Interleukin-1-induced nuclear factor κ B activation is inhibited by overexpression of phospholipid hydroperoxide glutathione peroxidase in a human endothelial cell line

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Oxygen radicals are commonly accepted mediators in the tumour necrosis factor-mediated nuclear factor κ B (NF κ B) signalling cascade, but evidence for their role during interleukin-1 (IL-1) signalling is lacking. To test the involvement of hydroperoxides we investigated whether IL-1-induced NF κ B activation could be influenced by glutathione peroxidases (GPx). These enzymes remove hydroperoxides with various specificities for the hydroperoxide substrate. By overexpressing phospholipid hydroperoxide glutathione peroxidase (PHGPx), which characteristically reacts with lipophilic hydroperoxides, the roles of H₂O₂ and lipidhydroperoxides were assessed. A human umbilical endothelial cell line, ECV 304, was stably transfected with the genes for both PHGPx and selenophosphate synthetase (*selD*), which provides selenophosphate for selenoprotein biosynthesis. When grown in selenium-deficient culture medium, the double-transfected clone (ECV^{PHGPx+selD+}) expressed 5-fold higher ($P < 0.005$) PHGPx activity (measured by phosphatidylcholine hydroperoxide removal) than controls. The rate of H₂O₂ removal was

also significantly ($P < 0.01$) higher in this clone. When grown with high levels of extracellular selenium (up to 100 nM selenite), PHGPx activity and H₂O₂ removal were enhanced substantially in control cells and transfected cells. Under these conditions, PHGPx activity was 1.7-fold ($P < 0.005$) higher in ECV^{PHGPx+selD+}, but H₂O₂ removal was the same as in controls. IL-1-induced NF κ B activation was inhibited by selenium supplementation in control cells. In ECV^{PHGPx+selD+} under conditions of selenium restriction, IL-1 induced NF κ B activation only to a similar extent as under conditions of selenium supplementation in controls, and activation was abolished with 50 nM sodium selenite. These results show that overexpressed PHGPx is sufficient to inhibit NF κ B activation, and suggests that NF κ B activation by IL-1 is mediated by a preferential substrate of PHGPx, such as a fatty acid hydroperoxide, rather than by H₂O₂, the preferred substrate of the more abundant cytosolic GPx.

INTRODUCTION

Increasing evidence suggests that reactive oxygen species (ROS), apart from being toxic, play an important role as mediators or modulators in cellular signalling. In this context, one of the most extensively studied pathways is the activation of nuclear factor κ B (NF κ B). It depends on a redox regulated cascade of phosphorylation and dephosphorylation processes, leading to a dissociation of the cytosolic NF κ B/I κ B complex and translocation of activated NF κ B to the nucleus. Neither the oxidants involved nor the targets in the signalling cascade are known with certainty, but thiol modification or oxidation of kinases and/or phosphatases upstream of I κ B phosphorylation are being discussed [1]. Cytokines, such as tumour necrosis factor (TNF) or interleukin-1 (IL-1), are the most significant physiological activators of NF κ B. In some cell culture systems NF κ B activation can also be triggered by H₂O₂ (reviewed in [2]), indicating that hydroperoxides may be involved in the signal transduction of these cytokines. Hydroperoxides or other ROS have therefore been implicated as common second messengers of NF κ B activation. Such ROS production by TNF has been well documented [3–5], but publications proving ROS production by IL-1 are scarce. Superoxide production upon IL-1 exposure has been demonstrated in fibroblasts [6] and mesangial cells [7]. Other redox events in IL-1 signalling may be inferred from indirect evidence, e.g. the inhibition of IL-1-induced T-cell responses by lipoxygenase inhibitors [8], the rapid increase in phospholipase A₂ activity in T-cells [9], the involvement of

reactive oxygen intermediates in IL-1-induced cyclooxygenase-2 expression [10], and the IL-1-induced stimulation of nerve growth factor secretion mediated by arachidonic acid oxidation in astroglial cultures [11]. If hydroperoxides are involved in NF κ B activation by IL-1, intracellular hydroperoxide-detoxifying enzymes, such as glutathione peroxidases (GPx), should influence the IL-1 response.

GPx are selenoenzymes that can reduce various hydroperoxides at the expense of glutathione (GSH). So far, overexpression of the cytosolic GPx (cGPx) in human T47D cells has been shown to depress TNF-induced NF κ B activation by inhibition of I κ B phosphorylation [12]. However, four distinct types of GPx are known in mammalian systems. They all use GSH as reducing substrate but, in part, differ in their specificity for hydroperoxides [13]. The phospholipid hydroperoxide GPx (PHGPx) is most intriguing because of its ability to reduce hydroperoxides, not only from soluble substrates (such as cGPx) but also from complex lipids within membranes. Unlike cGPx, PHGPx is partially membrane-associated [14]. The use of an individual GPx with a characteristic substrate specificity and subcellular distribution might therefore help to identify the nature and site of action of hydroperoxides putatively involved in signalling cascades.

Being selenoproteins, the activity of GPx depends on the amount of selenium available for their biosynthesis. Providing optimal selenium supply proved to be the best tool to increase the activity of selenoproteins in cultured cells. Thereby, however, all selenoproteins are affected, whereas the influence of an individual

Abbreviations used: cGPx, cytosolic GPx; GPx, glutathione peroxidase; IL-1, interleukin 1; NF κ B, nuclear factor κ B; PCOOH, phosphatidylcholine hydroperoxide; PHGPx, phospholipid hydroperoxide glutathione peroxidase; ROS, reactive oxygen species; TNF, tumour necrosis factor.

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selenoprotein on a specific process can only be investigated by genetic manipulation. Unfortunately, overexpression of selenoproteins is not trivial, due to the limitation of the complex machinery of selenium incorporation [15,16]. In fact, a substantial increase in GPx activity by transfecting cells with cGPx has only been obtained in few cases, and only if the parent cells had extremely low endogenous activity, as was the case in the T47D cells mentioned above [12,17] or in NIH3T3 and MCF-7 cells [18]. In addition, in the last two cell lines mentioned, the activity was lost after some passages [18]. PHGPx could be enhanced by transfection with the corresponding cDNA 2–10-fold in the case of the guinea pig cell line 104C1 [19] and 3.8-fold in RBL2H3 cells [20]. We consistently failed to observe any PHGPx-overexpression in human endothelial cells (ECV 304) transfected with the PHGPx gene alone [21], but we obtained a significant increase in PHGPx activity by co-transfection with *selD*, the gene of selenophosphate synthetase, providing selenophosphate for selenocysteine biosynthesis [22]. This cell line, which characteristically responds to IL-1 with the activation of NF κ B, was used here to investigate the possible involvement of hydroperoxides in the activation process. We tested whether overexpression of PHGPx is negatively correlated with IL-1-induced activation of NF κ B.

MATERIALS AND METHODS

Cells and cell culture

ECV 304 cells were grown in medium M199 EARLE (Seromed) supplemented with 5% fetal calf serum (Gibco)/0.68 mM L-glutamine/0.22% NaHCO₃/5 mM Hepes/penicillin-streptomycin 50 units/ml. Selenium was supplemented as sodium selenite (Gibco), as indicated, for 4 days.

Transfection with the PHGPx gene

A cDNA fragment of pig PHGPx, covering the complete coding and 3' untranslated region including the SECIS (selenium incorporation sequence), was isolated from plasmid pMM3 [23] by *KpnI*–*Bam*HI digestion and subcloned into the eukaryotic expression vector pcDNA III (Invitrogen) under the control of cytomegalovirus immediate early promoter. One day before transfection, ECV 304 cells were seeded in 6-well plates (Costar) at 1.5×10^5 cells per well. Transfections were performed with 1 μ g of the PHGPx–pcDNAIII construct and 10 μ l of lipid (Lipofectamin; Gibco) for 4 h according to the instructions of the manufacturer. At day 1 after transfection, selection was started by culturing cells in growth medium containing 800 μ g/ml Geneticin (Gibco). After 20 days, individual resistant colonies were selected and expanded. Then clones were harvested and tested for PHGPx activity. Since none of the clones expressed substantially higher PHGPx activities, one clone was used for subsequent transfection with *selD*.

Transfection with *selD*

The complete cDNA fragment coding for the human *selD* homologue was isolated from clone SelD4-cDM8 (a generous gift from M. J. Berry, Harvard Medical School, Boston, MA, U.S.A.) [22] by *XhoI* digestion, and subcloned into the eukaryotic expression vector pZeoSV (Invitrogen). ECV 304 cells and the selected clone previously transfected with the PHGPx gene were transfected with the SelD4–ZeoSV construct as described above. Clones were selected for Zeocin resistance (100 μ g/ml Zeocin; Invitrogen) and analysed for PHGPx activity. The resulting clones were named ECV^{PHGPx+SelD+} and ECV^{SelD+} respectively.

GPx activities

GPx activities were determined as described in [24]: $(1-2) \times 10^7$ cells were homogenized in 0.7–1.5 ml of buffer (100 mM Tris/HCl/300 mM KCl/0.1% peroxide-free Triton X-100, pH 7.6) by sonication. The test mix was (final concentrations): 100 mM Tris/HCl, pH 7.6/5 mM EDTA/1 mM sodium azide/3 mM GSH/0.1 mM NADPH/0.1% peroxide-free Triton X-100/600 m-units/ml glutathione reductase. Sample (100 μ l) was added to 890 μ l of the test mix and preincubated at 37 °C for 10 min. The reaction was started by the addition of the substrate specific for PHGPx, 10 μ l phosphatidylcholine hydroperoxide (final concentration 50 μ M), synthesized as described in [25]. The activity of cGPx was measured by addition of H₂O₂ (final concentration 50 μ M) as substrate. GPx activity was expressed as turnover of hydroperoxide/min per mg of protein as measured by consumption of NADPH.

IL-1-induced NF κ B-activation by electrophoretic mobility-shift assays

Nuclear protein extracts were prepared as described previously [26] with some modifications: ECV 304 or ECV^{PHGPx+SelD+} were grown in the respective media supplemented with or without 50 nM sodium selenite. After stimulation with recombinant human IL-1 (provided by the Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany) for 1 h in serum-free medium, cells (10^7) were collected with 13 ml of PBS and pelleted by centrifugation at 1000 *g* for 8 min. The cells were lysed with 700 μ l of lysis buffer (0.3% Brij 96/50 mM NaCl/50 mM Tris/HCl, pH 7.4) while rotating at 4 °C for 25 min. Nuclei were pelleted at 10000 *g* for 30 s and then extracted with 50 μ l of buffer C (20 mM Hepes, pH 7.9/400 mM NaCl/1 mM EDTA/1 mM EGTA/1 mM dithiothreitol/1 mM PMSF) for 15 min. The nuclear extract was cleared by centrifugation at 10000 *g* for 5 min, estimated for protein concentration [27], adjusted to 5 μ g of protein/ml and frozen in aliquots at –70 °C. A DNA fragment, representing the binding motif for NF κ B in the human IL-2 gene [28] flanked by *Bam*HI sites, was generated by annealing two complementary oligonucleotides (5'-GATCCACAAA-GAGGGATTTCACCTACATCCATTG-3'). The fragment was purified by gel chromatography and 5'-labelled with ³²P. The reactions were carried out in a volume of 10 μ l containing 15 mM Hepes, pH 7.9, 1 mM EDTA, 1 mM dithiothreitol, 10% (w/v) glycerol, 3 μ g of poly(dIdC) (Pharmacia) and 10 fmol of labelled DNA with 1 μ l of nuclear extract containing 5 μ g of protein. The samples were incubated at room temperature for 30 min and immediately electrophoresed on a native 4% (w/v) polyacrylamide gel in 1 $\times$ TBE buffer (0.09 M Tris-borate/0.02 M EDTA). Supershift experiments were carried out with antibodies against p50, p65 and c-Rel (Santa Cruz Biotechnology) as described [29].

RESULTS AND DISCUSSION

Overexpression and selenium-dependent activity of PHGPx in control and double transfected ECV cells

The successful transfection of ECV cells with the genes for PHGPx and selenophosphate synthetase has been demonstrated recently [21]. However, overexpression of PHGPx in human endothelial cells was not readily achieved by transfection of the pertinent gene under the control of the strong cytomegalovirus promoter, since, obviously, it is not the concentration of the transcript, but one or more steps of the selenium incorporation machinery, that limit expression [21]. As had been shown before

Figure 1 Selenum-dependent GPx activities in control and transfected cells

Top, PHGPx activity; bottom, activity measured with H_2O_2 as substrate, mainly representing cGPx activity. ECV 304 (control), $\text{ECV}^{\text{SelD}+}$ and double-transfected cells ($\text{ECV}^{\text{PHGPx}+\text{SelD}+}$) were grown in the respective media supplemented with sodium selenite as indicated. After 3–4 days cells were harvested and analysed for GPx activity. Values are means $\pm$ SD from 3–5 independently grown cell samples from which the GPx activities were measured in triplicate. * $P < 0.005$; + $P < 0.01$ compared with control. For further details see text and the Materials and methods section.

in analogous attempts to overexpress selenoprotein 5'-deiodinase [22], the block in the PHGPx overexpression could be partially overcome by simultaneous overexpression of selenophosphate synthetase. At least in selenium-deficient cells, this co-transfection lead to a substantial overexpression of PHGPx in the $\text{ECV}^{\text{PHGPx}+\text{SelD}+}$ cells, whereas at saturating selenium supplementation the increment of PHGPx expression was less, though significantly, pronounced, probably due to further limitations in the complex mechanism of selenoprotein biosynthesis. Irrespective of these unresolved problems of selenoprotein biosynthesis, we expected the double-transfected cells to be a useful tool to study the consequences of a selective increase of PHGPx on IL-1 signalling.

We first assayed the selenium dependency of GPx expression, measured as enzyme activity with specific substrates, to allow differentiation between PHGPx and cGPx present simultaneously. The selenium-deficient medium contained 5% of serum with $8.7 \mu\text{g}$ selenium/l, resulting in 5.5 nM selenium, which is, however, of unknown bioavailability for the synthesis of GPx [24]. PHGPx activity measured with the specific phosphatidylcholine hydroperoxide (PCOOH) (Figure 1, top) was 1–2 nmol PCOOH/min per mg of protein in control cells (ECV)

when grown in selenium-deficient medium, and increased up to 17 nmol PCOOH/min per mg of protein upon supplementation with 30–100 nM selenite. PHGPx activity in cells only transfected with *selD* ($\text{ECV}^{\text{SelD}+}$) was not different from control, indicating that an enhanced selenophosphatase synthetase activity per se did not facilitate selenoprotein biosynthesis in general. In double-transfected cells ($\text{ECV}^{\text{PHGPx}+\text{SelD}+}$) the PHGPx activity was increased 4–5-fold to about 7 nmol PCOOH/min per mg of protein under selenium limiting conditions (0–0.5 nM sodium selenite) and increased further to 30 nmol PCOOH/min per mg of protein upon supplementation with 50 nM selenite. Thus, at optimum selenium supply, PHGPx activity could be enhanced only about 1.7-fold by transfection.

Higher GPx activity in the double-transfected cells compared with controls was also observed when the non-specific substrate H_2O_2 was used (Figure 1, bottom), generally leading to higher turnover rates (compare y-axes in Figure 1, top and bottom). In control (ECV) cells, this activity rose from 7–10 nmol H_2O_2 /min per mg of protein under selenium-limiting conditions (0–1 nM selenite) to about 150 nmol H_2O_2 /min per mg of protein at 100 nM selenite. $\text{ECV}^{\text{SelD}+}$ did not differ significantly from controls. In $\text{ECV}^{\text{PHGPx}+\text{SelD}+}$, however, a significantly higher activity was observed under conditions of selenium restriction (0–0.5 nM selenite), where a turnover of 18–20 nmol H_2O_2 /min per mg of protein was measured. This became less significant with increasing selenite supplementation, and ended in values similar to controls (about 150 nmol H_2O_2 /min per mg of protein) at 30–100 nM selenite. Thus, the difference in H_2O_2 -reducing capacity between untransfected and double-transfected cells is pronounced only at low selenium concentration, where PHGPx contributes to H_2O_2 metabolism by a reasonable amount. At selenium saturation, H_2O_2 metabolism by PHGPx becomes negligible. This observation is in accordance with the generally higher levels of cGPx in selenium-adequate cells and implies that the contribution of the additionally transfected PHGPx to H_2O_2 metabolism is insignificant under such conditions.

Depending on the selenium supplementation, we have thus created four states which differ in the activities of GPx. (1) At low selenium, PHGPx and cGPx activities are very low in controls and $\text{ECV}^{\text{SelD}+}$. Therefore, putative effects of H_2O_2 or lipophilic hydroperoxides are not inhibited. (2) At high selenium, PHGPx and cGPx activities are enhanced in controls and $\text{ECV}^{\text{SelD}+}$. Thus no differentiation can be made between H_2O_2 and PCOOH removing capacity. (3) At low selenium, PHGPx activity in the double-transfected cells is high. Under these conditions the activity measured with H_2O_2 is also higher and represents PHGPx rather than cGPx, since PHGPx also reacts with H_2O_2 . In consequence, at low selenium, transfected PHGPx will be relevant to both H_2O_2 and lipid hydroperoxide removal in $\text{ECV}^{\text{PHGPx}+\text{SelD}+}$. Again, an unequivocal differentiation between the effects of H_2O_2 and lipid hydroperoxide is not possible, although the effect on lipophilic hydroperoxides may be more pronounced. (4) At high selenium the relative increase in PHGPx, measured with PCOOH, in $\text{ECV}^{\text{PHGPx}+\text{SelD}+}$ is less than under selenium-deficiency, but still significantly higher than in controls. In contrast, the H_2O_2 metabolizing capacity is the same as in untransfected cells at optimum selenium supply (state 2). Thus, effects observed in selenium-supplemented $\text{ECV}^{\text{PHGPx}+\text{SelD}+}$ cells can be attributed solely to the removal of lipophilic hydroperoxides.

Overexpression of PHGPx inhibits IL-1-induced activation of NF κ B

As shown previously, selenium supplementation suppresses TNF-induced activation of NF κ B [30,31]. The inhibition of TNF-signalling has been suggested to result from an optimization

Figure 2 IL-1-induced activation of NF κ B: effect of selenium, PHGPx and *seID*

Cell lines, ECV, ECV^{PHGPx+SeID+} and ECV^{SeID+}, were grown in the respective media with, indicated as +Se, or without sodium selenite (50 nM). After 4 days cells were stimulated with the indicated concentrations of IL-1 for 1 h in serum-free medium. Controls were left in serum-free medium for 1 h without IL-1. Then cells were harvested and nuclei were extracted as described in the Materials and methods section. The protein content of nuclear extracts was estimated and the extracts adjusted to 5 μ g of protein/ μ l. For each binding reaction 5 μ g of protein was used. The results shown are from two gels, run and autoradiographed under identical conditions simultaneously. Thus, results obtained from the two gels can be compared directly. For further details see text and Materials and methods section.

of H₂O₂ metabolism by cGPx [30,31] but could equally be attributed to the increase of other selenoproteins such as PHGPx or thioredoxin reductase [32–34], which is linked to hydroperoxide metabolism via the peroxiredoxins [35]. In view of the broad and different substrate specificity of selenium-dependent peroxidases, optimized H₂O₂ removal can, however, not be unequivocally interpreted as the cause of suppressed NF κ B activation by selenium, and even in TNF-signalling the unique role of H₂O₂ remains controversial, as recently reviewed [1]. We therefore made use of the cellular models described above to test whether hydroperoxides are involved in the IL-1-induced activation of NF κ B and what the nature of these hydroperoxides could be.

NF κ B activation and subsequent translocation into the nucleus was analysed by gel electrophoretic mobility-shift assays. Figure 2 shows the following features. (i) IL-1 activates NF κ B in a dose-dependent manner in all three cells tested, ECV, ECV^{SeID+}, and ECV^{PHGPx+SeID+}. (ii) There are two bands representing complexes of NF κ B species bound to the binding site for NF κ B on the oligonucleotide used. The upper band is p50/p65, as was determined by supershifting with the antibodies for p50, p65 and c-Rel respectively (results not shown). The lower band is present before IL-1 treatment in cells without increased PHGPx activity (ECV and ECV^{SeID+}), but is further enhanced by IL-1, and mainly consists of p50. It is absent in cells overexpressing PHGPx and only slightly stimulated by IL-1. (iii) NF κ B activation is more pronounced in all three cell types under conditions of selenium deficiency at all three concentrations of

IL-1. Selenium supplementation (indicated by +Se) reduced NF κ B activation in control and ECV^{SeID+} and almost abolished it in ECV^{PHGPx+SeID+}. (4) A marked suppression of IL-1-induced NF κ B activation in ECV^{PHGPx+SeID+} compared with controls occurred under conditions of both selenium restriction and selenium supplementation. (5) The small increase in NF κ B activation in ECV^{SeID+} cells under selenium-limiting conditions was observed in all experiments and can therefore not be ignored. Binding of NF κ B to its responsive elements in the nucleus depends on thiols in the NF κ B protein. These thiols are kept in the reduced form by thioredoxin [36], which, in turn, is reduced by thioredoxin reductase, a selenoenzyme [32,33]. An enhanced selenophosphate synthetase activity might also enhance thioredoxin reductase biosynthesis, explaining a stronger binding of NF κ B. However, this hypothesis has to be further validated by experimental data.

Interpretation of PHGPx gene transfection on IL-1-induced NF κ B activation has to consider the substrate specificity and the different GPx activities dependent on selenium supply (see states 1–4 above). PHGPx is particularly reactive with lipid hydroperoxides [37], whereas cGPx is most active with H₂O₂ [38]. Inhibition of NF κ B activation in ECV^{PHGPx+SeID+} under selenium-deficient conditions (state 3) is as pronounced as that of full selenium supplementation in control (ECV) cells alone (state 2). This indicates that either H₂O₂ or lipophilic hydroperoxide could be responsible for the activation of NF κ B. The strong inhibition in ECV^{PHGPx+SeID+} cells under conditions of optimum selenium supplementation (state 4), however, strongly suggests that it is not H₂O₂ but another hydroperoxide which mediates or facilitates

NF κ B activation by IL-1, since under these conditions the contribution of PHGPx to H₂O₂ metabolism was negligible due to the abundance of cGPx.

We thus have shown that, in activating NF κ B, IL-1 signalling parallels the TNF signalling in being mediated, or at least facilitated, by an oxidant. In the case of TNF, H₂O₂ has been proposed as a potential mediator of NF κ B activation. This is in accordance with the dampening of the TNF-induced NF κ B activation by overexpression of cGPx [12]. However, even in TNF signalling, other oxidants, such as GSSG [39] and redox-independent processes, have been proposed [40]. IL-1-induced signalling in the ECV cells appears not to depend critically on H₂O₂. The pronounced inhibitory effect of small increases in PHGPx in the double-transfected ECV cells, despite excess levels of cGPx, rule out a relevant role of H₂O₂. Considering that IL-1 activates the arachidonic acid cascade [9,10,41], a primary cyclo-oxygenase or lipoxygenase product might be considered a reasonable candidate for the oxidant involved in the IL-1-induced NF κ B activation. Lipoxygenases and cyclo-oxygenases need a certain 'peroxide tone' for activity [42]. If the peroxide tone is reduced, for example by overexpression of PHGPx, these enzymes are less active and NF κ B activation is impaired. This would explain the absence of NF κ B activation in ECV^{PHGPx+SeID+} constitutively before IL-1 stimulation (Figure 2). This hypothesis is further strengthened by the observation that, due to compartmentation, PHGPx, rather than cGPx, interferes with leukotriene formation [43].

Although the oxidant involved in the activation of NF κ B may differ depending on the signalling cytokine or cell type, the molecular target subject to redox regulation may again be identical. Ample evidence indicates that the phosphorylation and/or degradation of the inhibitory I κ B within the cytosolic NF κ B complex depends on the redox state [12,44]. How this is achieved is still unknown. Modulation of the activities of yet unidentified upstream protein kinases or phosphatases could in principle be brought about by H₂O₂, fatty acid hydroperoxides, hydroperoxides of membrane lipids, or by a product of the peroxidase reactions, such as GSSG or oxidized thioredoxin.

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