone (Fig. 4A). Incubation of the binding reaction with an antibody directed against the NF-κB subunit p65 reduced formation of the DNA–protein complex (Fig. 4B),

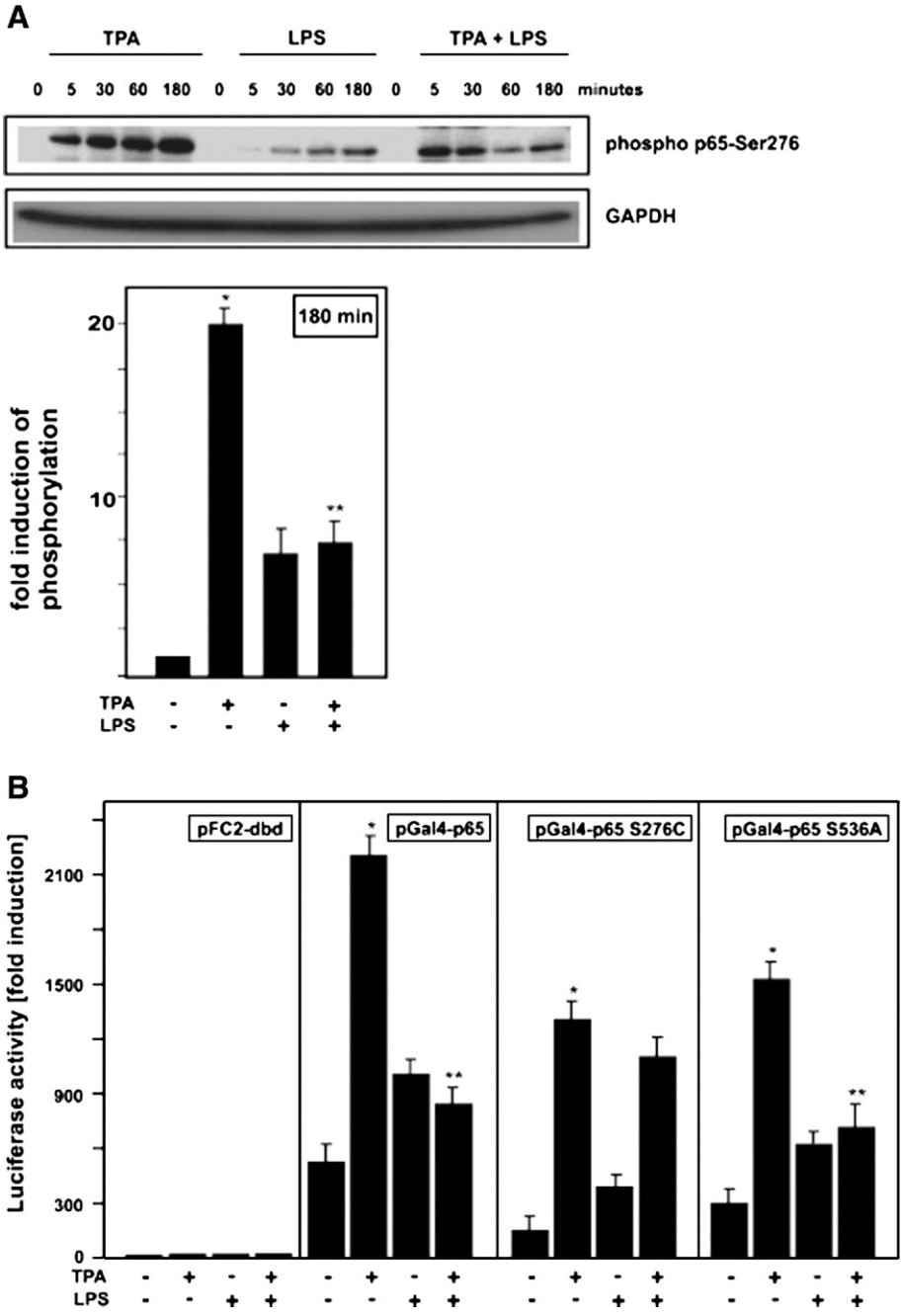


Fig. 6. Phosphorylation of p65 at serine 276 is critical for LPS-mediated inhibition of TPA-dependent induction in RAW264.7 cells. (A) RAW264.7 cells were treated with TPA (0.5 μM), LPS (1 μg/ml), TPA plus LPS, or control medium for the times indicated. Total protein (60 μg) was subjected to Western blot analysis and probed sequentially with antibodies against phosphorylated serine 276 of p65 and GAPDH. Similar results were obtained in three independent experiments and a representative autoradiogram is shown. The autoradiographic signals after 180 min were quantitated by videodensitometry. Values ± SE represent the fold- induction of phospho p65-Ser276 normalized to GAPDH from three independent experiments. Student's *t* test for paired values: significant differences, *TPA versus control, **TPA + LPS versus TPA, $p \leq 0.05$. (B) Cells were cotransfected with reporter gene constructs pFR-luc and pFC2-dbd, pGal4-p65, or the mutant pGal4-p65 S276C or pGal4-p65 S536A, as indicated. After transfection the cells were treated with TPA, LPS, or TPA plus LPS for another 18 h. Cell extracts were assayed for luciferase activity and the -fold induction relative to the control was determined. Values are means ± SE from three independent experiments with duplicates of each point. Student's *t* test for paired values: significant differences, *TPA versus control, **TPA + LPS versus TPA, $p \leq 0.05$.

suggesting the presence of p65. In competition EMSA studies the intensity of a DNA–protein complex which was formed with the Prx-κB oligonucleotide was abolished by an excess of unlabeled oligonucleotides with either the Prx-κB site or the NF-κB enhancer consensus sequence (Fig. 4B).

Thus, the data suggest that the Prx-κB site is a specific nuclear binding site for the NF-κB subunit p65.

IκB is not involved in LPS-mediated inhibition of TPA-dependent Prx I gene activation

Nuclear translocation of p65 occurs after release from the cytosolic inhibitor protein IκB, which is regulated via phosphorylation of specific regulatory serines in the N-terminal IκB region. This phosphorylation serves as a signal for subsequent proteasomal degradation of IκB and is referred to as the "canonical" or "classical" NF-κB pathway [31,32]. To investigate the regulatory potential of this pathway in RAW264.7 cells, we determined the phosphorylation of serine 32 of IκBα with a phosphospecific antibody. Phosphorylation of serine 32 was markedly up-regulated in response to TPA or LPS alone and simultaneous incubation with TPA plus LPS had a similar effect on the level of IκB phosphorylation compared to that observed for treatment with either compound alone (Fig. 5A). To determine the functional role of IκB on Prx I promoter regulation by TPA and LPS, we also applied an expression vector with a dominant negative mutant of IκB for cotransfection studies. Overexpression of this dominant negative IκB did not have a major effect on LPS-mediated repression of TPA-dependent Prx I promoter induction (Fig. 5B), but reduced LPS-dependent induction of the control reporter gene plasmid pNF-κB (data not shown).

Collectively, the data on IκB-mediated regulation of Prx promoter activity along with that of the EMSA studies (Fig. 4) suggest that the LPS-mediated inhibition of Prx I gene induction by TPA is not mediated via the classical NF-κB pathway.

LPS inhibits TPA-dependent phosphorylation of p65 at serine 276 and activity of a Gal4–p65 fusion protein

The NF-κB subunit p65 is a principal target for regulatory phosphorylation by stimulus-coupled kinase activity. Rapid and reversible phosphorylation of p65 at specific regulatory sites provides an important mechanism to modulate the transactivation capacity of NF-κB [31,33]. Because serine 276 plays a major regulatory role in gene activation by p65 [32], we examined the phosphorylation of serine 276 during treatment with TPA and LPS for up to 3 h in RAW264.7 cells with a phosphospecific antibody. Phosphorylation of serine 276 was visible as early as 5 min after exposure to TPA and the level of serine 276 phosphorylation was further up-regulated in a time-dependent manner (Fig. 6A). By contrast, treatment with LPS alone caused only a lower increase in serine 276 phosphorylation, and simultaneous treatment with TPA plus LPS reduced the level of serine 276 phosphorylation compared to treatment with TPA alone (Fig. 6A).

To investigate the functional relevance of serine 276 phosphorylation by TPA and LPS on p65 transactivation capacity, we also determined the regulation of the activity of a pGal4-p65 construct, in which p65 is fused to the DNA-binding domain of yeast Gal4. As monitored by luciferase activity of a cotransfected Gal4 reporter gene construct (pFR-luc), TPA significantly increased p65 transactivation. By contrast, LPS alone caused only a lower induction and the combination of TPA plus LPS led to a significant repression of luciferase activity compared with TPA alone (Fig. 6B). We also evaluated the regulation of a mutated Gal4–p65 construct, in which serine 276 of p65 was replaced by cysteine (Gal4-p65 S276C). Although basal luciferase activity of pGal4-p65 S276C was lower in comparison to that of wild-type pGal4-p65, treatment with TPA induced luciferase activity of this construct. Remarkably, and in contrast to wild-type pGal4-p65, simultaneous exposure of cells transfected with pGal4-p65 S276C to TPA plus LPS led to enhanced levels of luciferase activity, which were comparable to levels that were observed upon treatment with TPA alone (Fig. 6B). A similar regulatory pattern was observed for a pGal4-p65 mutant construct, in which serine 276 was replaced by alanine (data not shown). For comparison we examined the TPA- and LPS-dependent regulatory pattern of a Gal4–p65 fusion plasmid, in which serine 536 of the p65 transactivation domain was replaced by alanine. Similar to the pGal4-p65 S276C mutant, the basal activity of this construct was lower than that of wild-type pGal4-p65. Repression by LPS of TPA-dependent induction, however, was not lost with this construct, which is in contrast to the regulatory pattern observed for pGal4-p65 S276C mutant and indicates specificity for the effects observed with the latter construct.

Taken together, the data indicate that phosphorylation at serine 276 of p65 and transactivation of pGal4-p65 are induced by TPA and inhibited by simultaneous exposure to LPS.

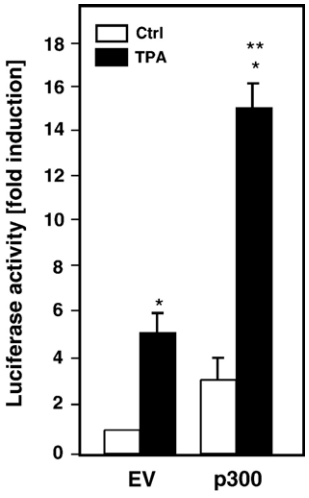


Fig. 7. Effect of overexpressed p300 on the regulation of Prx I promoter activity. RAW264.7 cells were cotransfected with pPrx-1750 and an expression vector for p300 or empty expression vector (EV). After transfection incubation of cells was continued with or without TPA (0.5 μM) for another 18 h. Cell extracts were assayed for luciferase activity and the -fold induction relative to the control was determined. Values are means ± SE from at least three independent experiments with duplicates of each point. Student's *t* test for paired values: significant differences, *TPA + EV versus control medium + EV and TPA + p300 versus control medium + p300, **TPA + p300 versus TPA + EV, $p \leq 0.05$.

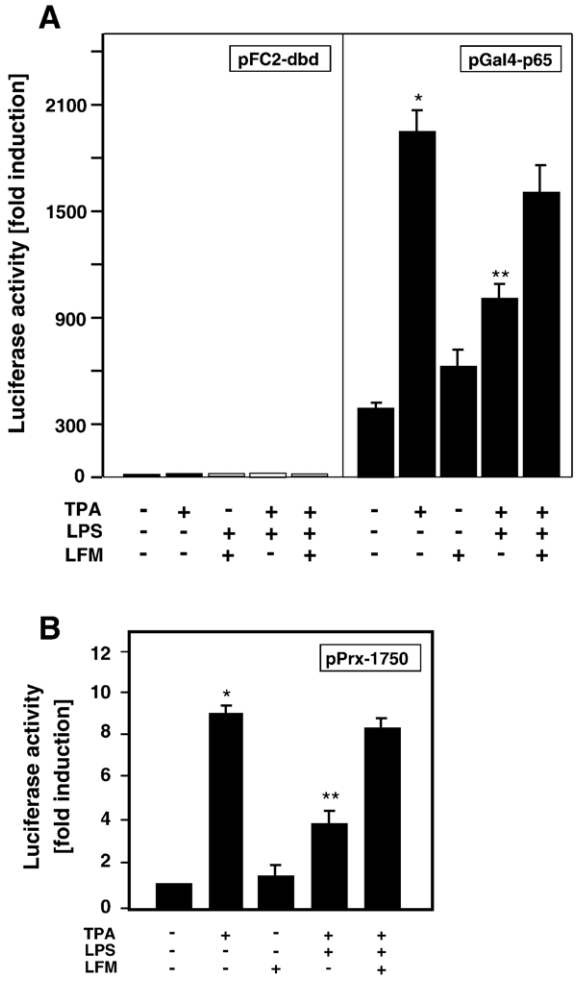


Fig. 8. Inhibition of Btk by LFM-A13 attenuates repression of TPA-dependent induction of the Prx I promoter by LPS. Cells were transfected (A) with pFR-luc and pFC2-dbd or pGal4-p65 and (B) with pPrx-1750. After transfection cells were pretreated with LFM-A13 (LFM; 100 μM) for 1 h, after which cell culture was continued for another 18 h with TPA (0.5 μM), LPS (1 μg/ml), or TPA plus LPS, as indicated. Cell extracts were assayed for luciferase activity and the -fold induction relative to the control was determined. Values are means±SE from at least three independent experiments with duplicates of each point. Student's *t* test for paired values: significant differences, *TPA versus control, **TPA + LPS versus TPA, $p \leq 0.05$.

Overexpression of p300 increases TPA-dependent Prx I promoter activity

Phosphorylation of serine 276 causes a conformational change in p65 that allows efficient recruitment of the co-activator p300/CREB-binding protein and increases gene transcription [31,32]. To explore the possibility that serine 276-dependent phosphorylation is involved in the regulation of Prx I gene expression, we determined the effect of a cotransfected expression vector for p300 on Prx I promoter activity. Overexpressed p300 enhanced the basal and the TPA-dependent induction of Prx I promoter activity (Fig. 7), suggesting that p300 is involved in the regulation of Prx I gene expression.

Inhibition of Bruton's tyrosine kinase blocks the repressive effect of LPS on TPA-dependent induction of p65 transactivation and phosphorylation of p65 at serine 276

Btk, which is a member of the Tec family of non-receptor tyrosine kinases, is involved in LPS signaling via TLR4 in mononuclear cells [34–36]. To investigate the potential role of Btk for Prx I gene expression, we determined the effect of the pharmacological Btk inhibitor LFM-A13 on the regulation of pGal4-p65 activity by TPA and LPS. Pretreatment with LFM-A13 markedly reduced the LPS-dependent repression of TPA-dependent transactivation of pGal4-p65 (Fig. 8A). Moreover, the effect of LFM-A13 was evaluated for Prx I promoter regulation by these compounds. Similar to the regulation of pGal4-p65 transactivity, exposure to LFM-A13 prevented the inhibitory effect of LPS on TPA-dependent induction of Prx promoter activity (Fig. 8B). To explore the possibility that the functional regulation of p65 transactivation and Prx I promoter activity in RAW264.7 cells would be mediated via Btk, we also determined the levels of p65 phosphorylation at serine 276 by TPA and LPS in the presence or absence of LFM-A13. Importantly, LPS-mediated inhibition of p65 phosphorylation by TPA at serine 276 was blocked by pretreatment with LFM-A13 (Fig. 9).

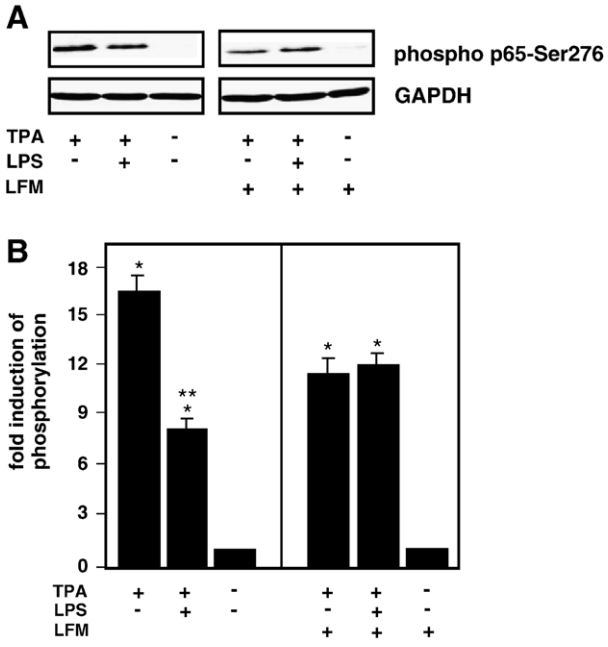


Fig. 9. LFM-A13 blocks the inhibitory effect of LPS on TPA-dependent induction of p65 phosphorylation at serine 276. (A) RAW264.7 cells were treated with TPA (0.5 μM) plus LPS (1 μg/ml) in the presence or absence of LFM-A13 (LFM; 100 μM) for 3 h, as indicated. Total protein (60 μg) was subjected to Western blot analysis and probed sequentially with antibodies against phosphorylated serine 276 of p65 and GAPDH. Similar results were obtained in three independent experiments and a representative autoradiogram is shown. (B) The autoradiographic signals of (A) were quantitated by videodensitometry. Values±SE represent the -fold induction of phospho p65-Ser276 normalized to GAPDH from three independent experiments. Student's *t* test for paired values: significant differences, *treatment versus control, **TPA + LPS versus TPA, $p \leq 0.05$.

The data indicate that the non-receptor tyrosine kinase Btk is involved in LPS-mediated inhibition of TPA-dependent induction of Prx I gene expression via specific regulation of p65 phosphorylation at serine 276.

Discussion

The antioxidant Prx I is a thioredoxin-dependent peroxidase which belongs to the Prx family. Members of this protein family have been identified in a variety of organisms ranging from bacteria to mammals [1–3,7]. Gene expression of Prx I has previously been shown to be induced by oxidative stress stimuli [7]. The major findings of the present study are that the prototypical proinflammatory mediator LPS inhibits the transcriptional induction of Prx I gene expression by the tumor promoter TPA in monocytic cells. NF-κB, which binds to a newly identified κB site of the Prx I gene promoter, is involved in this regulatory mechanism. Phosphorylation of the NF-κB subunit p65 on serine 276 is up-regulated by TPA and this TPA-dependent induction is inhibited by simultaneous exposure to LPS. LPS-dependent repression of Prx I gene induction by TPA is mediated via a signaling cascade downstream of Ras and involves the non-receptor tyrosine kinase Btk.

LPS inhibits the TPA-dependent transcriptional Prx I gene activation in monocytic cells

Prx I gene expression has previously been shown to be induced by the tumor promoter TPA in RAW264.7 cells [16] and by the proinflammatory stimulus LPS in primary cultured rat liver tissue macrophages [19]. Thus, it was unexpected that the up-regulation of the Prx I gene expression by TPA was markedly inhibited by simultaneous exposure to LPS in monocytic cells (Fig. 1). LPS-mediated repression of Prx I gene induction by TPA was cell-specific, because this inhibitory effect was not observed in non-monocytic cells (Table 1). Moreover, such a regulatory pattern did not apply to the regulation of the COX-2 and TNF-α genes by TPA and LPS (Fig. 1). Similar to the negative regulation of Prx I by LPS, an inhibitory effect of LPS has previously been reported for the constitutive and inducible expression of the microsomal epoxide hydrolase and glutathione *S*-transferase genes in rat liver [37].

Because luciferase activity of an AP-1 reporter gene construct was induced in an additive manner by TPA plus LPS (Fig. 1C), LPS-dependent Prx I gene repression seemed not to be regulated via the TF AP-1. Two previously identified AP-1 sites of the Prx I promoter, however, mediated the weak induction of Prx I by LPS (Fig. 1C). Therefore, we hypothesized that an enhancer element(s) independent of previously characterized Prx I AP-1 sites [16] would be involved in this regulatory mechanism. Accordingly, a functional κB site of the rat Prx I promoter was identified, which matches the κB-enhancer consensus sequence in 9 of 10 bp (Fig. 3A). The Prx-κB site conferred TPA-responsiveness to luciferase reporter gene constructs and deletion of this element attenuated LPS-mediated inhibition of TPA-dependent Prx I promoter induction (Fig. 3). The differential regulatory pattern in response to TPA and LPS of the Prx I, COX-2, and TNF-α promoters (Fig. 1B), all of which contain κB sites, suggested that the configuration of other promoter sequences may also play a role in the regulation of these genes. The Prx- κB site served as a nuclear target for the NF-κB subunit p65, which is synonymous with RelA (Fig. 4). It is remarkable, however, that TPA-inducible binding of nuclear proteins to the Prx-κB site was not changed by simultaneous treatment with TPA plus LPS (Fig. 4). Moreover, overexpressed dominant negative IκB had no appreciable effect on LPS-mediated repression of Prx I promoter induction by TPA (Fig. 5B). These findings indicate that the classical NF-κB pathway, which governs gene expression via inducible phosphorylation and degradation of IκB [31,32], does not play a major role in the LPS-mediated inhibition of Prx I gene activation by TPA. Because LPS markedly decreased TPA-induced phosphorylation of the NF-κB subunit p65 on serine 276 (Fig. 6A), the present data suggest that direct phosphorylation of p65 via an IκB-independent pathway mediates Prx I gene regulation by these two compounds. In line with such a conclusion, TPA-induced transactivation of a wild-type Gal4–p65 fusion protein was down-regulated by simultaneous exposure to LPS, but TPA-mediated up-regulation of a pGal4-p65 construct with a targeted serine 276 mutation of p65 (Gal4-p65 S276C) was not affected by LPS (Fig. 6B). The NF-κB subunit p65, which is a principal target of inducer-specific regulatory phosphorylation, is known to modulate the transcriptional activity of NF-κB [31,32]. Specifically, phosphorylation of p65 on serine 276 is known to cause a conformational change to make p65 more accessible for interaction with the transcriptional coactivator p300/CREB-binding protein, which increases transcriptional gene expression [32,38]. This mechanism may also be involved in transcriptional Prx I gene regulation, because basal and TPA-regulated Prx I promoter activity was strongly enhanced by overexpressed p300 (Fig. 7).

Inhibitory effects that are brought about by modulation of the transcriptional activity of NF-κB have previously been reported for the regulation of other genes. Waltner-Law et al. have shown that NF-κB represses the induction of phosphoenolpyruvate carboxykinase gene expression by glucocorticoids and cAMP in hepatoma cells [39]. Moreover, interleukin-1 has been shown to inhibit the up-regulation of the α_2-macroglobulin gene by interleukin-6 via interaction of NF-κB with the TF STAT3 [40]. More recently, activation of NF-κB by TNF-α has also been reported to decrease the insulin-mediated induction of glucose-6-phosphatase gene expression, independent of a direct DNA interaction with the promoter of this gene [26]. To our knowledge the present report is the first to demonstrate gene repression that is mediated by a stimulus-coupled inhibitory effect on p65 phosphorylation and adds to the multiple levels of gene regulation that are mediated via NF-κB [31–33,41].

Signaling pathways that mediate the LPS-dependent inhibition of Prx I gene induction by TPA

Activation of PKC plays a crucial role in signaling by the phorbol ester TPA [42]. Accordingly, Prx I gene induction by TPA has previously been shown to be regulated via a PKC/Ras-

mediated pathway in mononuclear cells [16]. Because LPS strongly reduced Prx I promoter activation by Ras (Fig. 2), the repression of Prx I gene induction by LPS is regulated via a signaling pathway downstream of Ras. A candidate molecule that could be involved in the inhibition of TPA-dependent Prx I gene induction via LPS is the non-receptor tyrosine kinase Btk [36]. This conclusion is supported by the finding that inhibition of Btk with the small-molecule inhibitor LFM-A13 reduced not only LPS-dependent repression of TPA-mediated induction of Prx I promoter activity and Gal4–p65 transactivation (Fig. 8), but also that of p65 phosphorylation at serine 276 (Fig. 9). Btk is an important regulator of B-cell signaling [36] and has also been shown to be involved in TLR4 signaling, which is activated by LPS in monocytic cells [18,34]. It is entirely possible, however, that other kinases such as the tyrosine kinase c-abl or the serine/threonine kinase Atm, which have recently been shown to affect Prx I gene expression [15], are involved in this regulatory pathway. NF-κB has previously been reported to be phosphorylated on serine 276 of p65 via an atypical protein kinase A [31] or mitogen- and stress-activated kinase-1 [25]. More recently, the latter kinase has also been shown to be involved in p65 phosphorylation via p38 MAP kinase by *Borrelia burgdorferi* antigens in RAW264.7 cells [43]. Because activation of TLR4 signaling by LPS in mononuclear cells is still incompletely understood, it is also conceivable that signaling molecules other than kinases may be involved in LPS-dependent gene regulation of Prx I. Potential candidates for such a mechanism are the adaptor proteins Dok-1 and Dok-2, which down-regulate LPS signaling in macrophages [44].

Physiological significance of Prx I gene regulation by TPA and LPS in monocytes

The present findings suggest that different signaling programs in mononuclear cells may be integrated at the level of Prx I gene expression. Prx I gene activation by the phorbol ester TPA, which primarily affects cellular proliferation and differentiation, is inhibited by the bacterial product LPS, which is a prototypical proinflammatory mediator. The exact regulatory role of Prx I with respect to carcinogenesis is contradictory. On the one hand observations in Prx I genetically deficient mice have suggested that this gene may be an important tumor suppressor. On the other hand, not only has it been shown that Prx I is highly expressed in various malignant tumors, it has also been demonstrated that Prx I may promote an aggressive survival type of tumor cells and resistance to tumor therapy [10,11]. Therefore, regulation of Prx I gene expression by TPA and LPS in mononuclear cells may have important implications for carcinogenesis as well as chronic inflammation. It is also remarkable that not only is Prx I gene expression regulated by NF-κB, as demonstrated in the present report, but also that Prx can modulate the activity of NF-κB via enzymatic degradation of hydrogen peroxide [45]. In conclusion, further studies are necessary to understand the regulatory mechanisms that govern Prx I gene expression. Such studies may ultimately help to develop novel therapeutic strategies for the treatment of cancer and inflammation.

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3.1. HO-1 gene activation by PMA via an atypical NF-κB pathway in monocytes (Paper II)

HO-1 gene expression was induced by PMA and LPS in monocytic cells. PMA and LPS, however, induced HO-1 gene expression with different time kinetics and had an additive effect on HO-1 gene expression when treated simultaneously (*Figure 1, paper II*). These observations suggest that distinct signaling pathways mediate HO-1 gene activation by PMA and LPS.

Although several earlier studies have implicated a regulatory role of NF-κB for the induction of HO-1 gene expression, the molecular basis of this regulation has remained elusive (Alam et al., 2007). The present paper identified a functional κB site of the rat HO-1 promoter that serves as a nuclear target for the NF-κB subunit p65. Functional studies and nuclear binding assays indicated that this κB site mediated the activation of HO-1 gene expression by PMA (*Figures 2 – 4, paper II*). A previously identified PMA-responsive element of the human HO-1 promoter (Muraosa et al., 1993) was not functional in the context of the rat HO-1 gene promoter (*Figure 2, paper II*). These potential species-specific differences of HO-1 gene expression were also supported by computational comparisons of DNA-sequences from rat, mouse and human HO-1 gene promoters, which exhibited significant structural discrepancies.

An IKK-independent atypical activation of NF-κB was involved in the regulation of HO-1 gene activation by PMA, because overexpression of IκBα, but not that of IKK2, attenuated PMA-dependent induction of HO-1 promoter activity (*Figure 6, paper II*). IKK-independent activation of NF-κB has previously been shown to be mediated via phosphorylation of IκB at tyrosine 42 in response to oxidative stress stimuli (Takada et al., 2003). Accordingly, treatment with PMA, a known inducer of ROS in monocytes (Datta et al., 2000) led to a transient phosphorylation of IκB at tyrosine 42 (*Figure 6C, paper II*). These weaker phosphorylation levels, however, do not correlate with the strong induction of HO-1 gene expression by PMA suggesting that IκB phosphorylation at tyrosine 42 does not play a major role for this induction. In addition, studies with the pharmacological inhibitors demonstrated that p38 MAPK and CK2 are involved in the regulation of PMA-dependent induction of HO-1 gene expression (*Figures 8 – 10, paper II*).

Therefore, it can be concluded that the PMA-dependent induction of HO-1 gene is regulated via an atypical NF-κB pathway that involves phosphorylation of IκB at the C-terminal domain in a p38 and CK2 dependent manner (Figure 6). A similar signaling cascade has previously been demonstrated for NF-κB activation by UV-light in HeLa cells (Kato et al., 2003).

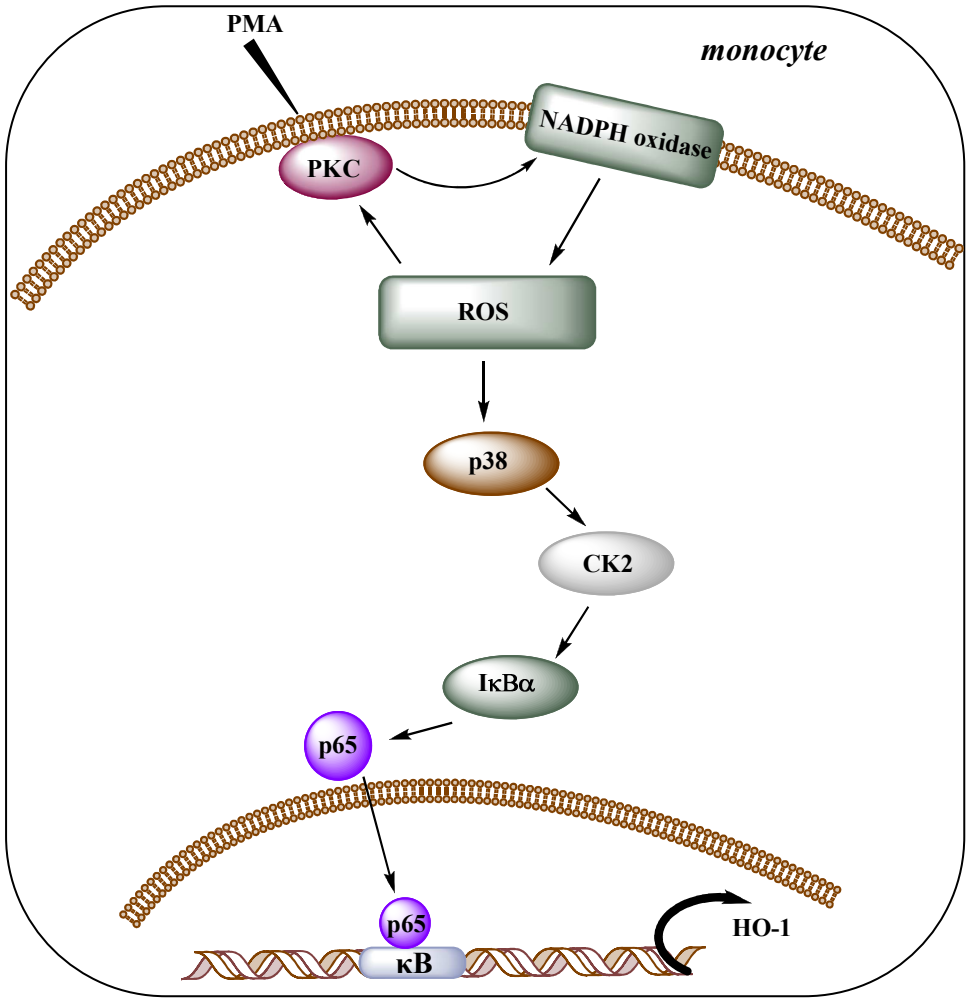

Figure 6: Signaling pathways of HO-1 gene activation by PMA

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

Srivatsava Naidu,* Nastiti Wijayanti,* Sentot Santoso,* Thomas Kietzmann,† and Stephan Immenschuh[2]*

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)[3]-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, $HO\text{-}1^{-/-}$ 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, $CD14^{+}$

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[3] Abbreviations used in this paper: HO, heme oxygenase; IKK, I-κB kinase; LTM, liver tissue macrophage; MEF, mouse embryonic fibroblast; MTE, macrophage-specific 12-*O*-tetradecanoyl-phorbol-13-acetate-responsive element; NAC, *N*-acetylcysteine; NE, nuclear extracts; Nrf2, NF-E2-related factor-2; PKC, protein kinase C; RE, regulatory element; RFBD, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; ROS, reactive oxygen species; TF, transcription factor.

immunomagnetic microbeads were from Miltenyi Biotec, and polyvinylidene difluoride membranes were from Millipore. All other chemicals were purchased from Sigma-Aldrich and Roche Applied Science unless otherwise indicated.

Cell isolation and culture

Liver tissue macrophages (LTM), peritoneal macrophages (24), rat hepatocytes (25) and human PBMC were isolated and cell culture was maintained 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 DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. All cell cultures were kept under air/CO_2 (19:1) at 100% humidity. Treatment of cells with PMA (0.5 μM) and LPS (*Escherichia coli* 0111:B4; 1 μg/ml) was performed with serum-free medium. Specific inhibitors of CK2, apigenin, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (RFBD) (Calbiochem), p38 MAPK inhibitor SB202190 (Calbiochem), IKK2 inhibitor SC-514 (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 performed 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 micrograms of total protein was separated on a 12% SDS-polyacrylamide gel and electroblotted onto polyvinylidene difluoride membranes. Membranes were blocked with Tris-buffered saline containing 5% skim milk or 5% BSA, 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.1% Tween 20 for 1 h at room temperature. The primary Abs against HO-1 (Stressgen), and GAPDH (Hytest) were used at 1/1000 dilutions. The primary Abs for the detection of phosphorylated IκBα serine 32 (Cell Signaling) and tyrosine 42 (ECM Biosciences) were applied at the concentrations recommended by the manufacturers. Secondary Abs were goat anti-rabbit IgG HRP and anti-mouse IgG HRP (DPC Biermann) and were used at 1/20,000 and 1/100,000, respectively. The ECL chemiluminescent detection system (Amersham Biosciences) was used for detection according to the manufacturer's instructions.

Plasmid constructs

The luciferase reporter gene constructs pHO-1338, pHO-754, and pHO-347 have been described previously (28), pTNF-585 was from Dr. G. Duff (University of Sheffield, Sheffield, U.K.), and pNF-κB was from Dr. L. Schmitz (University of Giessen, Giessen, Germany). The expression vector for dominant negative IκBα was from Dr. R. Gaynor (University of Texas Southwestern Medical Center, Houston, TX), the expression plasmid pRSV-NF-κB (p65/RelA) was from Dr. D. Schmoll (Sanofi Aventis Pharma) (30), and the expression vector for CK2α was from Dr. R. Kemler (Max-Planck-Institute for Immunobiology, Freiburg, Germany) (31). The plasmid pFA-CHOP with the transactivation domain of the TF CHOP fused with the DNA-binding domain of yeast Gal4 and the empty control vector pFC2-dbd were purchased from Stratagene. The reporter gene construct pHO-347 κBmut, which contains targeted mutations within the rat HO-1 proximal κB site, was generated with the template pHO-347 and the oligonucleotides mutκBfor (5′-GAATTGTCTCCTAGTTCTCCTACCTTGGAGATTCC TGAGAGGGC-3′) as forward primer and mutκBrev (5′-GCCTCTCAG GAATCTCCAAGGTAGGAGAACTAGGAGACAATTC-3′) as reverse primer. The reporter construct pHO-347 MTE was generated with the oligonucleotides (5′-GAGCTTGCCAGAGCTATACAATTTATC CCCATAC-3′) as forward primer and (5′-GTATGGGGATAAATTGTA TAGCTGTGGCAAGCTC-3′) as reverse primer and pHO-347 as template. Both plasmids were generated with the QuikChange XL site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. Plasmid pHO-20 was generated from synthetic oligonucleotides containing the rat HO-1 region from −20 to + 71 bp and *Xho*I or *Kpn*I restriction sites at their ends (MWG-Biotech). Annealed dsDNA was ligated into *Xho*I/*Kpn*I sites of pGL3basic (Promega) by using standard molecular cloning methods. All constructs were verified by DNA sequencing in both directions.

Transfection and luciferase assay

After growth for 24 h, transfection of plasmid DNA into RAW264.7 cells and MEF was performed by using FuGENE (Roche Applied Science) as described previously (29). Unless otherwise mentioned, cells were transfected with 0.5–1 μg of the reporter plasmid and, in cotransfection experiments, with 0.1–1 μg of the indicated expression vectors. Transfection efficiency was controlled using 0.1 μg of *Renilla* luciferase expression vector pRL-SV40 (Promega) as described previously (29). Cells were lysed with luciferase lysis reagent (Promega) and luciferase activity was determined with a commercial Dual-Luciferase reporter assay system (Promega) according to the manufacturer's instructions. Cells were either harvested 24 h after transfection or treated for the time points indicated with PMA or other reagents, as indicated. Relative light units of *Firefly* luciferase activity were normalized with *Renilla* luciferase activity.

Preparation of nuclear extracts (NE) and EMSA

NE were prepared as described previously (29). The sequences of the biotin-labeled oligonucleotides (MWG-Biotech) used for the EMSA are as follows: HO-κB-B, (5′-CCTAGTTCTGGAACCTTCCAGATTCCT GA-3′), HO-κBmutant (HO-κB-Bmut), (5′-CCTAGTTCTTTAACCG TTAAGATTCCTGA-3′), and NF-κB consensus oligonucleotide with sequence (5′-AGTTGAGGGGACTTTCCCAGGC-3′) with respective oligonucleotides of the noncoding strand. For competition assays, an excess of unlabeled oligonucleotide was added as indicated. After preincubation 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 interaction 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. 1*A*) and peritoneal macrophages (data not shown), but not in hepatocytes (Fig. 1*A*). Subsequently, we examined 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 induced by this treatment (Fig. 1*B*). It is also remarkable, that PMA-dependent induction of HO-1 gene expression was observed in human PBMC (Fig. 1*B*). 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 different from that 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 mechanism(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

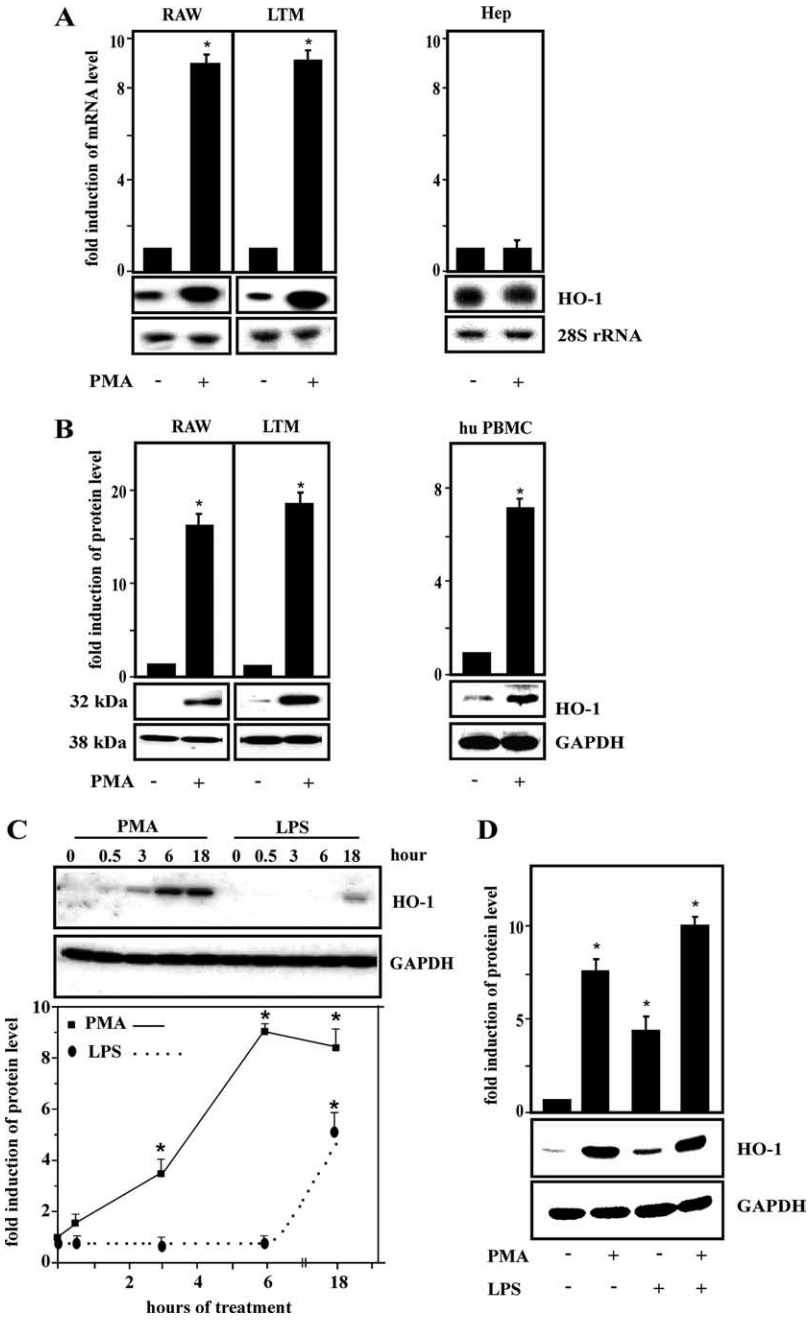

FIGURE 1. PMA-dependent induction of HO-1 gene expression in monocytic cells. RAW264.7 cells (RAW), LTM, hepatocytes (Hep), and human PBMC were cultured as described under *Materials and Methods*. *A*, Cells were treated with (+) or without (−) PMA (0.5 μM) for 6 h in serum-free medium, as indicated. Total cellular RNA (5 μg) was isolated and subjected to Northern blot analysis, and the blots were sequentially probed with a ^{32}P-labeled cDNA of rat HO-1 and a 28S rRNA oligonucleotide. *B*, RAW264.7 cells, LTM, cells and human (hu) PBMC were treated with PMA (0.5 μM) or control medium for 6 h in serum-free medium. *C*, RAW264.7 cells were treated with PMA (0.5 μM), LPS (1 μg/ml), or control medium for the times indicated. *D*, RAW264.7 cells were treated with PMA (0.5 μM) and/or LPS for 18 h in serum-free medium, as indicated. *B–D*, Total protein (50 μg) was subjected to Western blot analysis and sequentially probed with Abs against HO-1 and GAPDH. *A–D*, Autoradiograms from representative experiments are shown respectively. The autoradiographic signals were scanned by video densitometry and quantitated using ImageQuant software. *A*, The signal of the 28S rRNA band served as internal standard. Numbers show the fold induction rate relative to control HO-1 mRNA expression from at least three independent experiments ± SEM. *B–D*, Values ± SEM represent the fold-induction of HO-1 normalized to GAPDH from three independent experiments. Statistics and Student's *t* test for paired values: ∗, significant differences for treatment vs control, $p \leq 0.05$.

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. 1*C*). 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. 1*D*). 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. 2*A*). 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. 2*A*).

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

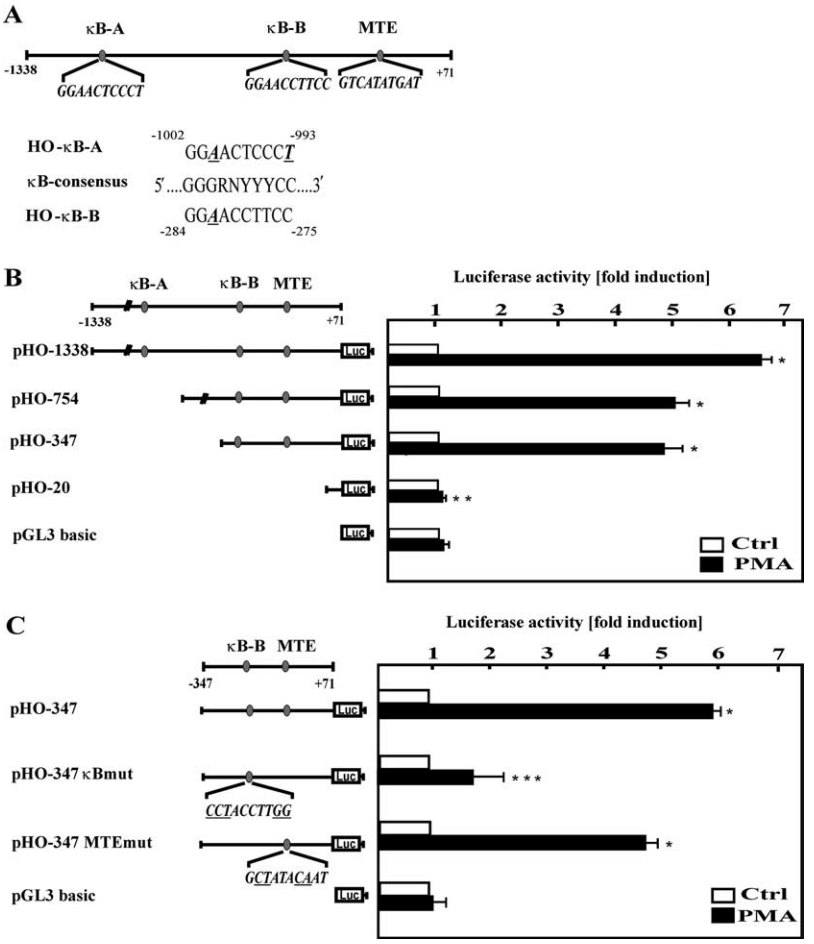

FIGURE 2. Identification of a functional κB element in the rat HO-1 promoter that mediates PMA-dependent induction. *A*, Localization of the HO-κB-A, HO-κB-B, and MTE sites within the rat HO-1 promoter and a sequence comparison with the NF-κB consensus sequence are shown. Mismatches to the prototypical NF-κB consensus sequence are bold and underlined. *B* and *C*, Luciferase (Luc) reporter gene constructs with the indicated rat HO-1 promoter fragments were transfected into RAW264.7 cells. Twenty-four hours after transfection, cells were treated with or without PMA (0.5 μM) for 18 h. Cell extracts were assayed for luciferase activity and the fold induction relative to the control was determined. Values are means ± SEM from at least three to four independent experiments with duplicates of each point. Student's *t* test for paired values: *, significant differences for PMA vs control (Ctrl); **, pHO-347 plus PMA vs pHO-20 plus PMA, $p < 0.05$ (*B*); ***, pHO-347 plus PMA vs pHO-347κBmut plus PMA, $p < 0.05$ (*C*).

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. 2*B*).

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 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 MTE sequence did not have a major effect on PMA-induced luciferase activity when compared with the wild-type construct (Fig. 2*C*). 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 with either 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. 3*A*). 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. 3*B*). 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. 3*C*). Moreover, incubation of the binding reaction with an Ab against the NF-κB subunit p65 caused a reduction of DNA-protein complex formation (Fig. 3*C*), 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

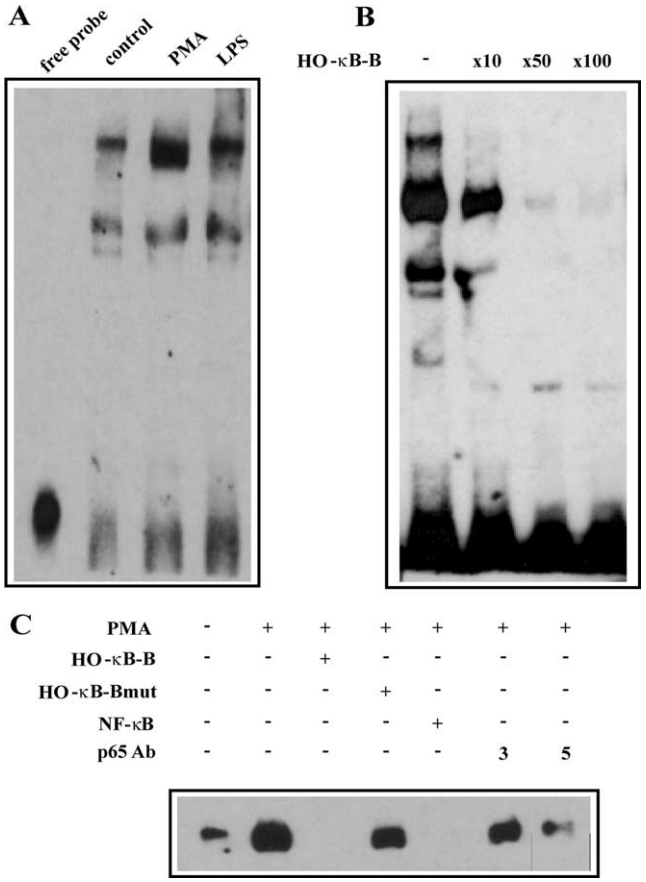

FIGURE 3. Binding of nuclear proteins to the HO-κB-B site. *A*, A biotin-labeled oligonucleotide with the HO-κB-B element was incubated with 7 μg of NE from control cells or from cells treated with PMA (0.5 μM), LPS (1 μg/ml), or without NE as a free probe. *B*, For competition analyses the biotin-labeled 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. *C*, Biotin-labeled HO-κB-B oligonucleotide was preincubated with 7 μg of 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 before the biotin-labeled HO-κB-B oligonucleotide was added. DNA-protein complexes were separated by electrophoresis on a 6% native polyacrylamide gel.

able to induce HO-1 expression in $p65^{-/-}$ MEF that lack p65, whereas PMA induced HO-1 expression in wild-type $p65^{+/+}$ MEF (Fig. 5*A*, *left panel*). Importantly, in $p65^{-/-}$ MEF in which p65 was reconstituted by stable transfection with an expression vector for p65 (27), PMA-dependent induction of HO-1 gene expression was similar to that in wild-type cells (Fig. 5*A*). We also determined PMA-dependent regulation of HO-1 promoter activity in $p65^{-/-}$ and $p65^{+/+}$ MEF. Luciferase activity of pHO-1338 was induced in $p65^{+/+}$ but not in $p65^{-/-}$ MEF. Similar to the observations on endogenous HO-1 gene regulation, PMA-dependent induction of HO-1 promoter activity was restored in p65-reconstituted $p65^{-/-}$ MEF (Fig. 5*B*). Taken together, the data confirm that p65 mediates the transcriptional induction of PMA-dependent HO-1 gene expression.

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

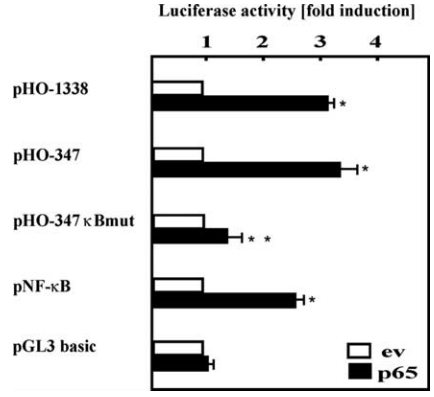

FIGURE 4. Effect of overexpressed p65 on HO-1 promoter activity. RAW264.7 cells were cotransfected with luciferase reporter gene constructs pHO-1338, pHO-347, pHO-347 κBmut, pNF-κB, and an expression vector for wild-type p65 or empty control expression vector (ev). Twenty-four hours after transfection, 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-347κBmut vs p65 plus pHO-347, $p < 0.05$.

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. 6*A*, 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. 6*B*, 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 not only treated with PMA but, for a comparison, also with LPS for various lengths of time, and cell extracts were analyzed for the phosphorylation of IκBα at serine 32. Treatment with LPS caused a marked increase of IκBα phosphorylation at serine 32 for up to 8 h. In contrast, PMA induced IκBα phosphorylation at serine 32 only to a minor extent (Fig. 6*C*, *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. 6*C*, *second panel from top*).

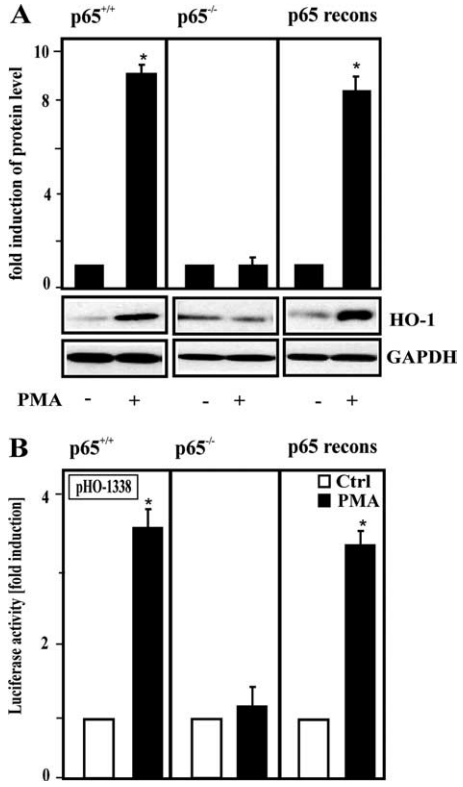

FIGURE 5. Regulation of HO-1 gene expression by PMA in p65-deficient MEF. *A*, $p65^{+/+}$, $p65^{-/-}$, and $p65^{-/-}$ MEF, in which p65 was reconstituted (p65 recons), were cultured as described under *Materials and Methods* and treated with PMA (0.5 μM) or control medium for 6 h. Western blot analysis and quantitation was performed as described in Fig. 1*B*. Values ± SEM represent the fold induction of HO-1 normalized to GAPDH from three independent experiments. Statistics and Student's *t* test for paired values: ∗, significant differences PMA vs control, $p \leq 0.05$. *B*, Cells were transiently transfected with the luciferase reporter gene construct pHO-1338 or empty control (Ctrl) vector pGL3basic. Twenty-four hours after transfection cells were cultured for another 18 h with or without PMA (0.5 μM). 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 PMA vs control, $p < 0.05$.

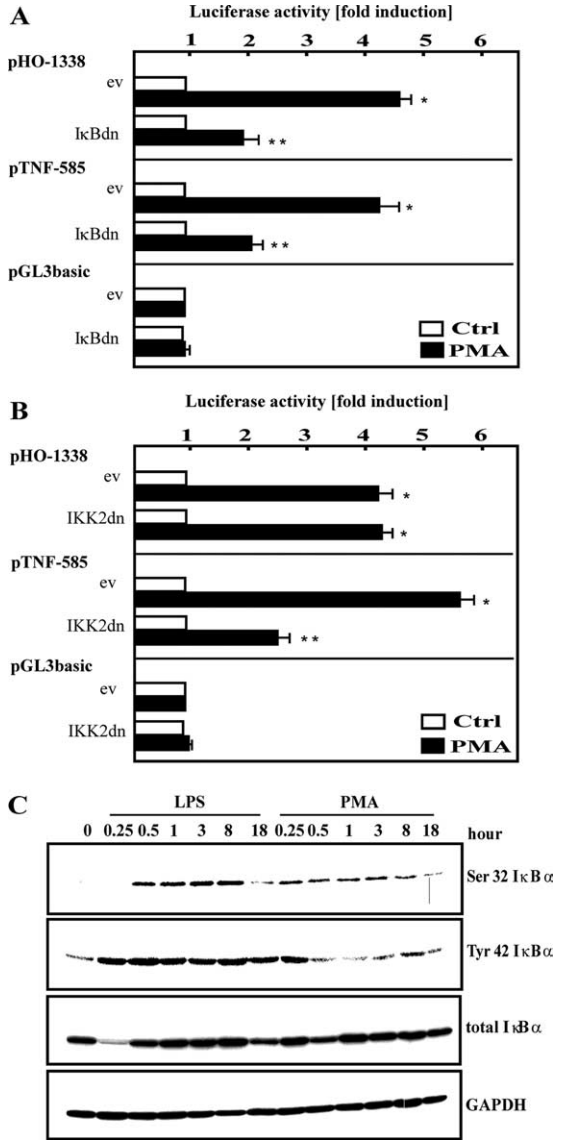

FIGURE 6. HO-1 promoter activation by PMA via an IκBα-dependent but IKK2-independent pathway. *A* and *B*, RAW264.7 cells were cotransfected with pHO-1338, pTNF-585, and an expression vector for dominant negative IκBα (*A*), dominant negative IKK2 (*B*) or empty control expression vectors (ev) (Ctrl, control), respectively. Twenty-four hours after transfection cells were treated for 18 h with or without PMA (0.5 μM). Luciferase assay and quantitation was performed as described in Fig. 2. Values are means ± SEM from at least three independent experiments with duplicates of each point. Student's *t* test for paired values: ∗, significant differences PMA vs control; ∗∗, PMA plus dominant negative IκBα (IκBdn) vs PMA plus empty vector or PMA plus dominant negative IKK2 (IKK2dn) vs PMA plus empty vector, $p < 0.05$. *C*, RAW 264.7 cells were treated with PMA (0.5 μM), LPS (1 μg/ml), or control medium for the times indicated. Total protein (50 μg) was subjected to Western blot analysis and probed sequentially with Abs against phosphorylated serine 32 (Ser 32 IκBα) and tyrosine 42 of IκBα (Tyr 42 IκBα), total IκBα, and GAPDH. Similar results were obtained in three independent experiments and a representative autoradiogram is shown.

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. 7*A*). 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 pHO-1338 reporter gene construct (Fig. 7*B*), 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

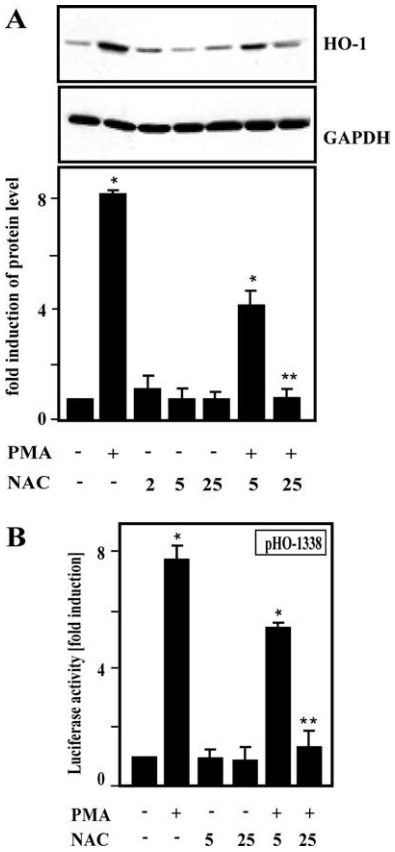

FIGURE 7. Effect of NAC on HO-1 gene activation by PMA. *A*, RAW264.7 cells were pretreated with NAC at concentrations of 2, 5, and 25 mM for 30 min, after which treatment was continued for another 6 h with (+) or without (−) PMA (0.5 μM). Western blot analysis and quantitation were performed as described in Fig. 1*B*. Values ± SEM represent the fold induction of HO-1 normalized to GAPDH from three independent experiments. Statistics and Student's *t* test for paired values: ∗, significant differences for PMA vs control; ∗∗, PMA plus NAC vs PMA, $p < 0.05$. *B*, RAW264.7 cells were transfected with pHO-1338. Twenty-four hours after transfection, cells were pretreated with NAC at concentrations of 5 or 25 mM for 30 min, after which treatment was continued for another 18 h with or without PMA (0.5 μM). Luciferase assay and quantitation was performed as described in Fig. 2. Values are means ± SEM from at least three independent experiments with duplicates of each point. Student's *t* test for paired values: ∗, significant differences for PMA vs control; ∗∗, PMA plus NAC vs PMA, $p < 0.05$.

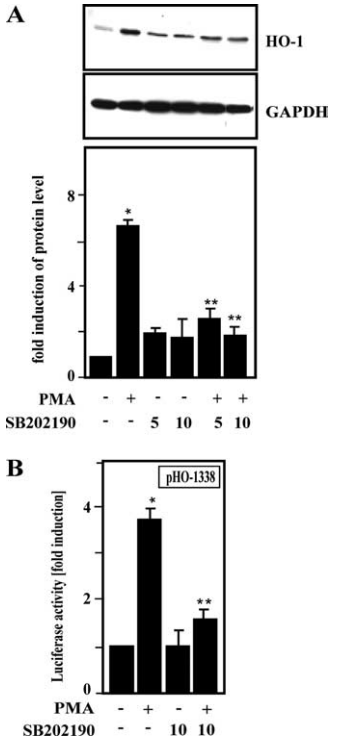

FIGURE 8. Effect of p38 MAPK inhibitor SB202190 on PMA-dependent HO-1 gene activation. *A*, RAW264.7 cells were pretreated with SB202190 (5 and 10 μM) for 30 min, after which treatment was continued for another 6 h with (+) or without−) PMA (0.5 μM). Western blot analysis and quantitation were performed as described in Fig. 1*B*. Values ± SEM represent the fold induction of HO-1 normalized to GAPDH from three independent experiments. Statistics and Student's *t* test for paired values: ∗, significant differences PMA vs control; ∗∗, PMA plus SB202190 vs PMA, $p < 0.05$. *B*, RAW264.7 cells were transfected with pHO-1338. Twenty-four hours after transfection cells were treated with SB202190 at a concentration of 10 μM for 30 min, after which treatment was continued for another 18 h with or without PMA (0.5 μM). Luciferase assay and quantitation were performed as described in Fig. 2. Values are means ± SEM from at least three independent experiments with duplicates of each point. Student's *t* test for paired values: ∗, significant differences PMA vs control; ∗∗, PMA plus SB202190 vs PMA, $p < 0.05$.

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. 8*A*). 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. 8*B*). 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. 9*C*). 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. 9*D*). 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

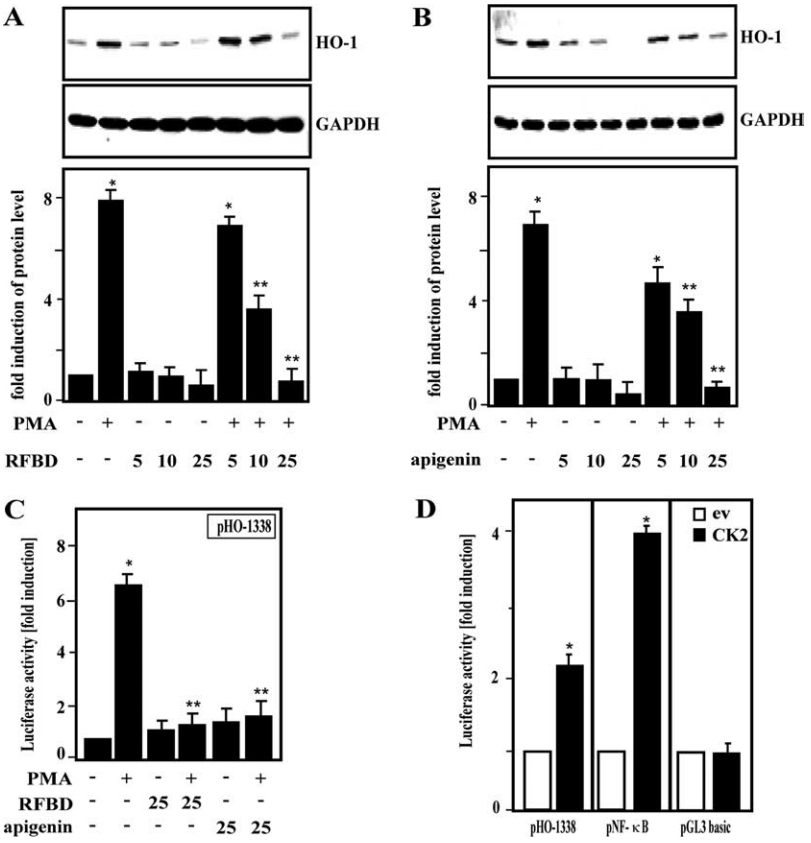

FIGURE 9. Effect of CK2 on PMA-dependent HO-1 gene activation. *A* and *B*, RAW264.7 cells were pretreated with RFBD (*A*) and apigenin (*B*) at increasing concentrations (μM) for 30 min, after which treatment was continued for another 6 h with (+) or without (−) PMA (0.5 μM). Western blot analysis and quantitation were performed as described in Fig. 1*B*. Values ± SEM represent the fold induction of HO-1 normalized to GAPDH from three independent experiments. Statistics and Student's *t* test for paired values: ∗, significant differences PMA *versus* control; ∗∗, PMA plus RFBD vs PMA and PMA plus apigenin vs PMA, $p < 0.05$. *C*, RAW264.7 cells were transiently transfected with pHO-1338. Twenty-four hours after transfection cells were treated with RFBD and apigenin (25 μM, respectively) for 30 min, after which treatment was continued for another 18 h with or without PMA (0.5 μM). Luciferase assay and quantitation were performed as described in Fig. 2. Values are means ± SEM from at least three independent experiments with duplicates of each point. Student's *t* test for paired values: ∗, significant differences PMA vs control; ∗∗, PMA plus RFBD vs PMA and PMA plus apigenin vs PMA, $p < 0.05$. *D*, RAW264.7 cells were cotransfected with pHO-1338, pNF-κB, and an expression vector for CK2 or empty control expression vector (ev). Twenty-four hours after transfection, luciferase assay and quantitation was performed as described in Fig. 2. Values are means ± SEM from at least three independent experiments with duplicates of each point. Student's *t* test for paired values: ∗, significant differences CK2 vs empty vector, $p < 0.05$.

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.

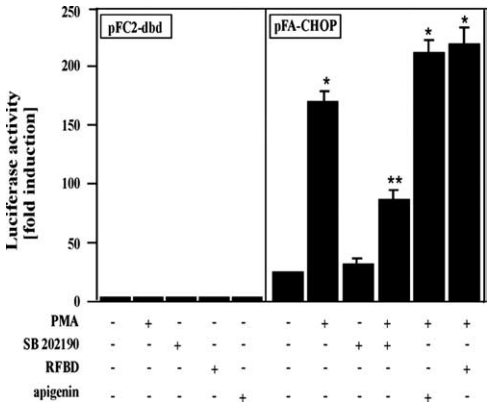

FIGURE 10. Regulation of PMA-dependent induction of CHOP transactivity by inhibition of p38 and CK2. RAW264.7 cells were cotransfected with the luciferase reporter gene construct pGal4-luc, pFC2-dbd, or pFA-CHOP, as indicated. Twenty-four hours after transfection, cells were treated with PMA (0.5 μM), SB202190 (10 μM), or the CK2 inhibitors RFBD (25 μM) and apigenin (25 μM), as indicated. Cell extracts were assayed for luciferase activity, and the fold induction relative to the control was determined. Values are means ± SE from at least three independent experiments with duplicates of each point. Student's *t* test for paired values: ∗, significant differences treatment vs control; ∗∗, SB202190 plus PMA vs PMA, $p < 0.05$.

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 κB site of the rat HO-1 promoter that is a nuclear target of p65/RelA; and 3) an IKK-independent, atypical NF-κB 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. 1*A*). 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. 1*C*). 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. 1*D*). Distinct kinetics of gene induction by PMA and LPS were also reported for cyclooxygenase-2 gene expression in monocytes (26, 45). The different kinetics of HO-1 gene induction by PMA and

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. 2*A*). 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 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. 6*C*).

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. 6*C*). 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 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 H_2O_2 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

The authors have no financial conflict of interest.

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3.1. Role of p38 MAPK for the regulation of HO-1 gene expression (Paper III)

Although p38 MAPK is considered to be a major mediator of HO-1 gene activation by a host of stimuli (Ryter et al., 2006), it has been suggested that p38 may play an inhibitory role for HO-1 gene expression (Yu et al., 2000, Keum et al., 2006). The present study demonstrated that pharmacological inhibition of p38 in monocytic cells and genetic deficiency of p38α in mouse embryonic fibroblasts (MEFs) up-regulated HO-1 gene expression, but not that of Cox-2 (*Figure 1, paper III*). Activation of HO-1 gene expression by inhibition of p38 was regulated at the transcriptional level and mediated via a StRE of the mouse HO-1 gene promoter that served as a nuclear target for the stress-inducible TF Nrf2 (*Figures 4, 6 and 7, paper III*). Thus, these findings would agree with the findings of Yu et al., 2000 and Keum et al., 2006.

Pharmacological inhibition or genetic deficiency of p38 MAPK up-regulated ERK activity. In addition, pharmacological blockage of ERK in p38α-deficient MEF attenuated HO-1 expression, suggesting cross-talk between these two signaling pathways for the regulation of HO-1 expression (*Figure 8, paper III*). In accordance with these findings, previous studies have independently demonstrated an inhibitory role of p38 for ERK (Singh et al., 1999) and JNK (Elbirt et al., 1998). These observations may suggest that p38 MAPK is an important up-stream regulator of ERK and JNK.

Recent studies have also indicated that p38 may serve as a sensor of the cellular redox balance (Dolado et al., 2007). Accordingly, deficiency of p38 has been shown to cause an increased generation of ROS, which agrees with the findings of the present study (*Figure 9, paper III*). It is conceivable that intracellular ROS levels that are increased by p38 deficiency are responsible for the activation of ERK. This hypothesis may be supported by a study showing that, ROS mediate sustained activation of ERK in human hepatoma HepG2 cells (Wu et al., 2006).

In summary, inhibition and genetic deficiency of p38 activates HO-1 gene expression via a transcriptional mechanism, which involves the redox-dependent TF Nrf2 (Figure 7), suggesting that p38 MAPK may play a dual role for the regulation of HO-1 gene expression.

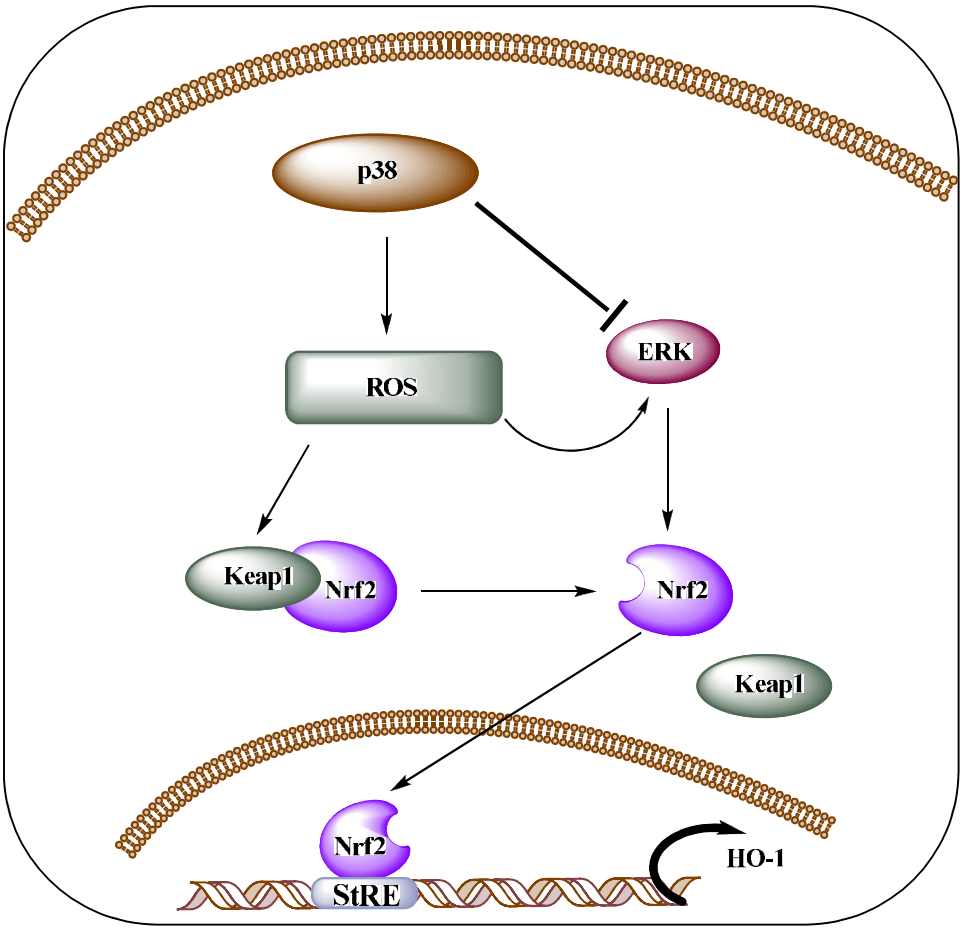

Figure 7: Inhibition of p38 MAPK activates HO-1 gene expression

Inhibition and genetic deficiency of p38 MAPK up-regulates heme oxygenase-1 gene expression via NF-E2-related factor-2 (Nrf2) °

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Running title: Up-regulation of heme oxygenase-1 via p38 inhibition

Key words: monocytes, gene regulation, transcription factors, MAPK, signal transduction

ABSTRACT

Heme oxygenase (HO)-1 is the inducible isoform of the first and rate-limiting enzyme of heme degradation. The HO products carbon monoxide and bilirubin not only provide antioxidant cytoprotection, but also have potent anti-inflammatory and immunomodulatory functions. Although HO-1 has previously been shown to be induced by various stimuli via activation of the p38 MAPK signaling pathway, the role of this protein kinase for HO-1 gene regulation is largely unknown. In the present study it is demonstrated that pharmacological inhibitors of p38 induced HO-1 expression in monocytic cells. Moreover, basal HO-1 gene expression levels were markedly higher in untreated murine embryonic fibroblasts (MEF) from $p38\alpha^{-/-}$ mice as compared to that from wild type mice. Transfection studies with luciferase reporter gene constructs indicate that increased HO-1 gene expression via inhibition of p38 was mediated by the transcription factor NF-E2-related factor- 2 (Nrf2), which is a central regulator of the cellular oxidative stress response. Accordingly, inhibitors of p38 induced binding of nuclear proteins to a Nrf2 target sequence of the HO-1 promoter, but did not affect HO-1 protein expression and promoter activity in $Nrf2^{-/-}$ MEF. Genetic deficiency of p38 led to enhanced phosphorylation of ERK and increased cellular accumulation of reactive oxygen species (ROS). In addition, pharmacological blockage of ERK and scavenging of ROS with N-acetylcysteine reduced HO-1 gene expression in $p38^{-/-}$ MEF, respectively. Taken together, it is demonstrated that pharmacological inhibition and genetic deficiency of p38 induce HO-1 gene expression via a Nrf2-dependent mechanism in monocytic cells and MEF.

INTRODUCTION

Heme oxygenase (HO)-1 is the inducible isoform of the first and rate-controlling enzyme of heme degradation (1). HO-1 is induced by a variety of oxidative stress stimuli and exerts potent antioxidant and anti-inflammatory effects via its products bilirubin and carbon monoxide (CO) (2, 3). The anti-inflammatory functions of HO-1 have been described in HO-1 knockout mice (4, 5) and have essentially been confirmed in a human case of genetic HO-1 deficiency (6). HO-1 deficient mice exhibit a disproportional activation of monocytes and are highly vulnerable to endotoxin-mediated toxicity (4, 7). The potential significance of HO-1 in the adaptive immune system has been implied by a recent report, in which genetic deficiency of HO-1 decreased the suppressive activity of regulatory T cells (8). Importantly, overexpression of HO-1 either by gene transfer or by pharmacological inducers has beneficial effects in various animal models of inflammatory disease (3, 9-13).

The up-regulation of HO-1 gene expression and that of other genes during inflammation and in response to pharmacological stimuli is mediated by a network of signaling pathways, among which MAPK play a central role (14). Three major subfamilies of MAPK are known: ERK, JNK and p38. While ERK mainly mediates cellular responses to hormones and growth factors, JNK and p38 are primarily activated by stress-related stimuli (14). Immunologically active cells such as monocytes and T cells display a high content of p38 MAPK (15) and it has initially been shown that p38 is activated by LPS stimulation of monocytes (16, 17). Subsequently, p38 has been shown to play a critcal role for the regulation of proinflammatory genes such as cytokines (18) and cyclooxygenase-2 (Cox-2)(19). Because targeted deletion of the p38α gene in mice causes embryonic lethality (20), p38α appears to be of major physiological significance among the known p38 isoforms. Although numerous studies have demonstrated that activation of p38

mediates HO-1 induction by a host of stimuli (2, 3, 9), an inhibitory role of p38 on HO-1 gene expression has previously been reported (21, 22). Thus, it was the primary goal of the present study to evaluate the role of p38 for HO-1 gene expression in further detail.

In the current report, it is shown that small molecule inhibitors of p38 induced HO-1 expression in monocytic cells and that basal HO-1 gene expression was constitutively high in murine embryonic fibroblasts (MEF) from p38$\alpha^{-/-}$ mice. Moreover, p38 inhibition-dependent HO-1 gene induction was regulated via the transcription factor (TF) NF-E2-related factor-2 (Nrf2), which is a central regulator of the cellular stress response. Activation of ERK and reactive oxygen species (ROS) were involved in this up-regulation of HO-1 gene expression.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium and RPMI 1640 were obtained from PAA Laboratories (Pasching, Germany), FCS was from Biochrom KG (Berlin, Germany), Ficoll-Paque was from Pharmacia (Freiburg, Germany), CD14+ immunomagnetic microbeads were from Miltenyi Biotech (Bergisch-Gladbach, Germany) and polyvinylidene diflouride membranes were from Millipore (Bedford, MA). All other chemicals were purchased from Sigma-Aldrich and Roche Applied Science, unless otherwise indicated.

Cell isolation and culture

RAW264.7 cells were from American Type Culture Collection (Manassas, VA), MEF from p38$\alpha^{-/-}$ mice were from Dr. Angel R. Nebreda (Spanish National Cancer Center, Madrid, Spain) (23), MEF from Nrf2$^{-/-}$ mice were from Dr. Larry Higgins (University of Dundee, UK)(24) and were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Human PBMC were isolated and maintained in culture as described previously (25). All cell cultures were kept under air/CO_2 (19:1) at 100% humidity. Treatment of cells with PMA (0.5 μM), LPS (*E. coli* 0111:B4; 1 μg/ml), inhibitors of p38 MAPK (SB202190 and SB 203580, Calbiochem), ERK (PD 98059, Calbiochem) or JNK (SP 600125, Calbiochem) was performed with serum-free medium. N-acetylcysteine (NAC) was added to the culture medium 30 min prior to treatment with PMA, as indicated.

Subcellular fractionation

p38$\alpha^{-/-}$ and p38$\alpha^{+/+}$ MEF were cultured overnight in complete media and were washed twice in ice-cold PBS. Thereafter, cells were incubated in 200 μl of ice-cold homogenizing buffer

containing 250 mM sucrose pH 7.4, 5 mM MOPS pH 7.4, 0.1% ethanol, 1 mM EDTA, 0.2 mM DTT, 1 mM 6-aminocaproic acid and protease inhibitor cocktail for 15 min. Cells were scraped from the dishes, dounce-homogenized with 50 strokes and centrifuged at 2500 x *g* for 20 min at 4°C to separate nuclei from cytosol. The supernatant was collected as cytosolic fraction, and the pelleted nuclear fraction was washed twice in homogenizing buffer and incubated in 100 µl of extraction buffer containing 25% glycerol, 20 mM Hepes, pH 7.9, 0.4 M NaCl, 1 mM EDTA, pH 8.0, 1 mM EGTA, 1 mM DTT and protease inhibitor cocktails. Samples were rocked at 4°C for 20 min and centrifuged at 12,000 x *g* for 10 min. This fraction was collected as nuclear extract (NE). All fractions were brought to equal volumes and protein content was determined for the cytosol and nuclear extract fractions.

Western blot analysis

Cells were washed with 0.9% NaCl and thereafter lysis and protein quantitation was essentially performed as described previously (25). 50 µg of total protein was separated on a 12% SDS-polyacrylamide gel and was blotted onto polyvinylidene difluoride membranes. Membranes were blocked with Tris-buffered saline containing 5% skim-milk or 5% BSA, 50 mM Tris/HCl (pH 7.6), 150 mM NaCl and 0.1% Tween 20 for 1 h at room temperature. The primary antibodies against HO-1 (Stressgen, Victoria, BC, Canada), peroxiredoxin 1 (26), Cox-2 (Alexis Biochemicals, Grünberg, Germany) and GAPDH (Hytest, Turku, Finland) were used at 1:1000 dilutions. The primary antibodies against Nrf2 and lamin B1 were from (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and were used at 1:2000 dilutions. The primary antibody against Bach1 was a gift from Dr. Kazuhiko Igarashi (Tohoku University School of Medicine, Sendai, Japan) and was used at 1:1000 dilution (27). Primary antibodies for detection of phosphorylated and total MAPK were from Cell Signaling and were applied at the

concentrations recommended by the manufacturer, respectively. Secondary antibodies were goat anti-rabbit IgG horseradish peroxidase and anti-mouse IgG horseradish peroxidase (DPC Biermann, Bad Nauheim, Germany) and were used at 1:20,000 and 1:100,000, respectively. The ECL chemiluminescent detection system (Amersham) was used for detection according to the manufacturer's instructions. The chemiluminescent autoradiographic signals were visualized and quantitated with the Fluorchem FC2 gel documentation system.

Plasmid constructs

The luciferase reporter gene constructs pHO-4045-luc was a gift from Dr. Mark A. Perella (Harvard Medical School, Boston, Massachusetts)(28), pE2-luc was a gift from Dr. Jawed Alam (Alton Ochsner Medical Center, New Orleans, LS)(29). Plasmid pFA-CHOP with the transactivation domain of the TF CHOP, plasmid pFA-Elk with the transactivation domain of the TF Elk each fused with the DNA-binding domain of yeast Gal4 and the empty control vector pFC2-dbd were purchased from Stratagene. The plasmid pAP-1-luc with three AP-1 repeats in front of a minimal fos promoter was a gift from Dr. Craig A. Hauser (The Burnham Institute, La Jolla, CA)(30).

Transfection and luciferase assay

After growth for 24 h, transfection of plasmid DNA into RAW264.7 cells and MEF was performed by using FuGENE (Roche Applied Science) as described previously (25). Unless otherwise indicated, cells were transfected with 0.5 to 1 μg of the reporter plasmid, and in cotransfection experiments with 0.1 to 1 μg of the indicated expression vectors. Transfection efficiency was controlled using 0.1 μg of *Renilla* luciferase expression vector pRL-SV40 (Promega) as described previously (25). Cells were lysed with luciferase lysis reagent (Promega), and luciferase activity was determined with the commercial Dual Luciferase reporter

assay system (Promega) according to the manufacturer's instructions. Cells were either harvested 24 h after transfection or treated for another 18 h with the indicated reagents, respectively. Relative light units of *Firefly* luciferase activity were normalized with *Renilla* luciferase activity.

Preparation of NE and EMSA

NE were prepared as described previously (25). The sequences of the biotin-labeled oligonucleotides (MWG, Ebersberg, Germany) used for the EMSA are as follows: E2, (5'-GTGCCTTTTCTGCTGAGTCAAGGTCCGGGG-3'), E2mutant (E2mut), (5'-GTGCCTTTTCT-GCTTATTTAAGGTCCGGGG-3') and NF-κB consensus oligonucleotide with sequence (5'-AGTTGAGGGGACTTTCCCAGGC-3') with respective oligonucleotides of the non-coding strand. For competition assays an excess of unlabeled oligonucleotide was added, as indicated. After preincubation for 10 min at room temperature, the biotin-labeled probe was added and incubation was continued for another 20 min. For supershift analysis 1 to 2 µl of antibody either directed against Nrf2 (Santa Cruz Biotechnology) or anti-sera raised against mouse NB1 protein (from Dr. Stroncek, NIH, Bethesda, NY), were added to the EMSA reaction, as indicated. The reaction mixture was loaded on a 6% native polyacrylamide gel in 0.5% TBE and was blotted onto nylon membranes (Pierce). After UV-cross-linking the LightShift Chemiluminescent EMSA kit (Pierce) was used to detect interaction between biotin end-labeled DNA and protein with a streptavidin-horseradish peroxidase conjugate and the chemiluminescent substrate.

Measurement of ROS

Intracellular generation of ROS was detected with dihydroethidium (DHE) dye (Invitrogen). The fluorescent byproduct ethidium, which is produced after DHE cleavage by ROS, was detected with a flow cytometer. Cells were incubated with or without SB 202190 (10 µM) for 1 h, after which they were washed and further incubated for 20 min with 5 µM DHE. Cells were then

washed with PBS and the levels of ROS were determined with a FACScalibur flow cytometer. DHE-detectable superoxide anion (FL2-H) fluorescent signals were displayed as histograms. Ratios of signals *versus* control data were calculated by mean fluorescence intensity.

RESULTS

Up-regulation of HO-1 gene expression via pharmacological inhibition and genetic deficiency of p38 MAPK

In agreement with earlier work the phorbol ester PMA and the proinflammatory mediator LPS induced HO-1 (25, 31) and Cox-2 (32) gene expression in cell cultures of RAW264.7 monocytes (Figure 1A). Previous reports have shown that p38 MAPK contributes to the PMA-dependent induction of these two genes (25, 31-33). Although pretreatment of RAW264.7 cells with SB 202190, which pharmacologically blocks p38α and p38β MAPK activity, reduced PMA-dependent induction of HO-1 and Cox-2, treatment with this compound alone markedly induced HO-1, but not Cox-2, gene expression (Figure 1B). This unexpected observation was confirmed in a second series of experiments, in which HO-1 gene expression in RAW264.7 cells was time-dependently up-regulated by SB 202190 and by a second p38α inhibitor, SB 203580 (Figure 1C). To specify these results we investigated PMA-dependent regulation of HO-1 and Cox-2 gene expression in cell cultures of MEF, that were genetically deficient for p38α ($p38\alpha^{-/-}$ MEF) in comparison to that in wild-type MEF ($p38\alpha^{+/+}$ MEF). While PMA induced HO-1 and Cox-2 expression in $p38\alpha^{+/+}$ MEF, it had only minor effects in $p38\alpha^{-/-}$ MEF. Importantly, the basal level of HO-1 protein expression was significantly higher in $p38\alpha^{-/-}$ MEF, if compared to that in $p38\alpha^{+/+}$ MEF (Figure 1D). In contrast, Cox-2 protein was hardly detectable in both $p38\alpha^{-/-}$ and $p38\alpha^{+/+}$ MEF (Figure 1D). For a comparison, we determined HO-1 gene expression in cell cultures of human PBMC. HO-1 gene expression was augmented in human PBMC in the presence of the p38 inhibitor SB 203580 (Figure 1E).

The data indicate that pharmacological inhibition and genetic deficiency of p38α causes an increased HO-1 gene expression.

Constitutive activation of p38 MAPK in RAW264.7 cells

It is generally accepted that numerous stimuli can activate p38 MAPK (14). Since pharmacological inhibition of p38 in RAW264.7 cells has an effect on HO-1 gene expression, which is similar to that in $p38^{-/-}$ MEF (Figure 1), we examined p38 activity in untreated RAW264.7 cells. Interestingly, p38 phosphorylation could be detected under basal conditions and exposure to SB 202190 and SB 203580 markedly attenuated this phosphorylation (Figure 2A). In addition, we verified the functionality of p38 in RAW264.7 cells by a cotransfection assay, in which a luciferase reporter construct with five binding sites for the yeast transcription factor Gal4 was used together with a construct allowing expression of a fusion protein consisting of the Gal4 DNA-binding domain and the transactivation domain of the TF CHOP (pFA-CHOP). Transactivation of CHOP is regulated by p38-dependent phosphorylation of regulatory serine residues within the CHOP transactivation domain (34). Similar to the data on p38 phosphorylation in Figure 2A, high luciferase activity of pFA-CHOP was observed under basal conditions and exposure of RAW264.7 cells to SB 202190 and SB 203580 reduced reporter gene activity (Figure 2B). When $p38^{-/-}$ and $p38^{+/+}$ MEF were transfected with pFA-CHOP, luciferase activity was low in $p38^{-/-}$ MEF, whereas it was significantly higher in $p38^{+/+}$ cells. Here, reporter gene activity was inhibited by SB 202190 and SB 203580 (Figure 2C). The data demonstrate that p38 MAPK activity is constitutively high in RAW264.7 monocytic cells.

HO-1 gene expression is not affected by inhibitors of JNK and ERK

To investigate whether inhibition of MAPK other than p38 would affect HO-1 gene expression, RAW264.7 cells were treated with specific inhibitors for JNK, SP 600125, and ERK, PD 98059, respectively. For a comparison cells were also treated with PMA. In contrast to p38 inhibitors, treatment with these inhibitors did not induce HO-1 gene expression (Figure 3) indicating that inhibition of JNK and ERK did not affect basal HO-1 gene expression in RAW264.7 cells.

Genetic deficiency and pharmacological inhibition of p38 induces HO-1 gene promoter activity

Nrf2, a member of the cap'n'collar family of basic leucine zipper proteins (35, 36), plays a major regulatory role for the stress-dependent transcriptional induction of HO-1 gene expression (37, 38). Although p38 has been shown to be involved in Nrf2 activation, the exact regulatory role of p38 for Nrf2 activation is not well understood (39). To further investigate this pathway, we determined the regulation of luciferase reporter gene constructs pHO-4045-luc and pE2-luc, both of which carry the proximal (E1) and distal (E2) enhancer sequences of the mouse HO-1 promoter (37). Both enhancer sequences contain multiple copies of StRE sites that are targeted by Nrf2 (2). For a comparison, we also transfected $p38^{-/-}$ and $p38^{+/+}$ MEF with the pAP-1-luc construct, which carries three copies of the consensus recognition sequence of AP-1 (30). Basal luciferase activity of the reporter gene constructs pHO-4045-luc and pE2-luc, but not that of pAP-1-luc, was markedly higher in $p38^{-/-}$ MEF as compared to that in $p38^{+/+}$ MEF (Figure 4A). Furthermore, treatment with SB 202190 up-regulated reporter gene activity of these constructs in RAW264.7 cells (Figure 4B). Luciferase activity of the pE2-luc construct was slightly higher in comparison to that of pHO-4045-luc in $p38^{-/-}$ MEF and in RAW264.7 cells (Figure 4). Taken

together, the data suggest that an Nrf2-dependent transcriptional mechanism is involved in HO-1 gene activation via inhibition of p38.

p38 contributes to nuclear localization of Nrf2

Nrf2 regulates HO-1 gene expression via shuttling between the nucleus and cytoplasm in response to various stress-stimuli (35, 36). To further investigate whether p38 may regulate Nrf2 localization, nuclear and cytoplasmic Nrf2 levels were determined in $p38^{-/-}$ and $p38^{+/+}$ MEF. For a comparison, we also determined protein levels of Bach1, which is a counter-regulator of Nrf2 and represses HO-1 gene expression (27). Nrf2 levels were higher in nuclear fractions of $p38^{-/-}$ MEF as compared to those in $p38^{+/+}$ MEF. By contrast, no appreciable difference of Nrf2 levels was observed in cytoplasmic fractions (Figure 5, upper panel). Remarkably, cytosolic and nuclear levels of Bach1 were inverse to those of Nrf2 with higher Bach1 levels in cytoplasmic fractions of $p38^{-/-}$ MEF as compared to those in $p38^{+/+}$ MEF.

Inhibitors of p38 do not up-regulate HO-1 gene expression in $Nrf2^{-/-}$ MEF

To further examine the role of p38 on Nrf2-dependent HO-1 gene regulation, $Nrf2^{-/-}$ and $Nrf2^{+/+}$ MEF were treated with small molecule inhibitors of p38 and PMA. Treatment with SB 202190 and SB 203580 significantly induced HO-1 gene expression in $Nrf2^{+/+}$ MEF, but not in $Nrf2^{-/-}$ MEF (Figure 6A) and luciferase activity of transfected pHO-4045-luc and pE2-luc constructs was markedly higher in $Nrf2^{+/+}$ MEF as compared to that in $Nrf2^{-/-}$ MEF (Figure 6B). Furthermore, treatment with SB 202190 up-regulated luciferase activity of two HO-1 gene constructs to a magnitude similar to that by PMA in $Nrf2^{+/+}$ MEF, but not in $Nrf2^{-/-}$ MEF (Figure 6B). In contrast, reporter gene activity of the pAP-1-luc construct was not affected by p38 inhibitors (Figure 6B). Taken together, the data suggest that inhibition of p38 induces HO-1 gene expression in a Nrf2-dependent manner.

Induction of DNA-binding of Nrf2 in response to p38 inhibitors

We also examined the binding activity of nuclear proteins from RAW264.7 cells to a biotin-labeled E2 oligonucleotide with a StRE site of the mouse HO-1 gene promoter (37) in EMSA studies. After treatment with p38 inhibitors NE from RAW264.7 cells formed an inducible DNA-protein complex, which was similar to that observed in response to treatment with LPS (Figure 7A). The intensity of the DNA-protein complex from SB 202190-treated cells was decreased by an unlabeled E2 oligonucleotide, but not by an oligonucleotide with a mutation of the StRE site (E2 mut, Figure 7B) or a NF-κB oligonucleotide (Figure 7C). Incubation of the DNA-protein binding reaction with an ab against Nrf2 reduced the intensity of DNA-protein complex formation (Figure 7C). Taken together, the data indicate that the StRE site of the E2 region is a nuclear binding site for Nrf2 and may be involved in the regulation of p38 dependent induction of HO-1 gene expression.

Role of ERK for HO-1 gene activation via p38 inhibition

ERK is involved in HO-1 gene induction by numerous stimuli (3, 9). Since p38 MAPK has previously been shown to have a negative regulatory effect on ERK activity (23), we investigated the effect of p38 MAPK inhibition on ERK phosphorylation and its potential effect on HO-1 gene expression in $p38^{-/-}$ and $p38^{+/+}$ MEF. As demonstrated in Figure 8A, ERK was strongly phosphorylated in $p38^{-/-}$ MEF, but not in $p38^{+/+}$ MEF. Serum starvation did not affect ERK phosphorylation in either cell type. Treatment with a specific inhibitor of ERK attenuated the increased levels of HO-1 in $p38^{-/-}$ MEF suggesting a regulatory cross-talk of these protein kinases for HO-1 gene regulation (Figure 8B). Subsequently, we determined the effect of p38 inhibition on ERK phosphorylation in RAW264.7 cells. Treatment with p38 inhibitors induced ERK phosphorylation in these cells similar to that by the known ERK activator PMA (Figure

8C). Finally, the reporter gene construct pFA-Elk which is specifically regulated via ERK, was markedly induced by p38 inhibitors in RAW264.7 cells. As a control, treatment with PMA strongly induced luciferase activity of pFA-Elk (Figure 8D). Taken together, the data suggests that genetic deficiency or pharmacological inhibition of p38 activates ERK and may be involved in the induction of HO-1 gene expression.

Genetic deficiency and pharmacological inhibition of p38 MAPK causes increased accumulation of cellular ROS

Genetic deficiency of p38α MAPK has previously been shown to lead to an increased accumulation of ROS (40). Accordingly, $p38^{-/-}$ MEF exhibited a two-fold increase of intracellular ROS levels in comparison to $p38^{+/+}$ MEF (Figure 9A, B). Moreover, treatment of $p38^{+/+}$ MEF with SB 202190 augmented intracellular levels of ROS (Figure 9A, B) and the generation of ROS was also enhanced in SB 202190-treated RAW264.7 cells (data not shown). ROS have previously been demonstrated to up-regulate HO-1 gene expression in immunologically active cells and have been proposed to function as putative second messengers for the induction of this gene (2, 9, 25, 41). To investigate whether ROS would directly affect HO-1 gene expression, we treated $p38^{+/+}$ and $p38^{-/-}$ MEF with the antioxidant NAC. NAC reduced basal HO-1 expression in $p38^{-/-}$ MEF, but had no effect in $p38^{+/+}$ MEF (Figure 9C). Similarly, treatment with NAC markedly attenuated HO-1 gene expression in RAW264.7 cells. In line, induction of HO-1 expression by PMA, a known inducer of ROS production in monocytes (42) was abrogated by NAC (Figure 9D). The data suggest that genetic deficiency and pharmacological inhibition of p38 increases the intracellular generation of ROS, which are involved in the up-regulation of HO-1 gene expression.

DISCUSSION

The enzyme HO-1, which catalyzes the first and rate-limiting step of heme degradation, has attracted major attention in recent years, because its products CO and biliverdin have potent antioxidant and anti-inflammatory functions. Activation of the p38 MAPK pathway has previously been shown to be involved in HO-1 induction by a wide variety of stimuli. In the present study it is demonstrated that: 1) pharmacological inhibition of p38 in monocytic cells and genetic deficiency of p38α in MEF cause an up-regulation of HO-1 gene expression; 2) the increased HO-1 expression by inhibition of p38 is mediated via the TF Nrf2; 3) activation of ERK and ROS are involved in this HO-1 gene regulation.

Up-regulation of HO-1 gene expression by pharmacological inhibition and genetic deficiency of p38 MAPK is transcriptionally regulated via Nrf2

Inhibition of p38 MAPK by small molecule inhibitors in monocytic cells and genetic deficiency of p38α in MEF led to increased gene expression of HO-1, but not that of Cox-2 (Figure 1). It is remarkable that HO-1 levels were constitutively high in $p38^{-/-}$ MEF, because basal HO-1 expression is known to be barely detectable in most cells and tissues under non-stimulated conditions (2, 3). The present findings are in keeping with a recent report demonstrating that basal expression of several genes was increased without external stress stimuli in immortalized $p38\alpha^{-/-}$ cardiomyocytes (43). Up-regulation of HO-1 gene expression by inhibition of p38, however, was unexpected, because this protein kinase is considered to play a major role for mediating HO-1 induction by multiple stress stimuli (2, 3, 9). Thus, the present observations suggest that p38α may have a dual function for HO-1 gene regulation.

p38 MAPK modulates gene expression via transcriptional and post-transcriptional mechanisms (44). The present study demonstrates that HO-1 gene expression by inhibition or genetic

deficiency of p38 occurs on the transcriptional level via the TF Nrf2 (Figures 4-7), which is a central regulator of the cellular oxidative stress response (35, 36) and plays a major role for the induction of HO-1 (3, 37, 38). Nrf2 targets to StRE, several of which are localized in the 5'-flanking sequence of the HO-1 gene promoter (3). StRE, also referred to as antioxidant response elements or electrophile response elements, have been identified in a large number of phase II detoxifying enzymes and antioxidant stress proteins (39). An inhibitory role of p38 on Nrf2-mediated gene regulation is also supported by the finding that expression of peroxiredoxin 1, which is coordinately regulated with HO-1 (26) via an Nrf2-dependent mechanism (38), was induced by inhibition of p38 (data not shown). Similar to the findings of our present report, chemical blockage of p38 has previously been shown to enhance StRE-mediated induction of phase II detoxifying enzymes (21) and the sulforaphane-dependent HO-1 activation via Nrf2 in human hepatoma HepG2 cells (22). Our observations also implicate that Bach1, which is a counter-regulator of Nrf2 and a repressor of HO-1 gene expression (27), may be involved in the transcriptional regulation of HO-1 via p38 MAPK (Figure 5). Finally, we cannot exclude the possibility that Nrf2-independent regulatory mechanisms are involved in the up-regulation of HO-1 gene expression via p38 MAPK.

Regulatory pathways of increased HO-1 gene expression by pharmacological inhibition and genetic deficiency of p38 MAPK

In accordance with an earlier report (23), the present study demonstrates that genetic deficiency of p38α caused an increased ERK activity (Figure 8). Since HO-1 gene expression in $p38^{-/-}$ MEF was blocked by the ERK inhibitor PD 98059, it is conceivable that the MAPK p38 and ERK have opposite regulatory roles for governing HO-1 gene expression. This assumption would correspond with a previous study, in which pharmacological inhibition of p38 induced low

density lipoprotein receptor expression via activation of ERK in HepG2 cells (45). Independently, others have demonstrated that blockage of p38 increased LPS-dependent inducible NO synthase gene expression and NO production via activation of JNK in macrophages (46). Considerable variations have been reported for the regulatory role of various MAPK on HO-1 gene expression. Elbirt *et al*., have reported that activation of ERK and p38 MAPK were required for the arsenite-dependent induction of HO-1 gene expression in avian hepatocytes (47). By contrast, both JNK and p38 MAPK were necessary for HO-1 induction by this compound in rat hepatocytes (48). Thus, a complex network of MAPK appears to modulate HO-1 gene expression in a stimulus-, cell- and species-specific manner.

p38 could be involved in the sensing of cellular oxidative stress, because transformed p38α-deficient cells have been reported to accumulate much larger amounts of ROS than wild type cells (40). This observation corresponds with our present findings (Figure 9) and ROS are likely mediating ERK activation in cells lacking p38α (23). The latter assumption would be supported by recent reports, which demonstrated that ROS mediate a sustained activity of ERK in human hepatoma cells (49) and in a model of cerebral ischemia (50). Accordingly, treatment with the antioxidant NAC attenuated the p38-dependent increase of HO-1 gene expression (Figure 9).

Physiological significance of HO-1 up-regulation by pharmacological inhibition and genetic deficiency of p38 MAPK

Since HO-1 has potent anti-apoptotic functions (3), the increase of HO-1 expression levels in $p38\alpha^{-/-}$ MEF corresponds with the observation that p38α deficiency makes these cells less sensitive to apoptosis via up-regulation of anti-apoptotic genes (23). It is also important to note that anti-apoptotic effects of HO-1 have recently been associated with the degradation of p38α in endothelial cells (51). Taken together these findings may suggest an intimate interaction of HO-1

and p38α regulation. HO-1 has attracted major attention in recent years, because it has antioxidant cytoprotective and anti-inflammatory and immunomodulatory functions (3, 9-13). In particular, the anti-inflammatory role of HO-1 has initially been shown in HO-1 deficient mice that exhibited a proinflammatory phenotype and were highly sensitive to LPS-mediated toxicity. Remarkably, these observations in HO-$1^{-/-}$ mice have essentially been confirmed in a human case of HO-1 genetic deficiency (6). More recently, it has been demonstrated that loss of HO-1 activity in APC is associated with impaired suppressor function of regulatory T cells (8).

In summary, the present study demonstrates that the HO-1 gene is activated via pharmacological inhibition and genetic deficiency of p38 MAPK. These findings not only give new insights into the regulatory mechanisms of HO-1 gene expression, but also show the complex regulatory network that modulates the inflammatory response in monocytic cells. These findings may ultimately help to develop novel therapeutic approaches for the treatment of inflammatory diseases.

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DISCLOSURE

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FOOTNOTES

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Abbreviations: CO, carbon monoxide; Cox-2, cyclooxygenase-2; DHE, dihydroethidium; HO, heme oxygenase; MEF, mouse embryonic fibroblast; NAC, N-acetylcysteine; NE, nuclear extracts; Nrf2, NF-E2-related factor-2; PKC, protein kinase C; ROS, reactive oxygen species; StRE, stress response element; TF, transcription factor

FIGURE LEGENDS

Figure 1: Pharmacological inhibition in monocytic cells and genetic deficiency of p38 up-regulate HO-1 gene expression

RAW264.7, $p38^{+/+}$, $p38^{-/-}$ MEF and human (hu) PBMC were cultured as described under *Materials and Methods*. (A) RAW264.7 cells were treated with or without PMA (0.5 μM) or LPS (1 μg / ml) for 18 h. (B) RAW264.7 cells were pretreated with SB 202190 (7.5 and 12 μM) for 30 min, after which treatment was continued with or without PMA (0.5 μM) for another 6 h, as indicated. (C) RAW264.7 cells were treated with SB 202190 (10 μM), SB 203580 (20 μM) or control medium for the times indicated. (D) $p38^{+/+}$ and $p38^{-/-}$ MEF were treated with PMA (0.5 μM) or control medium for 18 h, as indicated. (E) Human PBMC were treated with or without SB 203580 (20 μM) and PMA (0.5 μM) for 6 h. Total protein (50 μg) was subjected to Western blot analysis and sequentially probed with antibodies against HO-1, Cox-2 and GAPDH (A, B and D), or HO-1 and GAPDH (C and E), as indicated. Autoradiograms from representative experiments are shown in A-E, respectively. Autoradiographic signals were visualized and quantified using the Fluorchem FC2 gel documentation system. (D) Numbers represent the protein expression levels relative to $p38^{+/+}$ MEF normalized to GAPDH from at least three independent experiments ± SEM. (B and E) Values ± SEM represent the -fold induction of HO-1 normalized to GAPDH from three independent experiments. Statistics, Student's *t* test for paired values: *, significant differences treatment *versus* control; **, $p38^{+/+}$ control *versus* $p38^{-/-}$ control, $p \leq 0.05$. *RAW*, RAW264.7 cells.

Figure 2: Activity of p38 MAPK in RAW264.7 and MEF cell cultures

(A) RAW264.7 cells were treated with or without SB 202190 and SB 203580 for the times indicated. Western blot analysis was performed as described in Figure 1 and probed sequentially

with antibodies against phospho-specific p38 and total p38 MAPK. Similar results were obtained in three independent experiments and a representative autoradiogram is shown. (B, C) RAW264.7 cells and $p38^{+/+}$, $p38^{-/-}$ MEF were cotransfected with luciferase reporter gene construct pGal4-luc, pFC2-dbd, and pFA-CHOP, as indicated. 24 h after transfection cells were treated with SB 202190 (10 μM) and SB 203580 (20 μM) for 18 h. Cell extracts were assayed for luciferase activity, and the -fold induction relative to the control was determined. Values are means ± SEM from at least three independent experiments with duplicates of each point. Statistics, Student's *t* test for paired values: *, significant differences pFC2-dbd *versus* pFA-CHOP; **, treatment *versus* control, $p \leq 0.05$.

Figure 3: Effect of JNK and ERK inhibitors on HO-1 gene expression

RAW264.7 cells were treated with or without SB 202190, SP 600125, PD 98059 and PMA at the indicated concentrations (μM). Western blot analysis and quantitation was performed as described in Figure 1. Values ± SEM represent the -fold induction of HO-1 normalized to GAPDH from three independent experiments. Statistics, Student's *t* test for paired values: *, significant differences treatment v*ersus* control, $p \leq 0.05$.

Figure 4: Role of p38 MAPK for the regulation of HO-1 promoter activity

(A) $p38^{-/-}$ and $p38^{+/+}$ MEF were transfected with the indicated reporter gene constructs. 24 h after transfection cell extracts were assayed for luciferase activity. The -fold induction relative to $p38^{+/+}$ was determined. Values are means ± SEM from at least three independent experiments with duplicates of each point. Student's *t* test for paired values: *, significant differences $p38^{+/+}$ *versus* $p38^{-/-}$, $p \leq 0.05$. (B) RAW264.7 cells were transfected with the indicated reporter gene constructs. 24 h after transfection cells were treated with or without SB 202190 (10 μM) for 18 h. Cell extracts were assayed for luciferase activity, and the -fold induction relative to the control

was determined. Values are means ± SEM from at least three independent experiments with duplicates of each point. Statistics, Student's *t* test for paired values: *, significant differences treatment *versus* control, $p \leq 0.05$.

Figure 5: Subcellular localization of Nrf2 and Bach1 in $p38^{-/-}$ and $p38^{+/+}$ MEF

Cytosolic and nuclear fractions from cell cultures of $p38^{-/-}$ and $p38^{+/+}$ MEF were separated as described under *Materials and Methods*. Western blotting and quantitation was performed as described in Figure 1. Similar results were obtained in three independent experiments and representative autoradiograms are shown. Values ± SEM represent protein levels of Nrf2 and Bach1 in cytosolic and nuclear fractions relative to $p38^{+/+}$ MEF normalized to GAPDH from three independent experiments and purity of nuclear fraction was controlled with a lamin B antibody. Statistics, Student's *t* test for paired values: *, significant differences $p38^{+/+}$ v*ersus* $p38^{-/-}$, $p \leq 0.05$. *C*, cytosolic fraction; *N*, nuclear fraction.

Figure 6: HO-1 gene regulation in $Nrf2^{-/-}$ and $Nrf2^{+/+}$ MEF

(A) $Nrf2^{-/-}$ and $Nrf2^{+/+}$ MEF were cultured as described under *Materials and Methods*, and were treated with or without SB 202190 (10 μM) , SB 203580 (20 μM) and PMA (0.5 μM) for 18 h. Western blotting and quantitation was performed as described in Figure 1. Similar results were obtained in three independent experiments and a representative autoradiogram is shown. Values ± SEM represent the -fold induction of HO-1 normalized to GAPDH from three independent experiments. Statistics, Student's *t* test for paired values: *, significant differences treatment v*ersus* control, $p \leq 0.05$. (B) $Nrf2^{-/-}$ and $Nrf2^{+/+}$ MEF were transfected with the indicated reporter gene constructs. 24 h after transfection cells were treated with or without SB 202190 (10 μM) and PMA (0.5 μM) for 18 h. Cell extracts were assayed for luciferase activity, and the luciferase activity relative to $Nrf2^{-/-}$ was determined. Values are means ± SEM from at least three

independent experiments with duplicates of each point. Statistics, Student's *t* test for paired values: *, significant differences treatment *versus* control; **, activity of pHO-4045 or pE2-luc in $Nrf2^{-/-}$ MEF *versus* $Nrf2^{+/+}$ MEF, $p \leq 0.05$.

Figure 7: Binding of nuclear proteins to a StRE oligonucleotide is induced by p38 inhibitors

(A) A biotin-labeled oligonucleotide with a StRE element of the distal enhancer sequence (E2) of the mouse HO-1 promoter was incubated with 7 μg of NE from control RAW264.7 cells or from cells treated with SB 202190, SB 203580 and LPS. (B) For competition analyses the biotin-labeled E2 oligonucleotide was preincubated with 7 μg of NE from SB 202190 (10 μM) treated cells along with a 10-, 50- and 100-fold molar excess of unlabeled E2 oligonucleotide or a 100-fold molar excess of E2 mut oligonucleotide, as indicated. (C) For super-shift analysis 1 or 2 μl of an antibody directed against Nrf2 was preincubated with NE from SB 202190 treated cells before the biotin-labeled E2 oligonucleotide was added. For a comparison, a 50-fold molar excess of unlabeled oligonucleotide with the consensus κB sequence, or 1 μl of anti-sera raised against mouse NB-1 protein was added. DNA-protein complexes were separated by electrophoresis on a 6% native polyacrylamide gel.

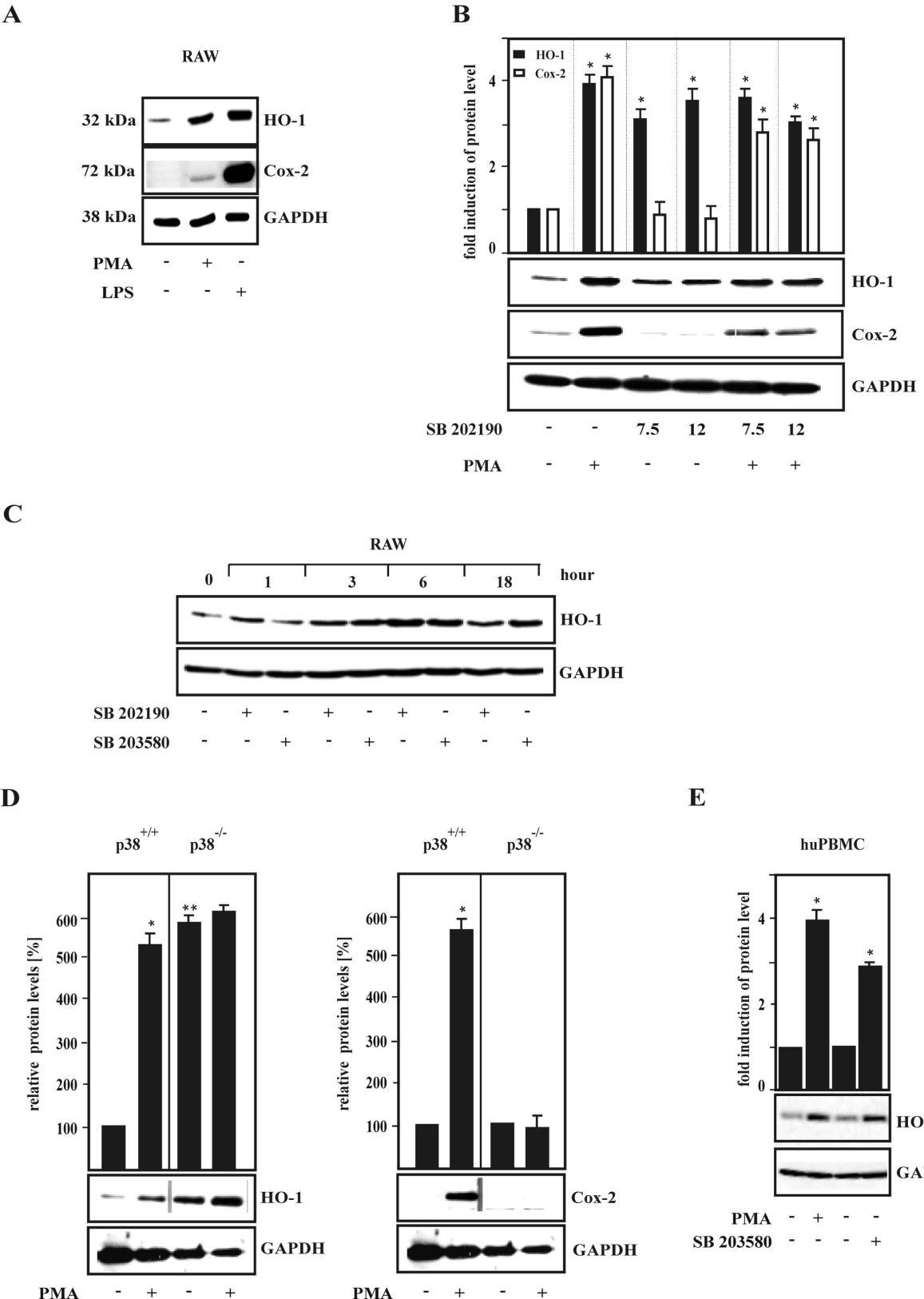
Figure 8: ERK activation is involved in p38-dependent HO-1 gene regulation

(A) $p38^{-/-}$ and $p38^{+/+}$ MEF were cultured with or without FCS and (B) $p38^{-/-}$ and $p38^{+/+}$ MEF were treated with or without PD98059 for 6 h. (C) RAW 264.7 cells were treated with SB 202190 (10 μM), SB 203580 (20 μM) and PMA (0.5 μM) or control media, as indicated. Western blot analysis was performed as described in Figure 1 and probed sequentially with antibodies (A, C) against phosphorylated ERK and total ERK, (B) HO-1 and GAPDH. Similar results were obtained in three independent experiments and representative autoradiograms are shown,

respectively. (D) RAW264.7 cells were cotransfected with luciferase reporter gene construct pGal4-luc, pFC2-dbd, and pFA-Elk. 24 h after transfection cells were treated with or without SB 202190 (10 μM), SB 203580 (20 μM) and PMA (0.5 μM) for 18 h. Luciferase assay and quantitation was performed as described in Figure 2. Values are means ± SEM from at least three independent experiments with duplicates of each point. Statistics, Student's *t* test for paired values: *, significant differences treatment *versus* control, $p \leq 0.05$.

Figure 9: Inhibition of p38 MAPK increases intracellular levels of ROS

(A) $p38^{-/-}$ and $p38^{+/+}$ MEF were cultured as described under *Materials and Methods* and $p38^{+/+}$ MEF were treated with SB 202190 (10 μM) for 1 h. Cells were washed and incubated with DHE stain (5 μM) for 20 min and analyzed by flow cytometry. (B) Numbers indicate mean fluorescence intensity relative to $p38^{+/+}$ MEF from at least three independent experiments ± SEM. Statistics, Student's *t* test for paired values: *, significant differences treatment v*ersus* control; **, $p38^{+/+}$ control *versus* $p38^{-/-}$ control, $p \leq 0.05$. (C) $p38^{-/-}$ and $p38^{+/+}$ MEF were treated with or without NAC for 6 h at the indicated concentrations (5 and 25 mM). (D) RAW264.7 cells were pretreated with NAC at the indicated concentrations (mM) for 30 min, after which treatment was continued for another 6 h with or without SB 202190 (10 μM) and PMA (0.5 μM). Western blot analysis and quantitation was performed as described in Figure 1. (C, D) Similar results were obtained in three independent experiments and representative autoradiograms are shown. (D) Values ± SEM represent the -fold induction of HO-1 normalized to GAPDH from three independent experiments. Statistics, Student's *t* test for paired values: *, significant differences treatment v*ersus* control; **, treatment *versus* NAC + treatment, $p \leq 0.05$.


A
RAW
32 kDa
HO-1
72 kDa
Cox-2
38 kDa
GAPDH
PMA - + -
LPS - - +
B
HO-1
Cox-2
fold induction of protein level
0
2
4
HO-1
Cox-2
GAPDH
SB 202190 - - 7.5 12 7.5 12
PMA - + - - + +
C
RAW
0 1 3 6 18 hour
HO-1
GAPDH
SB 202190 - + - + - + - + -
SB 203580 - - + - + - + - +
D
p38+/+ p38-/-
relative protein levels [%]
100
200
300
400
500
600
HO-1
GAPDH
PMA - + - +
p38+/+ p38-/-
relative protein levels [%]
100
200
300
400
500
600
Cox-2
GAPDH
PMA - + - +
E
huPBMC
fold induction of protein level
0
2
4
HO-1
GAPDH
PMA - + - -
SB 203580 - - - +

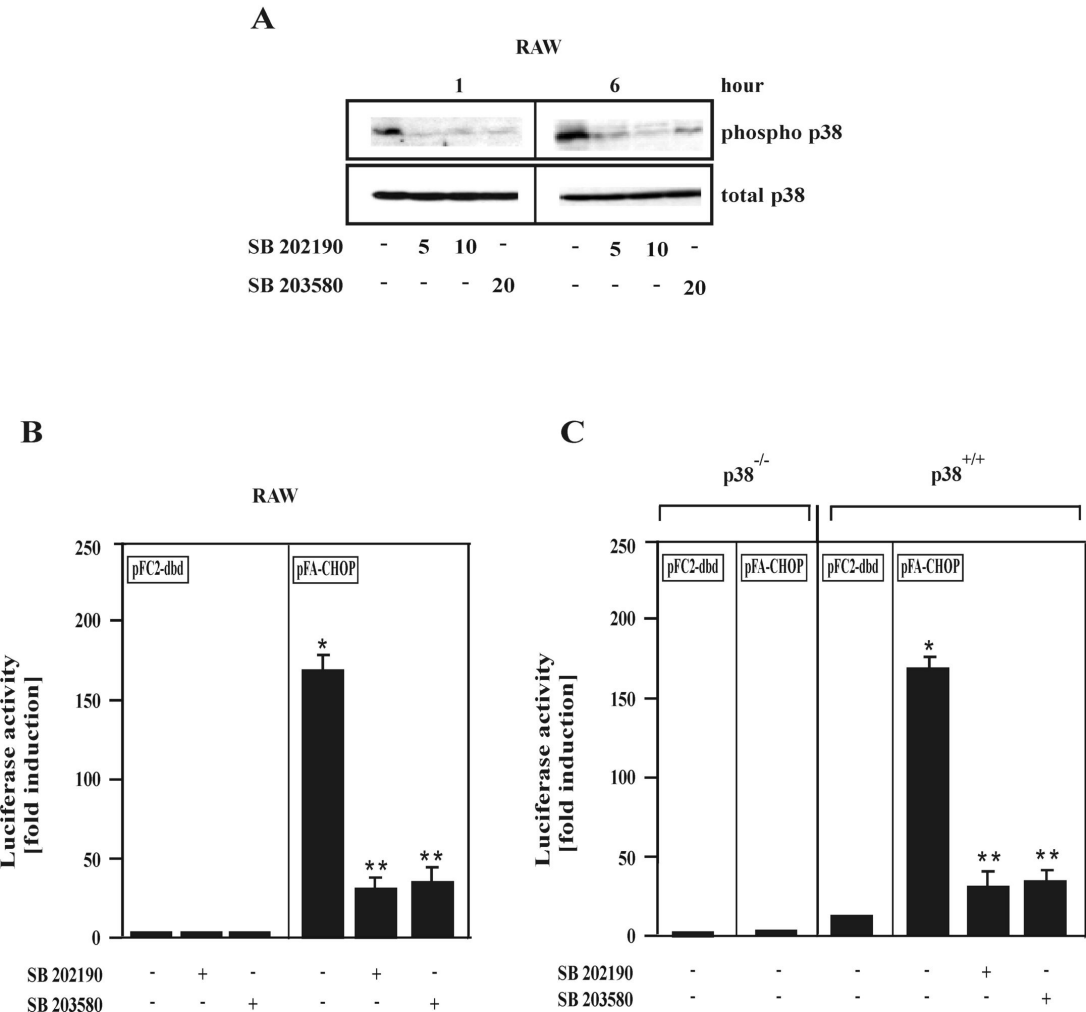

A
RAW
1
6
hour
phospho p38
total p38
SB 202190 - 5 10 - - 5 10 -
SB 203580 - - - 20 - - - 20
B
RAW
pFC2-dbd
pFA-CHOP
Luciferase activity [fold induction]
250
200
150
100
50
0
*
**
**
SB 202190 - + - - + -
SB 203580 - - + - - +
C
p38-/-
p38+/+
pFC2-dbd
pFA-CHOP
pFC2-dbd
pFA-CHOP
Luciferase activity [fold induction]
250
200
150
100
50
0
*
**
**
SB 202190 - - - - + -
SB 203580 - - - - - +

Figure 3

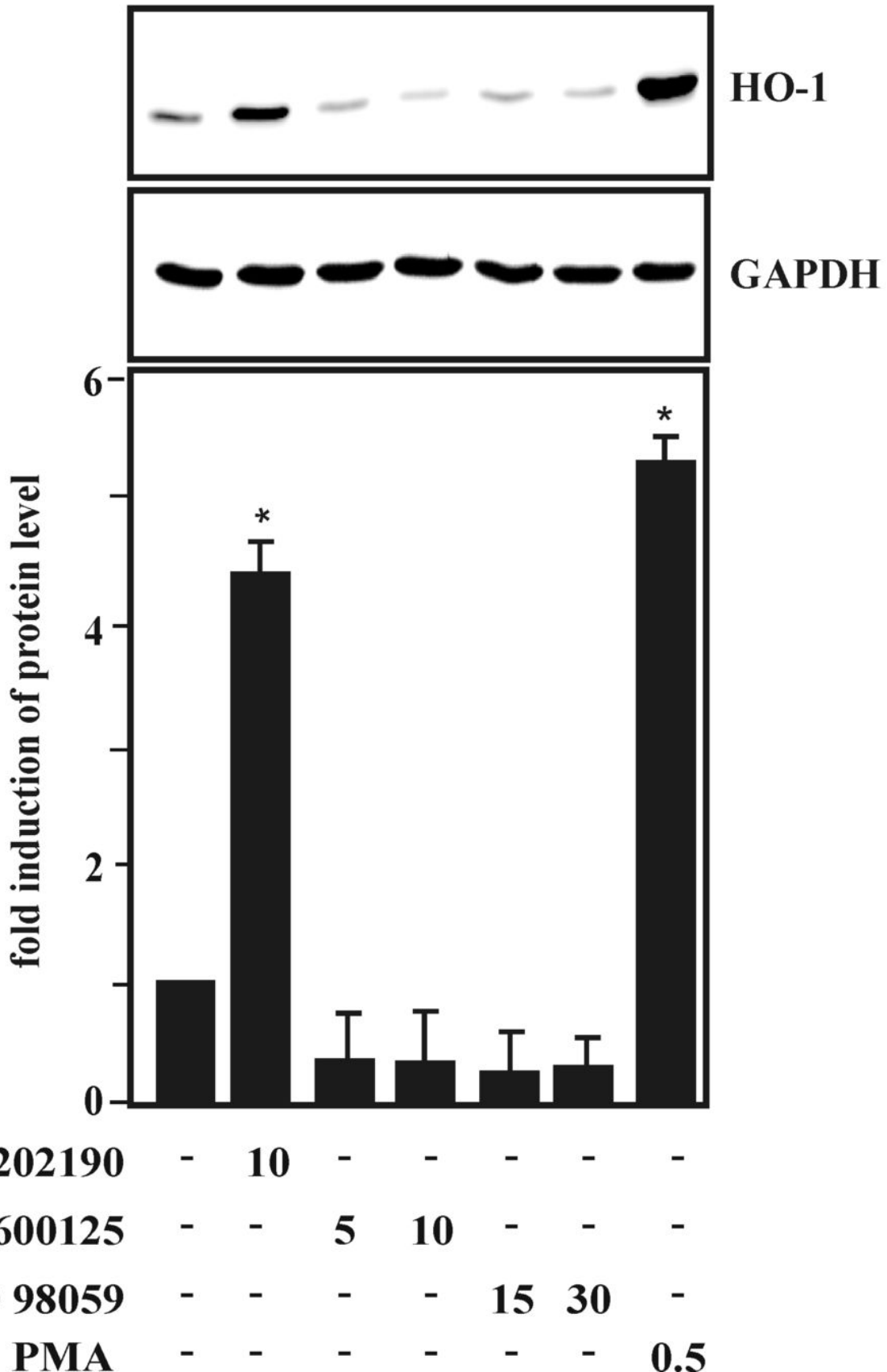

HO-1
GAPDH
fold induction of protein level
6
4
2
0
*
*
SB 202190 - 10 - - - - -
SP 600125 - - 5 10 - - -
PD 98059 - - - - 15 30 -
PMA - - - - - - 0.5

Figure 4

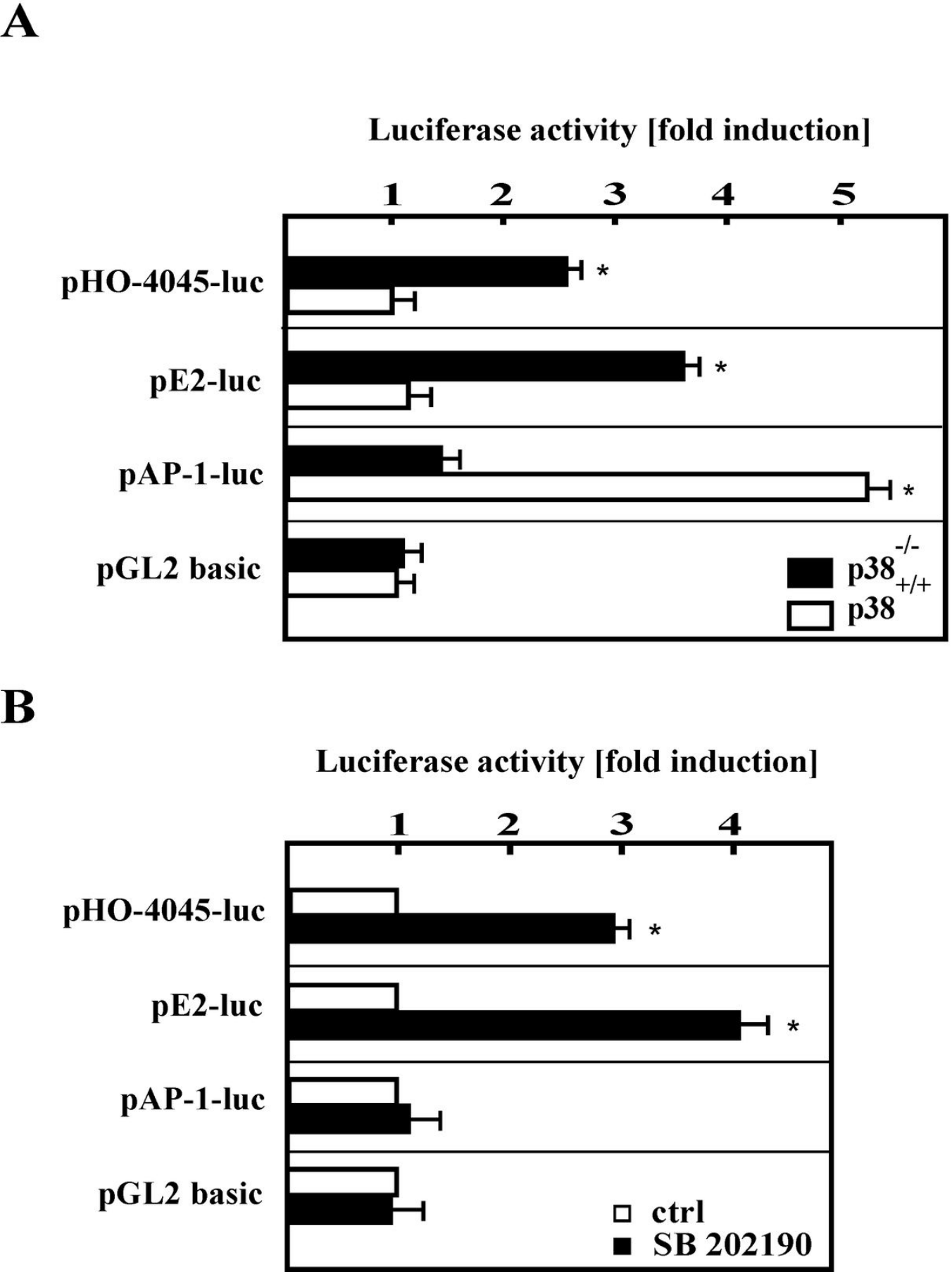
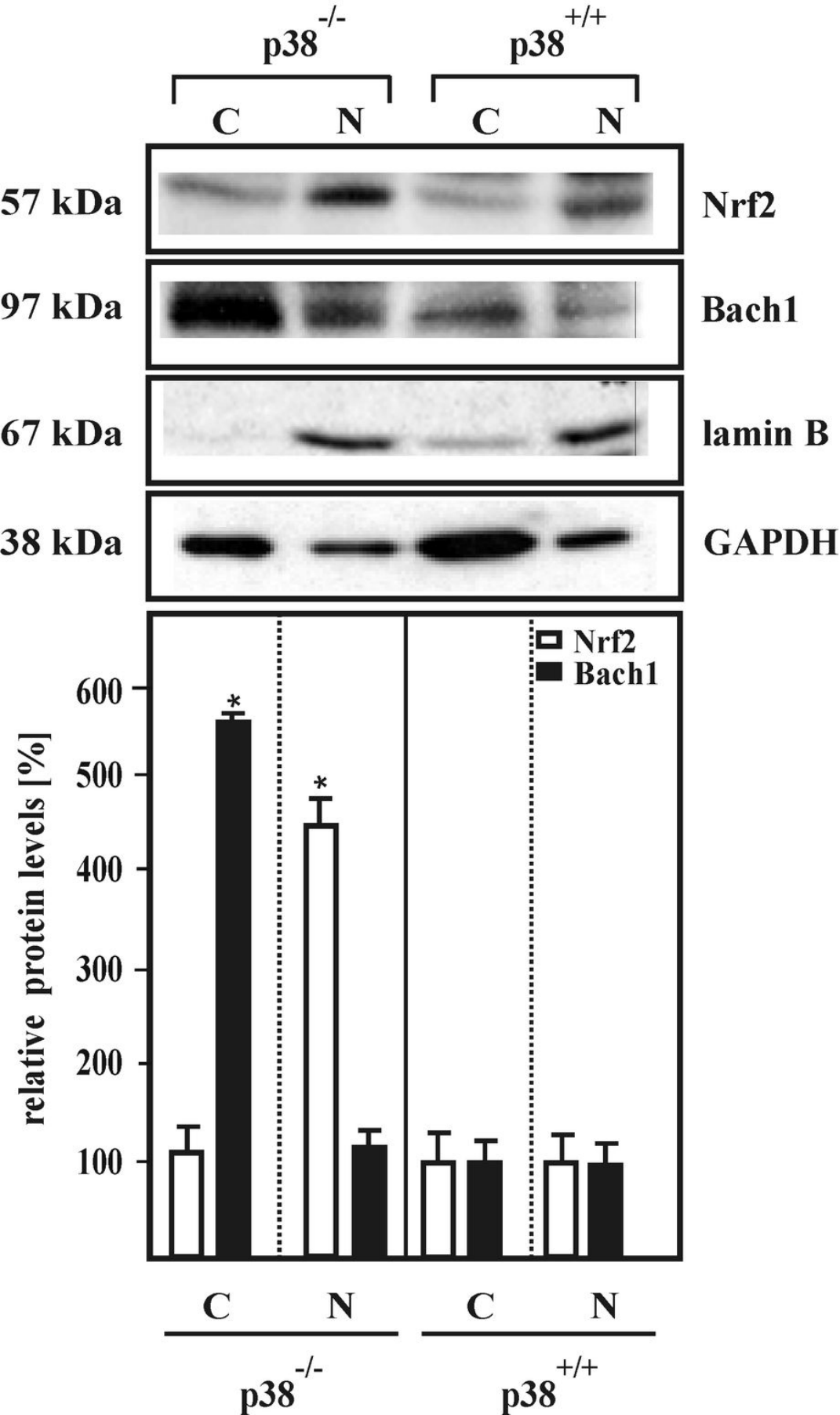
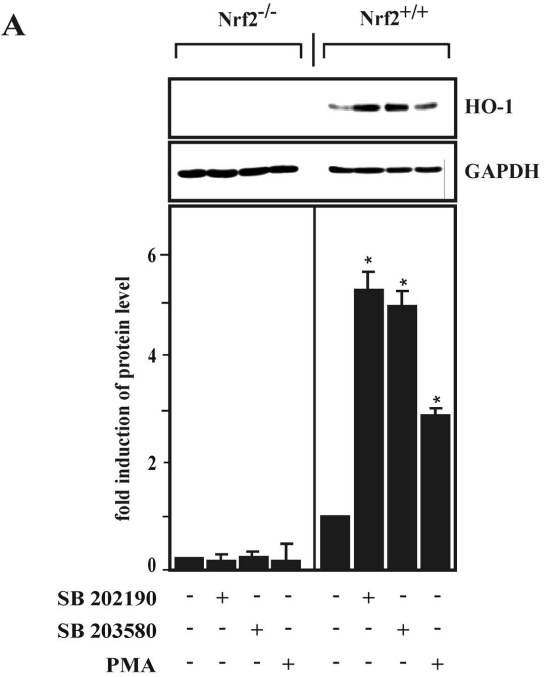
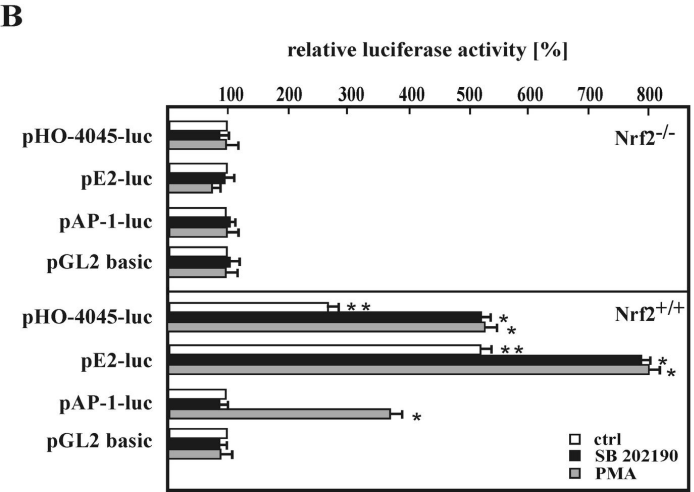

A
Luciferase activity [fold induction]
1
2
3
4
5
pHO-4045-luc
pE2-luc
pAP-1-luc
pGL2 basic
*
*
*
p38 -/-
+/+
p38
B
Luciferase activity [fold induction]
1
2
3
4
pHO-4045-luc
pE2-luc
pAP-1-luc
pGL2 basic
*
*
ctrl
SB 202190

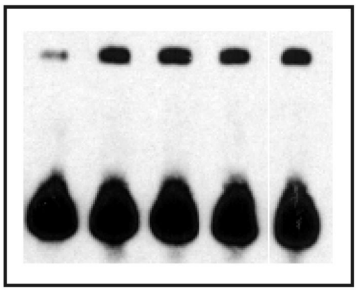
Figure 5


p38 -/-
p38 +/+
C
N
C
N
57 kDa
Nrf2
97 kDa
Bach1
67 kDa
lamin B
38 kDa
GAPDH
Nrf2
Bach1
relative protein levels [%]
600
500
400
300
200
100
*
*
C
N
C
N
p38 -/-
p38 +/+


A
Nrf2-/-
Nrf2+/+
HO-1
GAPDH
fold induction of protein level
0
2
4
6
SB 202190 - + - - - + - -
SB 203580 - - + - - - + -
PMA - - - + - - - +

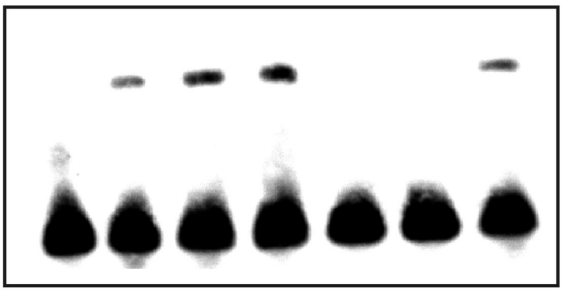

B
relative luciferase activity [%]
100 200 300 400 500 600 700 800
pHO-4045-luc
pE2-luc
pAP-1-luc
pGL2 basic
Nrf2-/-
pHO-4045-luc
pE2-luc
pAP-1-luc
pGL2 basic
Nrf2+/+
ctrl
SB 202190
PMA

A



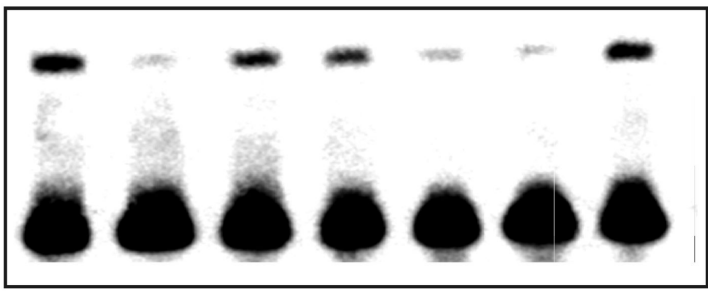
SB 202190	-	5	10	-	-
SB 203580	-	-	-	20	-
LPS	-	-	-	-	+

B

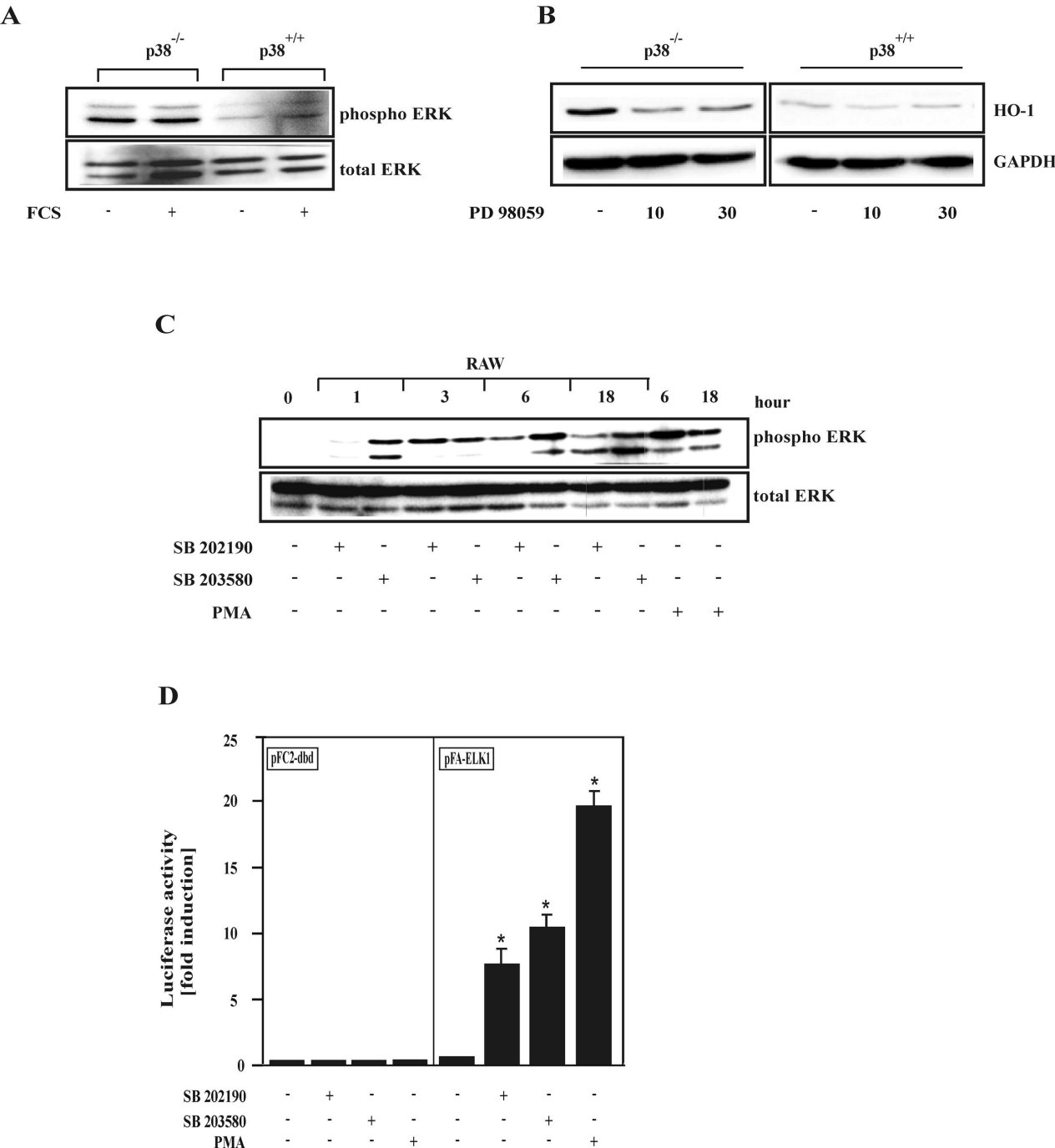


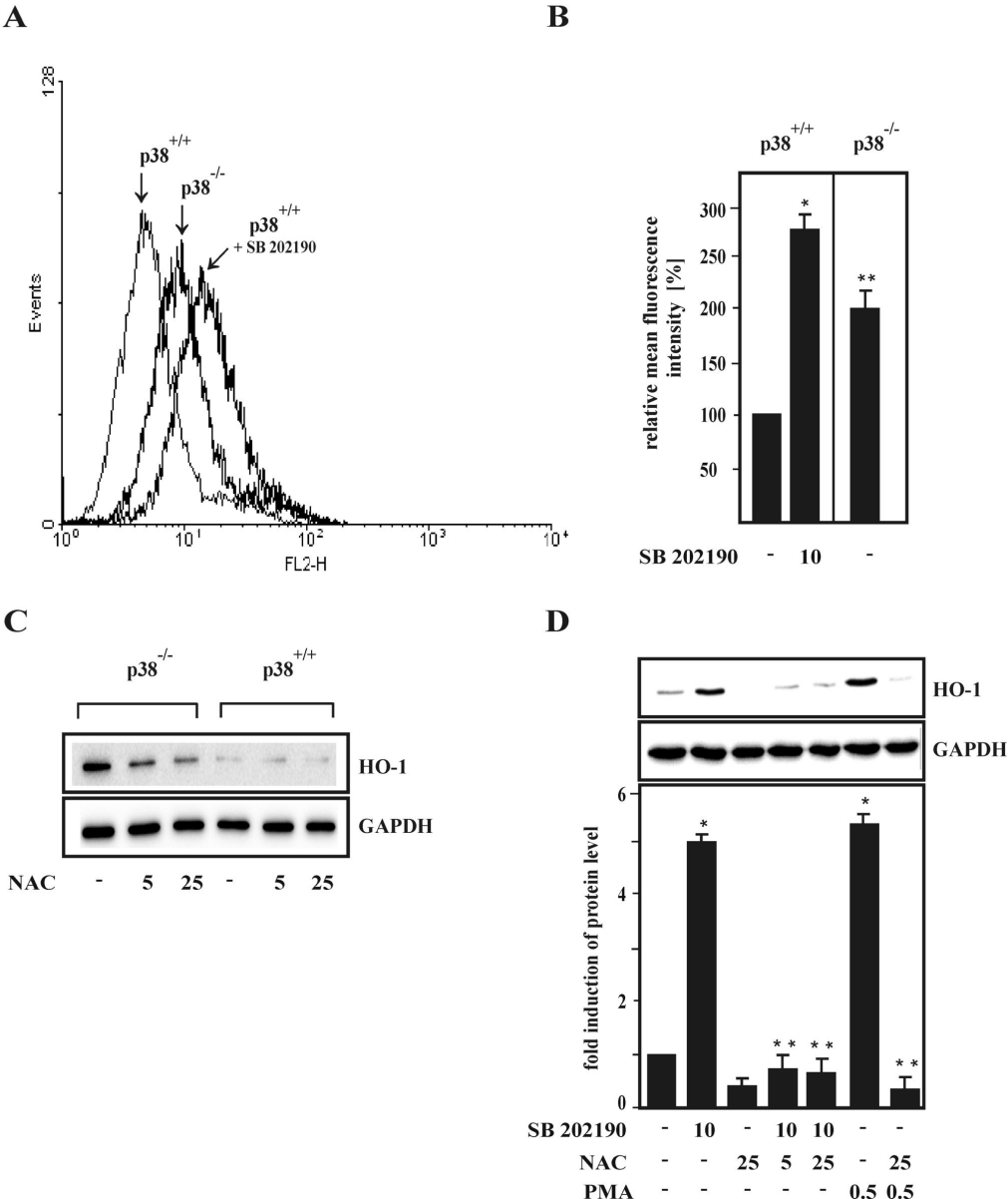
SB 202190	-	-	+	+	+	+	+
E2	-	-	-	X 10	X 50	X 100	-
E2 mut	-	-	-	-	-	-	X 100

C



SB 202190	+	+	+	+	+	+	+
E2	-	X 50	-	-	-	-	-
E2 mut	-	-	X 50	-	-	-	-
NF-kB	-	-	-	X 50	-	-	-
Nrf2 Ab	-	-	-	-	1	2	-
NB 1 sera	-	-	-	-	-	-	1


A
p38-/-
p38+/+
phospho ERK
total ERK
FCS - + - +
B
p38-/-
p38+/+
HO-1
GAPDH
PD 98059 - 10 30 - 10 30
C
RAW
0 1 3 6 18 6 18 hour
phospho ERK
total ERK
SB 202190 - + - + - + - + - - -
SB 203580 - - + - + - + - + - -
PMA - - - - - - - - - + +
D
pFC2-dbd
pFA-ELK1
Luciferase activity [fold induction]
25
20
15
10
5
0
SB 202190 - + - - - + - -
SB 203580 - - + - - - + -
PMA - - - + - - - +


A
128
Events
0
p38+/+
p38-/-
p38+/+
+ SB 202190
10⁰
10¹
10²
10³
10⁴
FL2-H
B
p38+/+
p38-/-
relative mean fluorescence intensity [%]
300
250
200
150
100
50
*
**
SB 202190 - 10 -
C
p38-/-
p38+/+
HO-1
GAPDH
NAC - 5 25 - 5 25
D
HO-1
GAPDH
fold induction of protein level
6
4
2
0
*
**
SB 202190 - 10 - 10 10 - -
NAC - - 25 5 25 - 25
PMA - - - - - 0.5 0.5

2. SUMMARY

Mononuclear phagocytes (monocytes, macrophages) play a pivotal role for the regulation of inflammation. An uncontrolled immunological response of activated mononuclear cells may cause irreversible tissue damage and chronic inflammatory disorders. Therefore, antioxidant and anti-inflammatory mechanisms of mononuclear cells are essential for the resolution of inflammation and restoration of immune homeostasis. Up-regulation of peroxiredoxin I (Prx I) and heme oxygenase-1 (HO-1) has been shown to have potent antioxidant and immunomodulatory properties.

This thesis integrates three original articles that demonstrate the signaling pathways involved in the regulation of the antioxidant genes Prx I and HO-1 by the prototypical monocyte activators phorbol myristate acetate (PMA) and lipopolysaccharide (LPS), in monocytic cells.

Prx I gene expression was up-regulated by PMA and was inhibited by simultaneous treatment with LPS in RAW264.7 monocytic cells. The repressive effect of LPS on Prx I gene activation by PMA was mediated via a newly identified κB site of the Prx I gene promoter. A 'non-classical' NF-κB pathway, that includes phosphorylation of the NF-κB subunit p65 at serine 276 and a non-receptor tyrosine kinase Bruton's tyrosine kinase, was involved in the regulation of LPS-dependent inhibition of Prx I gene activation by PMA (Paper I).

HO-1 gene expression was transcriptionally induced by PMA with a regulatory pattern different from that by LPS in RAW264.7 monocytic cells. PMA-dependent HO-1 gene activation was mediated via a newly defined κB element of the rat HO-1 promoter that serves as a nuclear target of p65. An IκB kinase-2-independent atypical NF-κB pathway, which was mediated via activation of p38 MAPK and CK2, was involved in HO-1 gene regulation by PMA (Paper II).

An unexpected up-regulation of HO-1 gene expression was observed by pharmacological inhibition of p38 in monocytes and by genetic deficiency of p38α in mouse embryonic fibroblasts (MEFs). The inhibitory effect of p38 on HO-1 gene expression was mediated via a stress response element (StRE) of the mouse HO-1 gene promoter that is a nuclear target of the transcription factor NF-E2-related

factor 2 (Nrf2). Activation of the ERK pathway and generation of reactive oxygen species (ROS) were involved in HO-1 gene activation by p38 inhibition (paper III).

Taken together, the thesis demonstrates the complex regulatory pathways that control the induction of Prx I and HO-1 gene expression in activated monocytic cells. Thus, further studies on the regulation of these two genes may help to develop novel therapeutic strategies for the treatment of inflammatory diseases.

3. Zusammenfassung

Mononukleäre Phagozyten (Monozyten, Makrophagen) spielen eine bedeutende Rolle bei der Regulation von Entzündungsreaktionen. Eine unkontrollierte Immunantwort von aktivierten mononukleären Zellen kann irreversible Gewebeschäden und chronische Entzündungen hervorrufen. Antioxidative und anti-inflammatorische Mechanismen von mononukleären Zellen spielen daher eine wesentliche Rolle bei der Rückbildung von Entzündungsreaktionen und die Wiederherstellung des immunologischen Gleichgewichtes. Es ist bekannt, dass die Heraufregulation von Peroxiredoxin (Prx) I und Häm Oxygenase (HO)-1 stark antoxidativ und immunmodulatorisch wirkt.

Die vorliegende Arbeit fasst den Inhalt von drei Originalpublikationen zusammen, die Signalwege beschreiben, die an der Regulation der antioxidativ wirksamen Gene HO-1 und Prx I durch die prototypischen Monozytenaktivatoren Phorbol-Myrisat Acetat (PMA) und Lipopolysaccharid (LPS) beteiligt sind.

Die Prx I Genexpression wurde durch PMA heraufreguliert und wurde durch die gleichzeitige Behandlung mit LPS in RAW264.7 Monozyten gehemmt. Der hemmende Effekt von LPS auf die PMA-abhängige Prx I Genaktivierung wurde über ein neu identifiziertes κB Element im Prx I Genpromotor vermittelt. Ein nicht-klassischer NF-κB Weg, der über Phosphorylierung der NF-κB Untereinheit p65 am Serinrest 276 und die Rezeptor-unabhängige Tyrosinkinase Bruton's Tyrosinkinase, vermittelt wurde, war für die LPS-abhängige Hemmung der Prx I Induktion durch PMA verantwortlich (Arbeit I).

Die HO-1 wurde in RAW264.7 Monozyten transkriptional durch PMA mit einem Genexpressionsmuster induziert, das sich von dem durch LPS hervorgerufenen Expressionsmuster unterschied. Die PMA-abhängige HO-1 Induktion wurde durch ein neu identifiziertes κB Element des Ratten HO-1 Genpromotors vermittelt, das als nukleäres Ziel für p65 diente. Ein IκB Kinase 2-unabhängiger atypischer NF-κB Weg, der über die Aktivierung von p38 MAPK und CK2 vermittelt wurde, war an dieser HO-1 Regulation durch PMA beteiligt (Arbeit II).

Eine unerwartete Heraufregulation der HO-1 Genexpression konnte durch pharmakologische Hemmung von p38 in Monozyten und bei genetischer Defizienz von p38α in embryonalen Fibroblasten der Maus (MEFs) beobachtet werden. Der hemmende Effekt von p38 auf die HO-1 Genexpression wurde über ein Stress-abhängiges Element (StRE) innerhalb des Maus HO-1 Promotors vermittelt, das als nukleäres Ziel für den Transkriptionsfaktor NF E2-related factor 2 (Nrf2) diente. Die Aktivierung des ERK Signalweges und die Entstehung von reaktiven Sauerstoffspezies (ROS) spielten dabei eine regulatorische Rolle (Arbeit III).

Zusammenfassend zeigt die vorliegende Arbeit die komplexen regulatorischen Signalwege, die die Induktion der Prx I und HO-1 Genexpression in Monozyten vermitteln. Ein besseres Verständnis der Regulation dieser beiden Gene könnte bei der Entwicklung neuer therapeutischer Strategien zur Behandlung entzündlicher Krankheiten helfen.

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