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NF-κB, Nrf2, and HO-1 Interplay in Redox-Regulated VCAM-1 Expression

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ABSTRACT

Oxidative processes are considered to play a crucial role in the induction of cell adhesion molecules, a key event in inflammatory processes. We recently reported on an unexpected unidirectional effect of an overexpressed antioxidant [phospholipid hydroperoxide glutathione peroxidase (PHGPx)] and an oxidant [15-lipoxygenase (15-LOX)] enzyme on the basal and interleukin-1 induced vascular cell adhesion molecule-1 (VCAM-1) expression in vascular smooth muscle cells (SMC). Both enzymes inhibited VCAM-1 expression and reduced the cellular protein thiol content, thus, both exerting an oxidant effect. We now investigated whether transcription factors known to be regulated by oxidation, *i.e.,* the nuclear factor-κB and the Keap1/Nrf2 system, were affected in our set of cells: SMC, SMC^{PHGPx}, and SMC^{LOX}, as well as ECV and ECV^{PHGPx}. PHGPx and 15-LOX inhibited nuclear factor-κB activation most efficiently at a step downstream of DNA binding, which explains their inhibitory effect on VCAM-1 expression. Both enzymes up-regulated endogenous heme oxygenase-1 most probably via activation of Nrf2. Transfected Nrf2 strongly inhibited VCAM-1 promoter activity, which could be reversed by cotransfection with Keap1. The key player in this complex cross-talk obviously is heme oxygenase-1, which is known to be induced by oxidant-activated Nrf2. The moderate oxidative stress initiated by enhanced PHGPx or 15-LOX activity appears to induce a defense system that diminishes the response to further proinflammatory stimuli. *Antioxid. Redox Signal.* 7, 889–899.

INTRODUCTION

Cellular adhesion molecules (CAM) are key players in initiating inflammatory responses of the vascular system (34, 36). Their expression in endothelial cells upon exposure to inflammatory stimuli causes the well-known phenomena of leukocyte rolling and sticking. In extreme acute inflammatory conditions such as septicemia, this leukocyte sticking finally results in endothelial damage and fatal occlusion of microcirculation. Certain CAM, such as vascular cell adhesion molecule-1 (VCAM-1), are also expressed on smooth muscle cells (SMC) where they contribute to leukocyte recruitment. The CAM-induced accumulation of activated polymorphonuclear leukocytes and macrophages inevitably leads to oxidative stress associated with tissue damage and proliferative responses. Inversely, expression of adhesion molecules, as well as SMC proliferation, is influenced by redox processes.

SMC respond to growth factor and cytokine stimulation with the production of hydrogen peroxide via NADPH oxidase, which stimulates cell growth (16). Accordingly, SMC up-regulate adhesion molecules when incubated with hydroperoxides (30). In endothelial cells, the expression of VCAM-1 is mediated by the activation of nuclear factor-κB (NF-κB) (39), and NF-κB may similarly be required for the VCAM-1 expression in SMC. A constitutive basic activation of NF-κB appears to be a prerequisite for proliferation of SMC (3), and the redox regulation of NF-κB is likely relevant to both VCAM 1 expression and proliferation of SMC. This redox regulation of the NF-κB system cannot be simply explained by a shift in the balance of cellular oxidants and antioxidants. Model systems, such as SMC stably transfected with "antioxidant" or prooxidant enzymes, yielded surprising results (2). Whereas overexpression of phospholipid hydroperoxide glutathione peroxidase (PHGPx) as a hydroperoxide-reducing enzyme resulted in the expected inhibition of VCAM-1

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expression, overexpression of 15-lipoxygenase (15-LOX) as a hydroperoxide-producing enzyme did not facilitate, but inhibited VCAM-1 expression (2). This unidirectional effect of otherwise contrarily working enzymes was unexpected and attributed to inhibition of the NF-κB system at a still unidentified site. Both enzymes significantly lowered the cellular protein thiol content and increased protein-glutathione mixed disulfides. These effects were more pronounced in SMC overexpressing 15-LOX. Essential functions in the signaling pathways leading to VCAM-1 expression via NF-κB were therefore suspected to be affected by thiol modification.

However, such thiol modification also affects other transcription factor systems, *e.g.,* the Kelch-like ECH-associated protein-1 (Keap1)/NF-E2-related factor-2 (Nrf2) system (12, 42, 44). In the absence of stimuli, which can be electrophiles or oxidants, Nrf2 is held in the cytoplasm by actin-associated Keap1. SH modification of Keap1 leads to a conformational change that liberates Nrf2. Nrf2 translocates into the nucleus and transactivates responsive genes via binding to the antioxidant responsive element (ARE). Phase II enzymes, γ-glutamylcysteine synthetase, and heme oxygenase-1 (HO-1) belong to Nrf2-responsive genes (22, 40, 41). We, therefore, investigated the involvement of the Keap1/Nrf2 system in VCAM-1 expression and its potential cross-talk to the NF-κB system. We report here that both PHGPx and 15-LOX inhibited the transactivating activity of NF-κB by a step downstream of DNA binding. Furthermore, both enzymes enhanced the activity of Nrf2. This Nrf2 activation led to an increased expression of HO-1, which might be responsible for the decreased proliferation and VCAM-1 expression via inhibition of NF-κB.

MATERIALS AND METHODS

Cell culture

Rabbit aortic vascular SMC, either wild type or stably transfected with PHGPx (8) or 15-LOX (2), were cultured as described. ECV304 cells were grown as previously described (7). Media for transfected cells contained 800 μg/ml Geneticin (Calbiochem, Schwalbach, Germany). Cells were studied from passages 2–10. All cells were supplemented with 100 n*M* sodium selenite.

Construction of PHGPx-overexpressing ECV304 cells

The *Eco*RI fragment from the clone containing the 10,000-bp insert with the genomic PHGPx obtained from a porcine genomic DNA library (6) was cloned into pUC18. From this plasmid, exon 1 and the first intron of PHGPx was isolated by *Eco*R47III × *Eco*RI digestion. The resulting 1,070-bp fragment was ligated into pcDNA3 × *Hind*III blunt-ended × *Eco*RI. The rest of the PHGPx coding sequence and the 3′-untranslated region was obtained from pMM3 (26) by *Eco*RI digestion and ligated into the intron-containing pUC18 linearized with *Eco*RI. Correct cloning was verified by sequencing (MWG Biotech, Ebersberg, Germany). ECV304 cells were transfected with pcDNA3 or pcDNA3-PHGPx using SuperFect (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Positive clones (ECV^{PHGPx}) grown with 100 n*M* sodium selenite generally had four times higher PHGPx activity than pcDNA3 transfectants (ECV).

Plasmids

κB-tk-Luc, containing one NF-κB site, has been described before (2). HO-ARE-pGL was generated by cloning a 29-bp oligonucleotide (5′-ggaattctgttttcgctgagtcatggttc-3′), containing the ARE of the murine HO-1-promoter (accession no. U70472), into *Eco*RI × *Hinc*II digested pBluescript II KS(+) (Stratagene, La Jolla, CA, U.S.A.). The resulting construct was ligated via *Sma*I and *Xho*I into pGL3-promoter (Promega, Mannheim, Germany). Expression plasmids for murine Nrf2 (pcDNA3-mNrf2) and Keap1 (pcDNA3-mKeap1) were kindly provided by M. Yamamoto (University of Tsukuba, Japan). Empty pcDNA3 (Invitrogen, Karlsruhe, Germany) served as control. pCH110, containing β-galactosidase (Amersham Biosciences, Freiburg, Germany), served as transfection control.

Isolation of the rabbit VCAM-1 promoter by genome walking

Approximately 2 kb of the sequence upstream of exon 1 of the VCAM-1 gene was isolated by genome walking (38). Genomic DNA of 15×10^6 SMC cells was digested with *Hinc*II or *Eco*RV to generate blunt-ended fragments that were ligated to an adaptor consisting of two annealed synthetic DNA oligonucleotides (forward adaptor: 5′-gtaatacgactcactatagggcacgcgtggtcgacggcccgggctggt-3′; reverse adaptor: 5′-accagccc-NH_2-3′). The target sequence was amplified by nested PCR with adaptor-specific primers (AP1, AP2) and gene-specific primers (GSP1, GSP2; accession no. AY212510) (Table 1). PCR products were cloned into pCR-II-Topo (Invitrogen). The longest sequence was deposited under accession no. AY699272.

The transcriptional start site was determined by 5′-rapid amplification of cDNA ends (5′-RACE) (SMART™ RACE Kit; Clontech, Heidelberg, Germany) with GSP-RACE (Table 1, accession no. AY212510).

VCAM-1 promoter constructs

Deletion constructs I–VI (Fig. 1A) were generated by standard PCR using the VCAM-1-promoter-pCRII-Topo-construct as template. For cloning into pGL3-basic, primers were designed to incorporate a *Mlu*I or *Kpn*I restriction site at the 5′-end and a *Mlu*I site at the 3′-end of the respective constructs (Table 1). All clones to be used were sequenced (MWG Biotech).

Site-directed mutagenesis

The VCAM-1 promoter was analyzed with the MatInspector program (35). NF-κB sites were changed from gggtttccc into gagtctcgc by "overlap-extension-PCR" (18) and the cor-

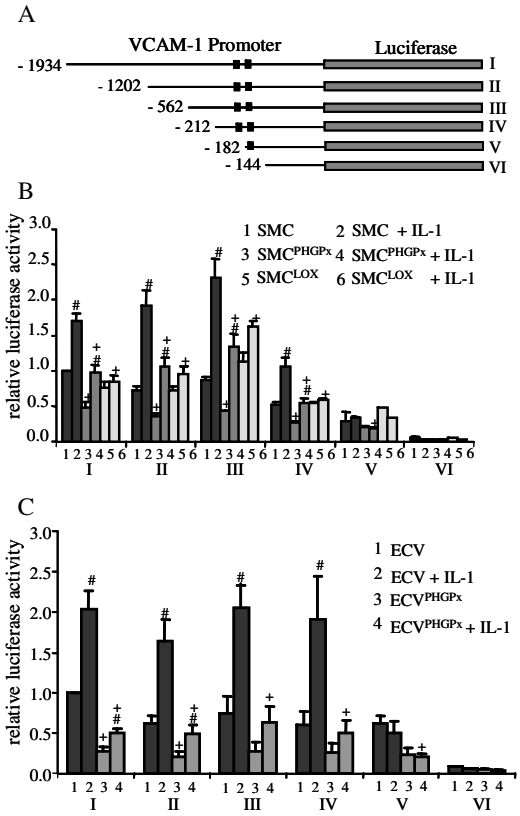

FIG. 1. Basal and IL-1-induced luciferase expression driven by rabbit VCAM-1 promoter fragments of varying length. (**A**) Length of VCAM-1 promoter fragments used for reporter gene constructs in pGL3-basic. NF-κB sites are indicated by boxes. (**B** and **C**) Relative luciferase activity in SMC, SMC^{PHGPx}, and SMC^{LOX} (B) or ECV and ECV^{PHGPx} (C) stimulated 24 h after transfection with IL-1 (1 ng/ml for 3 h) or not. Respective luciferase activity obtained with construct I in unstimulated SMC (B) or ECV (C) was used for normalization. Experiments were run in triplicate and performed at least three times. For further details, see Materials and Methods. #$p < 0.05$ versus the unstimulated control; +$p < 0.05$ versus the respective wild-type cell.

rectness verified by sequencing. For primer sequences, see Table 1.

Transfection and reporter gene assays

Cells (1×10^5), seeded onto 12-well plates 24 h before transfection, were transfected with 0.5 μg of pCH110, 0.8 μg of luciferase reporter plasmid, and if wanted 0.3 μg of expression plasmid using SuperFect (see above). Interleukin-1 (IL-1); 1 ng/ml) stimulation was started 24 h after transfection for 3–10 h. Cell lysis and determination of luciferase and β-galactosidase activity were performed as described (2). Relative luciferase activity obtained with the respective empty luciferase plasmids (pGL3-basic, tk-Luc, pGL3-promoter) was subtracted.

TABLE 1. OLIGONUCLEOTIDE SEQUENCES

Oligonucleotide	*Sequence (5′-3′)*
AP1	gtaatacgactcactatagggc
AP2	actatagggcacgcgtggt
GSP1	tgcaatcatcatccaaagtagagttg
GSP2	actccaaagaccaggaccatcttc
GSP-RACE	catcaatatgtaacttggcctgac
VCAM-promoter-down	accatcttcccacgcgttttaaatagct
VCAM-promoter I up	ctcttctgagaggtaccgggaggcatg
VCAM-promoter II up	actataggcacgcgtggt
VCAM-promoter III up	tgagtcatctgacgcgtgatctaaat
VCAM-promoter IV up	agtgtctgtaacgcgtttttccctggct
VCAM-promoter V up	ctggcccgggtttacgcgtgaagggatt
VCAM-promoter VI up	cttgcaacaagacgcgttataaagagca
VCAM κB-5′-Mut-up	ggctctggcccgagtctcgccttgaaggg
VCAM κB-5′-Mut-down	cccttcaaggcgagactcgggccagagcc
VCAM κB-3′-Mut-up	tgaagagatctcgctcctcctcttgc
VCAM κB-3′-Mut-down	gcaagaggaggagcgagatctcttca
HO-1 up	gagattgagcgcaacaagga
HO-1 down	agcggtagagctgcttgaact
GAPDH up	gggctgcttttaactctg
GAPDH down	agatgatgaccctttgg

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis

SDS-PAGE and western blot analysis were performed as described (4). Primary antibodies were NF-κB-p65-sc-109-G and NF-κB-p50-sc-1191 (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). The secondary antibody was peroxidase-conjugated chicken anti-(goat IgG) (Chemicon, Hofheim, Germany). To ensure equal blotting efficacy, blots were stained with fast green (Sigma, Taufkirchen, Germany). Quantification was performed densitometrically (Fuji LAS-1000-CCD camera system with AIDA 2.0 software; Raytest, Straubenhardt, Germany)

Electrophoretic mobility shift assays (EMSAs)

Cells were grown for 4 days in medium containing 10% fetal calf serum before IL-1 (10–500 pg/ml) stimulation for 10 or 30 min in serum-free medium. For nuclear protein extracts, cells were lysed with 50 m*M* NaCl, 50 m*M* Tris, pH 7.4, containing 0.3% (ECV304) or 1% (SMC) Brij97 (Sigma) for 25 min at 4°C. Nuclei were pelleted by centrifugation and lysed in 20 m*M* HEPES, 400 m*M* NaCl, 1 m*M* EDTA, 1 m*M* EGTA, 1 m*M* phenylmethylsulfonyl fluoride, pH 7.4, for 15 min at 4°C. Protein was determined in centrifuged nuclear extracts (5). EMSAs were performed as previously described (8). DNA oligonucleotides contained either the consensus element for NF-κB (5′-aattcacaaagagggactttcccctacatccattg-3′) or the ARE of the murine HO-1 promoter (same as for HO-ARE-pGL).

p65 [^{32}P] in vivo *labeling and immunoprecipitation*

Cells were cultured for 3 days in 25-cm^2 flasks. Prior to radioactive labeling, cells were incubated in phosphate-free

medium (Dulbecco's modified Eagle medium; GIBCO, Karlsruhe, Germany) for 1 h. [^{32}P]Orthophosphate (100 μCi/ml; Hartmann Analytic, Braunschweig, Germany) was added, and incubation continued for 4 h at 37°C before IL-1 was added (1 ng/ml) for another 30 min. Cells were washed with phosphate-buffered saline and lysed for 15 min on ice in RIPA buffer (50 m*M* Tris, 150 m*M* NaCl, 2 m*M* EGTA, 0.1% SDS, 0.5% sodium desoxycholate, 1% Nonidet P-40) supplemented with protease and phosphatase inhibitors. Cellular debris was removed by centrifugation. p65 was immunoprecipitated with an antibody against p65 (same as for western blotting). Protein (500 μg) was incubated for 2 h with 0.4 μg of antibody, followed by incubation with 30 μl of protein G-Sepharose slurry overnight. Immunoprecipitates were washed five times in RIPA buffer and once in 50 m*M* Tris, 50 m*M* NaCl, pH 7.4, before SDS-PAGE. Labeled proteins were visualized by autoradiography of dried gels. Quantification was performed densitometrically (GS-800; Bio-Rad, Munich, Germany).

RNA extraction and PCR of HO-1 cDNA

RNA and cDNA were prepared as previously described (2). PCR was performed in 25-μl reaction mixtures containing 25 pmol of each primer (Table 1), 200 μ*M* dNTP, 0.625 U of *Taq* DNA polymerase (Promega, Mannheim, Germany), 2.5 μl of 10× reaction buffer, 1 m*M* (HO-1) or 2 m*M* (GAPDH) $MgCl_2$, and 1 μl (HO-1) or 0.5 μl (GAPDH) of cDNA. Initial denaturation (4 min, 94°C) was followed by 25 cycles of 40 s at 94°C, 30 s at 60°C (HO-1) or 56°C (GAPDH), and 30 s at 72°C. HO-1 was normalized for GAPDH.

HO-1 activity

Cells were cultured for 4 days in 75-cm^2 flasks and sonicated in HO-1 buffer (100 m*M* potassium phosphate, 2 m*M* $MgCl_2$, pH 7.4). Supernatant (200 μl) was added to 800 μl of reaction mix (HO-1 buffer with 1 mg of rat liver cytosol, 0.8 m*M* NADPH, 2 m*M* glucose-6-phosphate, 0.25 U of glucose-6-phosphate dehydrogenase, and 25 μ*M* hemin; all Sigma). Samples were incubated at 37°C for 1 h. Bilirubin was extracted with an equal volume of chloroform and quantified by subtracting the extinction at 530 nm from that at 464 nm (ϵ = 40 mM^{-1} cm^{-1}). HO-1 activity is expressed as picomoles of bilirubin formed per hour and milligram of protein.

Cell proliferation

Cell proliferation was determined in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described (15).

RESULTS

Rabbit VCAM-1 promoter analysis

By genome walking, 1,934 bp of the rabbit VCAM-1 promoter was obtained. The transcription start site −107 bp downstream of the ATG translation start was identified by 5′-RACE. Alignment with the human and mouse promoter revealed a conserved region between bp −112 and −315 containing the tandem NF-κB site between bp −212 and −162: 5′-GGG*TTTCCCTTGAAGGGATTTCCCTCC-3′.

This sequence is identical to the human VCAM-1 promoter and only has a deletion of one G compared with the mouse promoter (indicated by the asterisk). The complete 1,934-bp sequence has been deposited under accession no. AY212510.

NF-κB is required for VCAM-1 promoter activity

To test the need for intact NF-κB sites in the rabbit VCAM-1 promoter, fragments of different lengths were placed in front of a luciferase reporter gene (Fig. 1A). The resulting plasmids were transfected into the set of SMC cells SMC, SMC^{PHGPx}, and SMC^{LOX}, as well as into ECV and ECV^{PHGPx}. VCAM-1 promoter activity in SMC (Fig. 1B) was comparable in constructs I, II, and III, started to decrease with construct IV, and was lost in construct VI, which no longer contained NF-κB sites. In construct IV also basal activity was decreased; the inducibility by IL-1, however, was similar to that in the larger constructs (I–III), indicating that for basal activity transcription factors other than NF-κB might be required. Inducibility, however, was dependent on NF-κB sites. In SMC^{PHGPx}, basal promoter activity was decreased. The IL-1 response was maintained, but absolute activities never reached those observed in

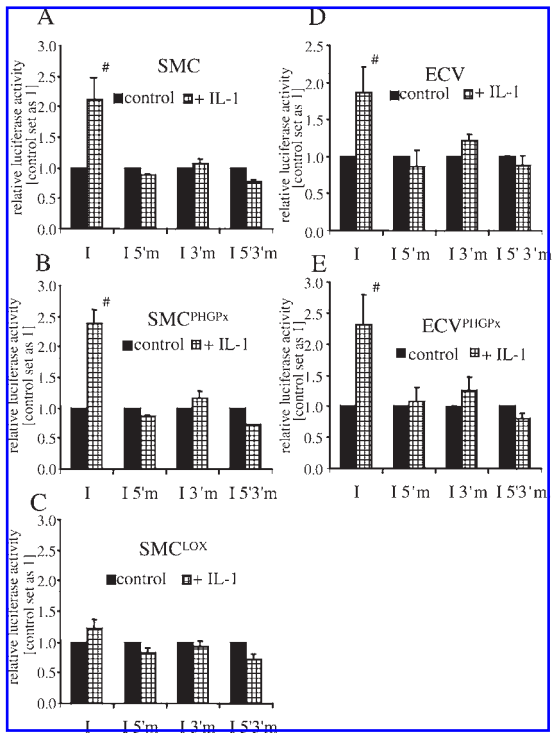

FIG. 2. Activity of wild-type and NF-κB-mutated VCAM-1 promoter in SMC and ECV cells. Either 5′ or 3′ or both NF-κB sites were mutated in construct I as described in Materials and Methods. SMC (**A**), SMC^{PHGPx} (**B**), SMC^{LOX} (**C**), ECV (**D**), and ECV^{PHGPx} (**E**) were transiently transfected with the respective constructs. IL-1 stimulation (1 ng/ml) 24 h after transfection lasted 10 h. Luciferase activities are expressed in relation to the respective unstimulated control. All experiments were run in triplicate and performed two to four times. #$p <$ 0.05 versus control.

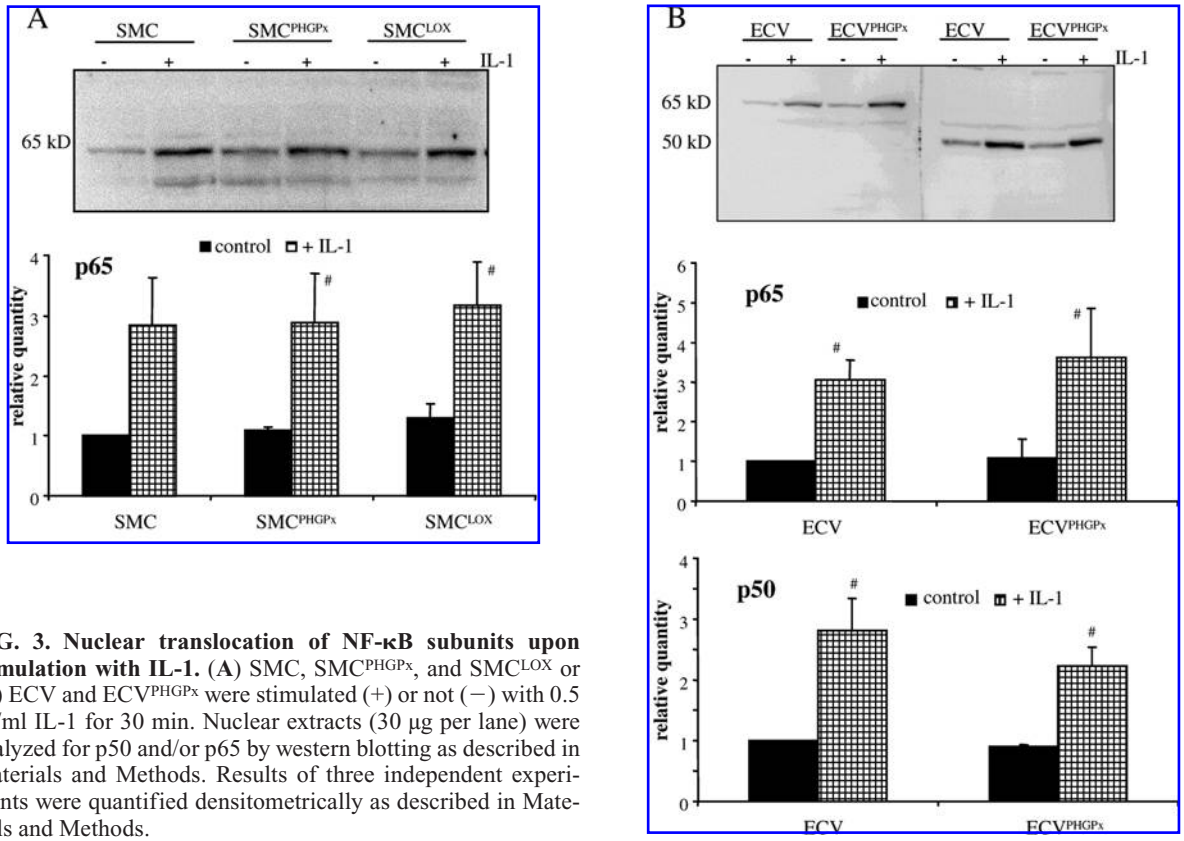

FIG. 3. Nuclear translocation of NF-κB subunits upon stimulation with IL-1. (**A**) SMC, SMC^{PHGPx}, and SMC^{LOX} or (**B**) ECV and ECV^{PHGPx} were stimulated (+) or not (−) with 0.5 ng/ml IL-1 for 30 min. Nuclear extracts (30 μg per lane) were analyzed for p50 and/or p65 by western blotting as described in Materials and Methods. Results of three independent experiments were quantified densitometrically as described in Materials and Methods.

SMC. Basal promoter activity in SMC^{LOX} was not further enhanced by IL-1. Thus, promoter activity reflects endogenous VCAM-1 mRNA and protein expression (2). VCAM-1 promoter activity was additionally investigated in ECV and ECV^{PHGPx} (Fig. 1C). It was active in ECV and to a much lesser extent in ECV^{PHGPx}. It could be stimulated by IL-1 as long as two NF-κB sites were present (constructs I–IV). In contrast to SMC, basal activity of construct IV in ECV was still the same as with constructs I–III. This might again indicate that other factors apart from NF-κB are required for basal expression, which probably are different in SMC and ECV cells; however, this remains speculative at present. Inducibility was lost with the first NF-κB site deleted (construct V). Without NF-κB sites (construct VI), activity was no longer detected. Thus, activation of the VCAM-1 promoter by IL-1 required NF-κB sites in SMC and ECV cells. This was further confirmed by mutation of NF-κB elements individually or both together in construct I. Each mutation resulted in a complete abrogation of the IL-1 response in all cells investigated (Fig. 2A–E).

PHGPx and 15-LOX affect DNA binding and transactivation ability of NF-κB

Having confirmed that intact NF-κB sites are required for VCAM-1 expression in SMC and ECV systems, we studied which of the individual steps of NF-κB-dependent gene expression was influenced by PHGPx and 15-LOX. The transfer of NF-κB subunits into the nucleus was not affected in ECV cells (Fig. 3B). Nuclear levels of both p50 and p65 were similar, irrespective of an overexpressed PHGPx. In nuclei from rabbit SMC, p50 was not recognized by available antibodies; therefore, only the transfer of p65 could be investigated. As in ECV cells, the transfer of p65 into the nucleus was influenced neither by PHGPx nor by 15-LOX (Fig. 3A). For control, nuclear expression of Fos was analyzed and was found to be equal irrespective of IL-1 treatment (data not shown).

IL-1-induced DNA binding of NF-κB was inhibited by PHGPx (Fig. 4). However, DNA binding of NF-κB in SMC was diminished only after short times of IL-1 stimulation or at lower doses (Fig. 4A). Identical results were obtained in SMC^{LOX} (data not shown). In contrast, the inhibition was clearly visible in ECV (Fig. 4B).

Phosphorylation of p65 is required for its transcriptional activity (45). We therefore tested the IL-1-induced phosphorylation of p65 in ECV and ECV^{PHGPx} cells. Again, we did not find any significant difference (Fig. 5).

As the marginally diminished DNA binding of NF-κB appeared not to suffice to explain the strongly decreased VCAM-1 expression, transactivation activity of NF-κB was tested in our set of cells. To this end, an NF-κB responsive element was placed in front of the thymidine kinase promoter, and the transactivating ability of IL-1-activated NF-κB measured in our cell systems transiently transfected with the NF-κB reporter plasmid. As obvious from Fig. 6, PHGPx and 15-LOX strongly inhibited the transactivating activity in ECV and SMC. Taken together, our data indicate that PHGPx and 15-LOX mainly inhibit NF-κB at a level downstream of DNA binding. The effects on DNA binding (Fig. 4) might be not sufficiently large to explain the dramatic inhibition of NF-κB's transactivation activity.

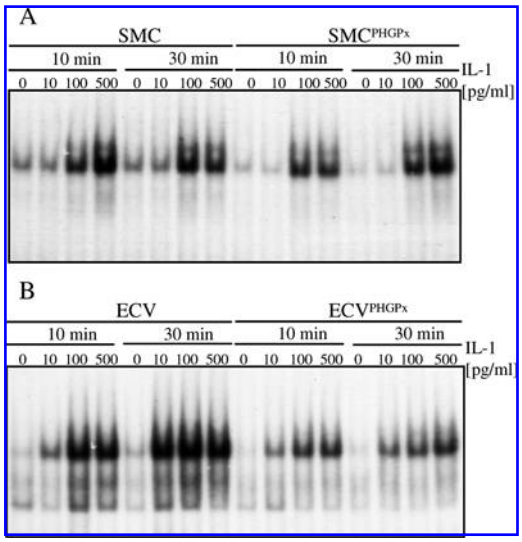

FIG. 4. Inhibition of IL-1-mediated activation of NF-κB DNA binding. (**A**) SMC, SMC^{PHGPx}, and SMC^{LOX} were incubated with IL-1 at concentrations and for times indicated. EMSAs were performed with an NF-κB oligonucleotide as described in Materials and Methods. (**B**) ECV and ECV^{PHGPx} were treated and analyzed as SMC. Results are representative of two independent experiments.

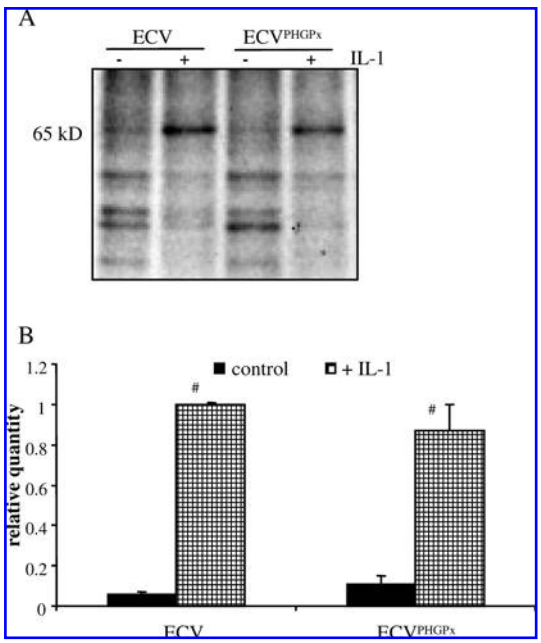

FIG. 5. Phosphorylation of p65 is not affected by PHGPx. ECV and ECV^{PHGPx} were labeled *in vivo* with [^{32}P]ATP (see Materials and Methods) and incubated with IL-1 (1 ng/ml) for 30 min. p65 was immunoprecipitated and analyzed by SDS-PAGE and autoradiography. (**A**) A representative film. **(B)** Densitometric quantification of three independent experiments.

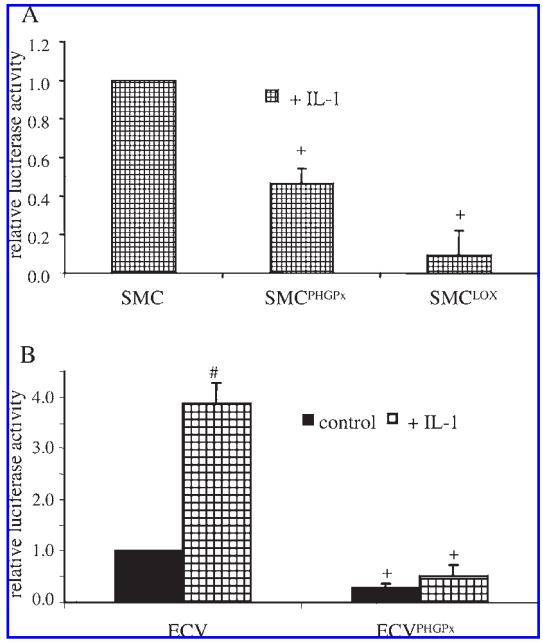

FIG. 6. Overexpression of PHGPx and 15-LOX inhibits transactivation activity of NF-κB. SMC, SMC^{PHGPx}, and SMC^{LOX} (**A**) and ECV and ECV^{PHGPx} (**B**) were transiently transfected with an NF-κB reporter plasmid. Transfected cells were incubated with IL-1 (1 ng/ml) for 10 h. In SMC, luciferase activity of IL-1-treated SMC was set to 1 and the activity in SMC^{PHGPx} and SMC^{LOX} related to SMC. This had to be done because basal activity without IL-1 stimulation was below the detection limit and could, therefore, not be taken as reference. In ECV cells, basal luciferase activity was set to 1. Values are means ± SD (n = 3). #$p < 0.05$ versus control; +$p < 0.05$ versus wild-type.

Overexpression of PHGPx and 15-LOX induces HO-1 via Nrf2

Another transcription factor that is activated by thiol modification is Nrf2. Nrf2 mediates induction of HO-1, which in SMC inhibits proliferation (24). Proliferation rate differed highly between the SMC where it was highest for SMC and lowest for SMC^{LOX} (Fig. 7A). Intrigued by this observation, we reasoned that the altered proliferation rate might result from HO-1 induction. We indeed found the proliferation rates to be inversely correlated with HO-1 expression. HO-1 mRNA was low in SMC and highest in SMC^{LOX} (Fig. 7B). mRNA levels correlated with HO-1 activity, which was 197 ± 62 pmol of bilirubin/h/mg of protein in SMC, 250 ± 100 in SMC^{PHGPx}, and 340 ± 143 in SMC^{LOX} (n = 6).

HO-1 can be induced by heme. We, therefore, tested whether a direct HO-1 induction can influence VCAM-1 expression to obtain more hints of the mechanism by which VCAM-1 is reduced by PHGPx and 15-LOX. In SMC treated with heme, VCAM-1 was indeed no longer detectable (Fig. 7C). This can be taken as strong evidence for an involvement of HO-1 in the regulation of VCAM-1 in SMC.

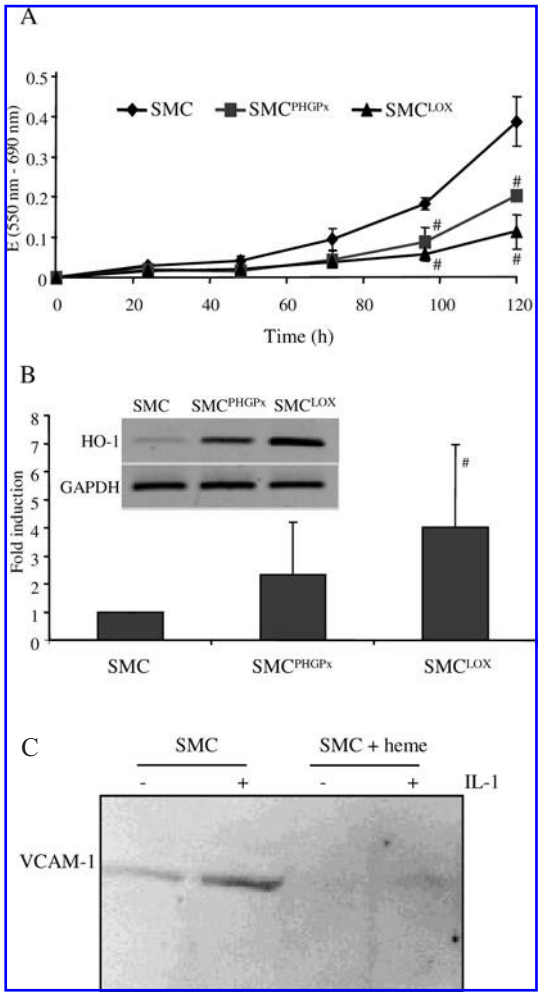

FIG. 7. Overexpression of PHGPx and 15-LOX inhibits proliferation of SMC and induces heme oxygenase, which inhibits VCAM-1 expression. (**A**) Proliferation: Cells were seeded into 96-well plates. After the times indicated, proliferation was assayed with MTT as described in Materials and Methods. Values are means ± SD (n = 3). #$p < 0.05$ versus SMC. (**B**) Induction of heme oxygenase: Cells (1.3×10^5) grown for 4 days were harvested and extracted RNA amplified by RT-PCR with primers specific for human HO-1 also recognizing rabbit HO-1. PCR products were separated on gel electrophoresis and quantified densitometrically. (**Insert**) One gel representative of six experiments. #$p < 0.05$ versus SMC. (**C**) Heme-induced repression of VCAM-1: Cells were stimulated (+) or not (−) with 1 ng/ml IL-1 for 24 h in the presence or absence of 10 μ*M* heme. Whole-cell extracts (60 μg/lane) were analyzed for VCAM-1 by western blotting as described in Materials and Methods. Results are representative of two independent experiments.

Nrf2 inhibits VCAM-1 promoter activity

HO-1 is a typical target for Nrf2. To test whether the Keap1/Nrf2 system was involved in the differential VCAM-1 expression and responsiveness to IL-1 in the SMC model system, SMC were transfected with the VCAM-1 promoter construct I and an expression plasmid for Nrf2 (pcDNA3-mNrf2) alone or in combination with pcDNA3-mKeap1. Whereas cells transfected with the empty vector showed the usual behavior, an ~2.5-fold reporter gene activation upon IL-1 stimulation, cotransfection with pcDNA3-mNrf2 not only reduced the basal activity, but also abolished the response to IL-1. Cotransfection with pcDNA3-mKeap1 partially reversed the suppression of basal and inducible activity by Nrf2 (Fig. 8) Thus, Nrf2 inhibited VCAM-1 promoter activity and, with respect to the IL-1 response, mimicked the effect of overexpression of 15-LOX and even exceeded that of PHGPx (see Fig. 1A).

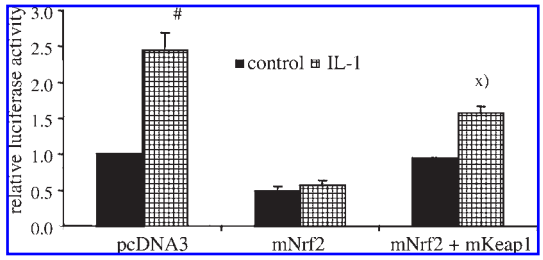

FIG. 8. Modulation of VCAM-1 promoter activity by the Keap-1/Nrf2 system. SMC transiently transfected with the VCAM-1 promoter construct I were cotransfected with 300 ng of the expression plasmid for Nrf2 (pcDNA3-mNrf2) or together with pcDNA3-mKeap1. Twenty-four hours after transfection, IL-1 treatment was started for 3 h. β-Galactosidase-normalized luciferase activity was related to pcDNA3-transfected unstimulated cells set to 1. Values are means ± SD from three individual experiments. #$p < 0.05$ versus respective controls; x), only one experiment was run in triplicate.

Overexpression of PHGPx and 15-LOX modulates the endogenous Keap1/Nrf2 system

EMSAs with an oligonucleotide containing the HO-1 promoter Nrf2 site and nuclear extracts from SMC, SMC^{PHGPx}, and SMC^{LOX} revealed a slightly enhanced Nrf2 activation in SMC^{PHGPx} and a distinctly enhanced activation of Nrf2 in SMC^{LOX} (Fig. 9A). Specificity was confirmed with SMC^{LOX} nuclear extracts and a 100-fold excess unlabeled Nrf2 (lane 4) and an unspecific NF-κB probe (lane 5). Preincubation with an antibody against Nrf2 abolished Nrf2 DNA binding (data not shown). Nrf2 activates genes via binding to the ARE. To test whether the binding is affected by PHGPx or 15-LOX, SMC were transiently transfected with a plasmid containing the ARE from HO-1, cloned in front of a pGL3 promoter-regulated luciferase, together with either pcDNA3 or pcDNA3-mNrf2. Cotransfection with Nrf2 revealed an enhanced reporter gene activity in SMC, which was more pronounced in SMC^{PHGPx} and SMC^{LOX} (Fig. 9B). This activation of Nrf2 in the PHGPx- or 15-LOX-overexpressing cells may be caused by inactivation of Keap1 by oxidation of critical SH groups (44).

DISCUSSION

The VCAM-1 promoter of rabbits contains the tandem NF-κB site already identified in humans and mice (10, 19).

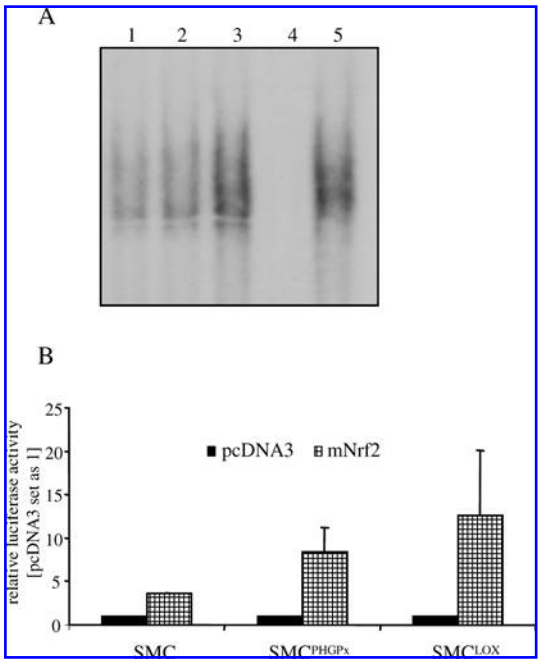

FIG. 9. Activation of Nrf2 in PHGPx- and 15-LOX-overexpressing SMC. (**A**) Nuclear extracts of respective SMC were incubated with an oligonucleotide containing the responsive element for Nrf2 of HO-1 (see Materials and Methods) and analyzed by EMSA. Lane 1, SMC; lane 2, SMC^{PHGPx}; lane 3, SMC^{LOX}; lane 4, specificity control (nuclear extracts of SMC^{LOX} were incubated with the labeled Nrf2 oligonucleotide in the presence of 100-fold excess of the same unlabeled specific oligonucleotide); lane 5, an unspecific oligonucleotide containing the NF-κB site. Results are representative of two independent experiments. (**B**) SMC, SMC^{PHGPx}, and SMC^{LOX} were transiently transfected with the luciferase reporter driven by the pGL3 promoter and the HO-1-specific ARE (HO-ARE-pGL). The ARE was activated by cotransfection with the expression plasmid for Nrf2 (pcDNA3-mNrf2). pcDNA3 transfection was for control. β-Galactosidase-normalized luciferase activity was related to pcDNA3-transfected cells set to 1. Values are means ± SD ($n = 3$).

In this study, we demonstrate the functional relevance of the NF-κB site: its deletion or mutation completely abrogates the up-regulation of a VCAM-1 promoter-driven reporter gene in SMC, and NF-κB proved to be essential for the activation of the VCAM-1 promoter also in a second cell line (ECV).

The NF-κB system has amply been shown to be modulated by redox events, the best documented one being the enhanced phosphorylation of IκB by oxidants that favors IκB degradation and facilitates nuclear translocation of the active p50/p65 complex (14, 32). This regulatory mechanism cannot account for the blunted VCAM-1 expression in our SMC model, because (a) the documented shift toward a more oxidized state of the transfectants would predict an enhanced NF-κB activation and (b) nuclear translocation was found to be unchanged. Another cytosolic event reported to contribute to NF-κB transactivation, the phosphorylation of p65, and presumed to be subject to redox regulation was equally unaltered. Thus, neither the hydroperoxide-reducing or the postulated thiol-modifying activity of PHGPx (28) nor the hydroperoxide-producing activity of 15-LOX affected the cytosolic oxidative NF-κB activation. Instead, the molecular basis for the refractory state of the transfectants must be sought downstream of the nuclear translocation and p65 phosphorylation.

Over the last few years, the understanding of how NF-κB regulates transcription has substantially increased, but is far from being clear. The p50 subunit of the active p50/p65 complex has to be reduced by thioredoxin to bind to DNA (17). p50/p50 homodimers are present in the nucleus and bind to DNA as histone deacetylase-1 complexes, thereby inhibiting gene expression (45). Signal-induced phosphorylated and CBP/p300-associated p65 can displace p50–histone deacetylase-1 complexes from DNA. CBP/p300 acetylates DNA-associated histone, which allows liberation of histone and accessibility of DNA (37, 45). This brief compilation may suffice to show that there are many more proteins involved that could be modified by PHGPx or 15-LOX.

In this context, it may be important to mention that overexpressed cytosolic proteins may also be found in the nucleus, as has been shown, *e.g.,* for cellular glutathione peroxidase (23). In fact, the PHGPx expression vector used allows the use of an alternative promoter in the first intron that encodes the nuclear form of PHGPx (27, 29). We thus have to consider the possibility that the overexpressed enzymes also affect transcription by modulating the nuclear redox balance. Therefore, the best documented nuclear event that is redox-modulated, *i.e.,* DNA binding of NF-κB, was investigated. In fact, binding of NF-κB to its specific binding site was slightly inhibited in the PHGPx- and 15-LOX-transfected SMC, but hardly to an extent that could explain the lack of any inducibility of VCAM-1 expression in SMC^{LOX}. In short, therefore, PHGPx and 15-LOX overexpression inhibits VCAM-1 expression downstream of cytosolic NF-κB activation, nuclear translocation, and DNA binding.

Overexpression of PHGPx or 15-LOX affects the activation of Nrf2

In contrast to the NF-κB system that is only modulated by thiol modification, Nrf2 activation requires an oxidative modification of essential cysteines in Keap1 to allow translocation of Nrf2 to the nucleus (20, 21). Accordingly, activators of Nrf2 are electrophilic compounds, which react with thiols in a Michael reaction, or oxidants (11). An important target gene of Nrf2 is HO-1 that degrades b-type hemes producing equimolar amounts of carbon monoxide, iron, and biliverdin (1). Beyond, HO-1 is considered to act as an antioxidant enzyme: it is induced by oxidative stress and various inflammatory stimuli (25). It stimulates apoptosis (24), inhibits proliferation (13), and protects against experimental endotoxemia (31). In our SMC system, Nrf2 is activated by PHGPx and 15-LOX, and this Nrf2 activation was relevant in terms of HO-1 expression. Interestingly, the enhanced HO-1 expression correlated with decreased VCAM-1 expression, VCAM-1 promoter activity, and NF-κB transactivation (Fig. 10).

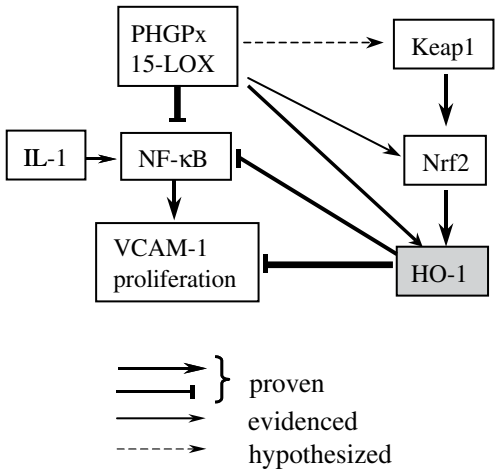

Fig. 10. Proposed mechanism by which PHGPx and 15-LOX inhibit VCAM-1 expression and proliferation in SMC. There is strong evidence for an induction of HO-1 and an inhibition of NF-κB translational activity. Both events lead to an inhibition of VCAM-1 expression and proliferation of SMC. There are hints for an activation of Nrf2, leading to HO-1 expression. This must be due to thiol modification of Keap1, which remains to be demonstrated. The key player in this cross-talk most probably is HO-1.

The link between the Keap1/Nrf2 system and NF-κB-mediated VCAM-1 expression might be HO-1

We here observe an activation of Nrf2 and a simultaneous inhibition of NF-κB transactivation capacity. A direct interference of Nrf2 with the NF-κB-binding site is not very likely because both factors bind to very different sequences. Also, a competition for reaction partners is not likely either, because Nrf2 heterodimerizes with other basic leucine zipper proteins such as small Maf (42), whereas the most common NF-κB heterodimer consists of the subunits p65 and p50. Common coactivators, or corepressors are not known yet. Thus, knowledge on a cross-talk between NF-κB and Nrf2 is scarce and circumstantial, but points to HO-1 as an essential link: (a) Chen *et al.* (9) reported that shear stress induces HO-1 in endothelial cells via activation of Nrf2 in a Keap1-dependent manner. In parallel, Nrf2 inhibited tumor necrosis factor-α (TNFα)-induced VCAM-1 expression. (b) A prominent feature of a human case of genetic deficiency in HO-1, apart from iron deposition, was endothelial damage (43), a phenomenon typically caused by adhering activated phagocytes. (c) HO-1$^{-/-}$ mice were reported to be highly susceptible to oxidative stress, in particular to endotoxin challenge (33), and the commonly accepted cause of death in such modeled and clinical septicemia is collapse of the microcirculation due to adhering white blood cells and more or less complicating intravascular coagulation, *i.e.,* phenomena that ultimately depend on IL-1 and/or TNFα-induced, NF-κB-mediated CAM expression. (d) Most importantly, Soares *et al.* (39) showed that overexpression of HO-1 inhibited the TNFα-dependent activation of NF-κB and VCAM-1 expression in aortic endothelial cells. HO-1 was suggested to inhibit NF-κB activity downstream of IκB phosphorylation/degradation and nuclear translocation.

The mild oxidative challenge caused by overexpression of PHGPx and 15-LOX in SMC strikingly mimics the effects exerted by overexpressed HO-1 in aortic endothelial cells. We are therefore tempted to share the conclusion of Soares *et al.* (39) that HO-1 can interfere with the transcriptional machinery of NF-κB in the nucleus. The molecular mechanism of this interference could not be elucidated by us either. We could rule out, however, that p65 phosphorylation contributes to an HO-1-mediated cross-talk between the Nrf2 and NF-κB systems, as had been suggested by Soares *et al.* (39).

Conclusions

In conclusion, similar changes in the cellular SH/SS balance can be induced by overexpression of seemingly antagonizing enzymes such as 15-LOX and PHGPx. The persisting oxidative shift thus induced activates the Keap1/Nrf2 system rather than the cytosolic cascade leading to NF-κB activation. Nevertheless, NF-κB-dependent transcriptions, exemplified by cytokine-induced VCAM-1 expression, are dramatically inhibited. Circumstantial evidence suggests that Nrf2-induced HO-1 blocks the NF-κB-dependent transcriptional machinery under these conditions in a still unknown manner. The Keap1/Nrf2 couple thus proves to be a more sensitive redox sensor than the cytosolic NF-κB system. In an 'antioxidant' response to a mild oxidative stress, Nrf2 activation may dampen NF-κB-dependent responses to proinflammatory cytokines.

In this respect, the data here presented may be viewed as a novel lead to understand the tolerance and cross-tolerance development against hyperbaric oxygen, inflammatory cytokines, endotoxins, and the like by a low dose of the same or an equivalent challenge.

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ABBREVIATIONS

AP, adaptor-specific primer; ARE, antioxidant response element; CAM, cell adhesion molecule(s); EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSP, gene-specific primer; HO-1, heme oxygenase-1; IL-1, interleukin-1; Keap1, Kelch-like ECH-associated protein-1; 15-LOX, 15-lipoxygenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor-κB; Nrf2, NF-E2-related factor-

2; PAGE, polyacrylamide gel electrophoresis; PHGPx, phospholipid hydroperoxide glutathione peroxidase (GPx4); RACE, rapid amplification of cDNA ends; SDS, sodium dodecyl sulfate; SMC, smooth muscle cell(s); TNFα, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule-1.

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