Peroxiredoxins are a widely distributed superfamily of peroxidases such as those of lung surfactant. Prx 6 is enriched in the lung compared to other organs and is expressed at highest levels in alveolar epithelial type II cells, bronchiolar Clara cells, and alveolar macrophages [6–8]. A recent study by Diet et al. [9] and own previous work [10] indicated that expression of Prx 6 is increased by LPS and IFN-γ in murine bone marrow-derived macrophages (BMM). Moreover, we could demonstrate that the LPS- and IFN-γ-induced cyclooxygenase-2 (COX-2) expression and secretion of prostaglandin E2 (PGE2) leads to increased Prx 6 gene expression. Inhibition experiments revealed that the LPS- and IFN-γ-mediated Prx 6 gene induction is in addition to a NO-dependent mechanism, regulated in a NO-independent manner by both COX-1 and COX-2 [10].

Three kinds of enzymes, phospholipase A2, COX, and terminal PG synthases, are involved in the biosynthesis of the conventional prostaglandins PGD2 and PGE2. Cytosolic phospholipase A2 (cPLA2) catalyzes the hydrolysis of the sn-2 acyl bond of membrane phospholipids, resulting in production of lysophospholipids and release of free arachidonic acid, which is then supplied to either of the two COX isoenzymes, constitutive COX-1 (PGH synthase 1) or inducible COX-2 (PGH synthase 2). The COX metabolite PGH2 is subsequently isomerized to PGD2 or PGE2 by terminal PG synthase enzymes, such as PGD synthase and PGE synthase, respectively. The cyclooxygenase prostaglandin 15d-PGJ2, the dehydration end product of PGD2, has been known to display multifaceted cellular functions, including anti-inflammatory and cytotoxic effects [11,12]. Interestingly, a previous study using mouse peritoneal macrophages demonstrated that 15d-PGJ2 is able to increase gene expression of Prx 1 [13].
PGD₂ and PGE₂ exert their effects through different G-protein-coupled receptors, DP1-2 or EP1-4 [14]. Among these, the DP1, EP2, and EP4 receptors increase cAMP via activation of adenylate cyclase [15]. To date, most cAMP-mediated effects of PGD₂ or PGE₂ have been explained by the classic downstream target, protein kinase A (PKA), which phosphorylates the cAMP response element-binding protein (CREB) in a variety of mammalian cells [16,17], or by a novel target for cAMP, exchange protein directly activated by cAMP (Epac) [18,19].

So far, the regulatory mechanisms controlling Prx 6 expression are not known. Thus, the aim of this study was first to investigate gene expression of peroxiredoxins in primary macrophages by conventional and cyclopentenone prostaglandins and second to characterize the role of various protein kinases and transcription factors on Prx 6 mRNA expression.

Materials and methods

Materials

PGA₁, PGA₂, PGE₂, PGF₂α, 15d-PGJ₂, dibutylryl-cAMP, forskolin, IBMX, H-89, KT5720, MDL 12,330A, LY294002, Ro31-8220, Gö6976, SB202190, SP600125, PD98059, AG490, MAPK, AAOCCF3, sulforaphane, caffeic acid phenethyl ester (CAPE), tert-butylhydroquinone (tBHQ), triplitzazone, ciglitazone, GW-9662, and L-NIL were obtained from Enzo Life Sciences (Lorrach, Germany). PGD₂ and PGE₁ were from Calbiochem (Darmstadt, Germany). Griess reagent (modified) and LPS (Escherichia coli serotype O55:B5) were from Sigma-Aldrich (Taufkirchen, Germany) and mIFN-γ was from Roche (Mannheim, Germany). 6-Bnz-cAMP and 8-pCPT-2′O-Me-cAMP were purchased from Calbiochem (San Francisco, USA). 6-Bnz-cAMP and 8-pCPT-2′O-Me-cAMP were purchased from Biolog Life Science Institute (Bremen, Germany). Polyclonal antibody against GAPDH and monoclonal antibody against Prx 6 were from AbFrontier (Acris Antibodies, Herford, Germany), polyclonal antibody against Nrf2 (IF) was from Santa Cruz Biotechnology (Heidelberg, Germany) and against Nrf2 (WB) was from Bioworld Technology (St. Louis Park, MN, USA). Cy2-conjugated anti-rabbit IgG was from Dianova (Hamburg, Germany), polyclonal antibodies against histone H3 and horseradish peroxidase (HRP)–conjugated anti-mouse or anti-rabbit IgG were purchased from Cell Signaling (Frankfurt am Main, Germany).

Generation, cultivation, and stimulation of BMM

C57BL/6 mice were obtained from Charles River (Wiga Sulzfeld, Germany) and C57B/SV129 Nrf2 knockout mice as well as C57B/SV129 wild-type mice [20] were kindly provided by Thomas Herdegen (Institute of Experimental and Clinical Pharmacology, University of Kiel, Germany) and C57B/SV129 Nrf2 knockout mice as well as C57B/SV129 wild-type mice [20] were kindly provided by Thomas Herdegen (Institute of Experimental and Clinical Pharmacology, University of Kiel, Germany). Nrf2 knockout mice were crossed onto a C57B/SV129 background [20]. BMMs were generated and cultivated in a serum-free cell culture system as recently described [21]. Briefly, tibias and femurs were aseptically removed and bone marrow cells were flushed with sterile phosphate-buffered saline (PBS) and then centrifuged at 150 g for 15 min. The cells were resuspended in RPMI medium containing 5% Panexin BMM (PAN Biotech, Aidenbach, Germany), 2 ng/ml recombinant murine GM-CSF (PAN Biotech), and 50 µM mercaptoethanol and cultivated at least 10 days at 37 °C in a humidified atmosphere containing 5% air and 5% CO₂. Twenty-four hours before stimulation experiments, 2.5 × 10⁵ BMMs were seeded in 12-well plates. When indicated, BMMs were preincubated for 1 h with appropriate inhibitors followed by treatment with prostaglandins, LPS, and IFN-γ or corresponding vehicle. The final concentrations of the vehicles were 0.1% for dimethyl sulfoxide (DMSO), ≤0.32% for methyl acetate, and ≤0.17% for acetone.

Nitrile assay

Nitrile, the end stable product of nitric oxide, was quantified in cell culture medium using the Griess reagent [modified]. Briefly, 500 µl of each supernatant was mixed with an equal volume of Griess reagent and absorbance was measured at 540 nm after 15 min. Nitrile concentration was determined from a sodium nitrite standard curve.

RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated using the TRizol reagent (Invitrogen, Karlsruhe, Germany) following the manufacturer's instructions. Reverse transcription of 1 µg of total RNA was performed using the Moloney murine leukemia virus reverse transcriptase (Promega, Mannheim, Germany) and 0.5 µg oligo(dT) primer (Invitrogen). qRT-PCR was performed using the LightCycler 480, and detection of amplification products was done using the LightCycler 480 Probes Master Kit (Roche). TaqMan PCR probes and gene-specific primer pairs were generated by Microsynth (Balgach, Switzerland). Data were analyzed with LightCycler software version 1.5. The reference gene RPLP0 served for the standardization of the individual PCRs. All assays were performed in duplicate and repeated four to six times as indicated.

Western blot analysis

Proteins were prepared using the TRizol reagent (Invitrogen) according to the manufacturer's instructions. Protein content was determined using the Bradford method. Equal amounts of protein were separated by SDS–PAGE and transferred onto nitrocellulose membranes by electroblotting. Membranes were blocked with 1× Roti-Block (Roht, Karlsruhe, Germany) for 1 h at room temperature and subsequently incubated overnight at 4 °C with a rabbit anti-GAPDH, mouse anti-Prx 6, rabbit anti-Nrf2, and rabbit anti-histone H3 concentration (in 20 mM Tris, 138 mM NaCl, pH 7.6, 5% (w/v) bovine serum albumin (BSA), 0.1% (w/v) Tween 20), HRP-conjugated anti-mouse or anti-rabbit IgG (in 1× Roti-Block) was used as a secondary antibody for 1 h at room temperature. The LumiGLO system (Cell Signaling) was used for detection. All experiments were performed at least twice. Densitometric measurements for relative quantification were done using Kodak software.

Immunofluorescence staining

Nuclei of living primary macrophages, cultured on coverslips, were stained with the blue fluorescent Hoechst 33342 dye (Invitrogen) for 10 min at 37 °C. Macrophages were washed with ice-cold PBS, incubated for 10 min in ice-cold methanol, and washed three times with IF buffer (0.2% (w/v) BSA, 0.05% (w/v) saponin, 0.1% (w/v) sodium azide in PBS, pH 7.4). To block nonspecific antibody binding, cells were incubated for up to 1 h in IF buffer followed by an overnight incubation at 4 °C in a humidity chamber with polyclonal rabbit anti-Nrf2 antibody (in IF buffer). After a wash in IF buffer, the immunoreacted primary antibody chamber was visualized with green fluorescent Cy2-conjugated goat anti-rabbit IgG (in IF buffer) by incubation for 1 h at room temperature in the dark. After another wash in IF buffer, slices were covered with Fluoresp (BioMérieux, Nütingen, Germany) and observed by fluorescence microscopy with a BZ-9000 microscope (Keyence Corp., Neu-Imsenburg, Germany).

Preparation of nuclear extracts

Cells were harvested in ice-cold Dulbecco's PBS (D-PBS; Invitrogen). After being washed, the cells were incubated in hypomolar Hepes buffer (10 mM Hepes, pH 7.6, 15 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol, Complete Mini EDTA-free protease inhibitor cocktail...

Prostaglandins increase mRNA expression of Prx's in primary macrophages. BMMs were cultured for 18 h in the presence of increasing concentrations of PGA1 (12.5–50 μM), PGE2 (12.5–50 μM), or 15d-PGJ2 (2.5–10 μM). As shown in Fig. 2, Prx 6 gene expression was induced at the mRNA level by concentrations of PGA1 (12.5–50 μM); PGE2 (50 μM), PGA2 (50 μM), 15 d-PGJ2 (10 μM); or corresponding vehicle (DMSO, methyl acetate). After 18 h of stimulation, nitrite was quantified and mRNA expression of Prx's 1–6 was measured by qRT-PCR in agreement with Itoh et al. [13], gene expression of Prx 1 was significantly increased by 15d-PGJ2, whereas Prx 2, 3, and 4 mRNA levels were not changed or even decreased by PGs. Moreover, Prx 5 was slightly increased by PGE1, PGE2, and PGF2α. In contrast, Prx 6 was enhanced by all PGs used in this study, most notably PGD2, PGE2, PGA1, and 15d-PGJ2 (Fig. 1). Nitrite was not changed by any of the PGs (data not shown), and PG-induced Prx 6 gene expression was not altered by the iNOS inhibitor L-NIL (Supplementary Fig. 1), indicating that induction of Prx's by PGs is NO-independent. These results imply that in addition to the selective induction of Prx 1 gene expression by 15d-PGJ2, mRNA expression of Prx 5 is slightly enhanced by PGs of the E and F classes, whereas Prx 6 transcription is strongly induced by various PGs in primary murine macrophages.

**Fig. 1.** Prostaglandins increase mRNA expression of Prx's in primary macrophages. BMMs were cultured in the presence or absence of PGA1 (50 μM), PGE2 (50 μM), PGE1 (50 μM), PGF2α (50 μM), cyclopentenone PGs, i.e., PGA1 (50 μM), PGA2 (50 μM), 15 d-PGJ2 (10 μM); or corresponding vehicle (DMSO, methyl acetate). After 18 h of stimulation, nitrite was quantified and mRNA expression of Prx's 1–6 was measured by qRT-PCR. The fold difference in mRNA expression, normalized to RPLP0, is indicated. Data are presented as means with SEM (n=6). Comparison of groups was performed using one-way ANOVA followed by the Bonferroni posttest (*P<0.05, **P<0.01, ***P<0.001).

In addition, we performed a dose- and time-dependent analysis of Prx 6 mRNA expression after stimulation with conventional prostaglandins. BMMs were cultured for 18 h in the presence of increasing concentrations (12.5–50 μM) of PGD2 or PGE2. As shown in Figs. 3A and D, treatment with both PGs resulted in significantly higher Prx 6 mRNA levels, depending on the concentration used. A sixfold (PGD2) or fourfold (PGE2) increase was reached with 50 μM corresponding PG. Subsequent time-course experiments (9–24 h) revealed that PGD2 elicited Prx 6 gene induction 9 h after stimulation, with a maximum after 18 h (Fig. 3B). In contrast, mRNA levels of Prx 6 were increased by PGE2 from 18 to 24 h (Fig. 3E). The following gene expression experiments were conducted at 18 h after stimulation with 50 μM PGD2 or PGE2.

To determine whether the Prx 6 mRNA induction in PGD2- and PGE2-stimulated BMMs results in an enhanced protein amount, we performed Western blot analyses. Protein expression of Prx 6 was time-dependently increased in response to PGD2 with a maximum after 12–18 h (Fig. 3C) and PGE2 with a maximum after 48 h (Fig. 3F).

Adenylate cyclase is involved in the up-regulation of the Prx 6 gene in response to PGD2 and PGE2

To study the molecular signaling mechanisms implicated in the PGD2- and PGE2-dependent Prx 6 mRNA induction, we first elucidated the role of adenylate cyclase (AC)-generated cAMP. Thus, BMMs were treated with either the AC activator forskolin (10–100 μM) in the presence of

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presence of the phosphodiesterase inhibitor IBMX (100 μM) or the
276 cAMP agonist and activator of cAMP-dependent protein kinases
dibuturyl-cAMP (100–400 μM) for 18 h. In addition, time-course
277 experiments with 100 μM forskolin/IBMX or 200 μM dibuturyl-cAMP
278 were carried out over a period of 24 h. Figs. 4A and C show that mRNA
279 levels of Prx 6 were enhanced by forskolin or dibuturyl-cAMP, with a
280 maximum at 100 or 200 μM, respectively. Moreover, stimulation with
281 forskolin/IBMX (Fig. 4B) or dibuturyl-cAMP (Fig. 4D) resulted in a
time-dependent increase in Prx 6 gene expression, with the strongest
282 induction after 24 h.

283 We further determined whether pharmacological inhibition of AC
284 by MDL 12,330A could prevent the stimulatory effect of both PGs and
285 LPS/IFN-γ on Prx 6 mRNA expression. qRT-PCR experiments demon-
286 strated that preincubation with increasing concentrations of MDL
287 12,330A (5–20 μM) significantly suppressed induction of Prx 6 mRNA
288 after stimulation with PGD2 (Fig. 4E), PGE2 (Fig. 4F), or LPS/IFN-γ
289 (Supplementary Fig. 2) in a dose-dependent manner. These data
290 indicate that adenylate cyclase-generated cAMP is involved as a
291 second messenger in the PGD2-, PGE2-, and LPS/IFN-γ-induced
292 regulation of Prx 6 mRNA.

PGD2 and PGE2 up-regulate Prx 6 through a cAMP-dependent but
PKA- and Epac-independent mechanism

To define the downstream targets of the cAMP signaling pathway,
we examined the possible involvement of PKA in the induction of
the Prx 6 gene in PG- as well as LPS/IFN-γ-stimulated BMMs using
the PKA-selective inhibitors H-89 (5–20 μM) and KT5720 (1.25–5 μM). As
shown in Fig. 5, pretreatment of BMMs with 20 μM H-89 only slightly
decreased PGE2-induced Prx 6 expression (Fig. 5B), whereas the
PGD2- (Fig. 5A) or LPS- and IFN-γ-dependent (Supplementary Fig. 3)
Prx 6 mRNA increase was not significantly altered. Moreover,
experiments performed using the PKA inhibitor KT5720 were without
significant effect on PGD2- and PGE2-induced Prx 6 gene expression
(Figs. 5 C and D). These data suggest that a PKA-independent
mechanism probably underlies the PGD2- and PGE2-dependent Prx
6 increase.

Recently, Epac has been identified as a novel target for cAMP
[18,19]. To assess whether Prx 6 induction is linked to PKA- and/or
Epac-mediated signaling pathways in BMMs, we examined the effects
of two highly specific cAMP analogs, PKA-specific 6-Bnz-cAMP (10–
Neither the PKA-specific cAMP analog (Fig. 5E) nor the Epac-specific cAMP analog (Fig. 5F) had an effect on Prx 6 gene expression, indicating that the cAMP-dependent induction of Prx 6 mRNA expression is mediated neither by PKA nor Epac signaling in BMMs. JAK2, phosphatidylinositol 3-kinase (PI3K), and p38 MAPK participate in the PGD2- and PGE2-dependent induction of Prx 6 mRNA expression. Previous work in our lab had shown that the LPS- and IFN-γ-dependent induction of Prx 6 was dependent on JAK2, PI3K, and MAPKs, particularly p38 MAPK [10]. To examine the roles of these protein kinases in the PG-mediated Prx 6 increase, we performed qRT-PCR experiments using selective inhibitors of JAK2 (AG490, 10–40 μM), PI3K (LY294002, 10–40 μM), and MAPKs (p38 MAPK, SB202190, 4 μM; JNK, SP600125, 20 μM; p44/42 MAPK, PD98059, 20 μM). Fig. 6 illustrates that the JAK2 inhibitor AG490 significantly reduced the PGD2- (Fig. 6A) and PGE2- (Fig. 6B) mediated induction of Prx 6 gene expression. Similarly, inhibition of PI3K by LY294002 resulted in a dose-dependent decrease in Prx 6 mRNA expression in response to both PGD2 (Fig. 6C) and PGE2 (Fig. 6D). Furthermore, SB202190, an inhibitor of p38 MAPK, diminished the PG-dependent Prx 6 mRNA induction, whereas inhibitors of p44/42 MAPK and JNK were without any effect (Figs. 6E and F). Therefore, our findings suggest that JAK2, PI3K, and p38 MAPK are upstream signaling events required for PGD2- and PGE2-inducible Prx 6 gene expression.

Induction of Prx 6 gene expression by PGD2 or PGE2 is mediated by protein kinase C

PI3K increases the activity of PI3-dependent kinase 1 (PDK-1), which is crucial for the activation of protein kinase B (PKB) and many...
other AGC kinases including protein kinase C (PKC) [22]. As determined by Western blot analysis we could demonstrate that PKB is not activated in response to PGD2 or PGE2 (data not shown). Therefore, we asked whether PGD2, PGE2, or LPS/IFN-\(\gamma\) transduces its effects on Prx 6 mRNA induction through activation of PKC. Consequently, we used the PKC pan inhibitor Ro31-8220 (1–4 \(\mu\)M) and the PKC inhibitor Gö6796 (0.5–2 \(\mu\)M) with high selectivity for PKC isotypes α, β, and γ. As shown in Fig. 7, the general PKC inhibitor Ro31-8220 dose-dependently blocked the PGD2- (Fig. 7A) and PGE2- (Fig. 7B) as well as the LPS/IFN-\(\gamma\) - (Supplementary Fig. 4) stimulated Prx 6 induction, indicating that regulation was dependent on PKC.

This finding led us to evaluate whether Gö6796 was sufficient to decrease Prx 6 gene induction. However, Gö6796 (0.5–2 \(\mu\)M) had no effect on the Prx 6 mRNA levels after stimulation with PGD2 (Fig. 7C) or LPS/IFN-\(\gamma\) (Supplementary Fig. 4) and had only a slight effect in the highest concentration after PGE2 stimulation (Fig. 7D). This set of data indicates that induction of Prx 6 mRNA expression by PGD2 and PGE2 is dependent on PKCs, in particular Ca\(^{2+}\)-independent PKCs.

PPAR\(\gamma\) is not involved in PGD2- or PGE2-induced Prx 6 gene expression

15d-PGJ2, the dehydration end product of PGD2, is a natural ligand of peroxisome proliferator-activated receptor \(\gamma\) (PPAR\(\gamma\)), a member of the nuclear receptor superfamily of ligand-dependent transcription factors [11] that is also activated by antidiabetic thiazolidinediones such as troglitazone or ciglitazone [23]. To determine whether Prx 6 mRNA expression is regulated by PPAR\(\gamma\) activation, we examined the effects of the synthetic PPAR\(\gamma\) agonists troglitazone (5–20 \(\mu\)M) and ciglitazone (5–20 \(\mu\)M). In addition, BMMs were treated with the PPAR\(\gamma\) antagonist GW-9662 (2.5–10 \(\mu\)M) followed by stimulation with PGD2, PGE2, or LPS/IFN-\(\gamma\). As shown in Fig. 8, the PPAR\(\gamma\) antagonist GW-9662 did not alter Prx 6 gene induction in response to PGD2 (Fig. 8A), PGE2 (Fig. 8B), or LPS and IFN-\(\gamma\) (Supplementary Fig. 5). Furthermore, neither troglitazone (Fig. 8C) nor ciglitazone (Fig. 8D), up to a concentration of 20 \(\mu\)M, had an effect on Prx 6 gene expression. Altogether, these results imply that PPAR\(\gamma\) does not contribute to the PGD2- or PGE2-mediated Prx 6 gene induction.

**Fig. 5.** Induction of Prx 6 gene expression by PGD2 or PGE2 is not mediated by PKA or Epac. BMMs were preincubated for 1 h with increasing concentrations of the PKA inhibitors (A, B) H-89 (5–20 \(\mu\)M) and (C, D) KT5720 (1.25–5 \(\mu\)M) before (A, C) PGD2 (50 \(\mu\)M) or (B, D) PGE2 (50 \(\mu\)M) was added for another 18 h. BMMs were stimulated for 18 h with increasing concentrations (10–100 \(\mu\)M) of (E) the PKA-specific cAMP analog (6-Bnz-cAMP) or (F) the Epac-specific cAMP analog (8-pCPT-2'-O-Me-cAMP). RNA was analyzed for Prx 6 gene expression by quantitative real-time PCR. The fold difference in mRNA expression, normalized to RPLP0, is indicated. Data are presented as means with SEM (n ≥ 4). (A–D) Comparison of groups was performed using one-way ANOVA followed by Bonferroni’s posttest (*P < 0.05, **P < 0.01, ***P < 0.001). (E, F) Statistical analysis was performed using Kruskal–Wallis test followed by Dunn’s posttest.
Nuclear factor erythroid-derived 2p45-related factor-2 (Nrf2) plays an important role in the regulation of Prx 6 gene expression in response to prostaglandins. 15d-PGJ2 has also been reported to elicit the activation of the redox-sensitive transcription factor Nrf2 [11]. An Nrf2-binding motif has also been found in the promoter of the human Prx 6 gene [24,25].

Using immunofluorescence staining and subcellular fractionation we investigated the nuclear accumulation of Nrf2 in primary macrophages by stimulation with PGD2 (50 μM), PGE1 (50 μM), PGE2 (50 μM), PGF2α (50 μM), PGA1 (50 μM), PGA2 (50 μM), and 15d-PGJ2 (10 μM); Nrf2 activators sulforaphane (20 μM), tBHQ (50 μM), and CAPE (40 μM); LPS (100 ng/ml) and IFN-γ (100 U/ml); and corresponding vehicle (DMSO, acetone, methyl acetate). As shown in Fig. 6A immunocytochemical staining indicated that in response to vehicle Nrf2 is localized in both the cytoplasm and the nucleus. However, after stimulation with PGs, Nrf2 activators, or LPS and IFN-γ (Supplementary Fig. 6A), Nrf2 accumulates within the nucleus. Western blot analyses of corresponding nuclear extracts confirmed nuclear accumulation of Nrf2 in response to PGD2, PGA1, PGA2, 15d-PGJ2, Nrf2 activators (Fig. 9B), and LPS and IFN-γ (Supplementary Fig. 6B).

To assess whether Nrf2 activation is essential for the PG- or LPS- and IFN-γ-induced increase in Prx 6 mRNA expression, BMMs derived from C57B/SV129 Nrf2 knockout, C57B/SV129 wild-type, and C57BL/6 mice were exposed to PGs or LPS and IFN-γ for 18 h. Fig. 9 demonstrates that induction of Prx 6 mRNA expression was completely abolished in C57B/SV129 Nrf2 KO BMMs compared to C57B/SV129 wild-type BMMs (Fig. 9C) as well as PGA1 and PGA2 (Fig. 9D). In addition, the LPS- and IFN-γ-dependent gene expression of Prx 6 was significantly diminished in C57B/SV129 Nrf2 KO BMMs compared to the wild type (Supplementary Figs. 6C and D). The basal expression levels of Prx 6 mRNA did not significantly differ among the macrophages of the three mouse strains (Supplementary Fig. 7).

To verify the obtained results, BMMs of C57BL/6 mice were treated with increasing concentrations of the Nrf2 activators sulforaphane [26] (5–20 μM), tBHQ [26] (12.5–50 μM), and CAPE [27] (10–40 μM) for 18 h. As expected, the Prx 6 gene expression was dose-dependently increased by all Nrf2 activators used in this study.
Fig. 7. PKC participates in the PGD2- or PGE2-dependent induction of Prx 6 mRNA expression. BMMs were preincubated for 1 h with increasing concentrations of (A, B) the PKC pan inhibitor Ro31-8220 (1–4 μM) or (C, D) the PKC α, β, and γ inhibitor Gö6796 (0.5–2 μM) before (A, C) PGD2 (50 μM) or (B, D) PGE2 (50 μM) was added for another 18 h. RNA was analyzed for Prx 6 gene expression by quantitative real-time PCR. The fold difference in mRNA expression, normalized to RPLP0, is indicated. Data are presented as means with SEM (n = 5). Comparison of groups was performed using one-way ANOVA followed by Bonferroni’s posttest (*P < 0.05, **P < 0.001).

Fig. 8. PGD2- or PGE2-induced gene expression of Prx 6 is not mediated by PPARγ. (A, B) BMMs were preincubated for 1 h with increasing concentrations of the PPARγ inhibitor GW-9662 (2.5–5 μM) before (A) PGD2 (50 μM) or (B) PGE2 (50 μM) was added for another 18 h. (C, D) BMMs of C57BL/6 mice were stimulated for 18 h with PPARγ agonists (C) troglitazone (5–20 μM) or (D) ciglitazone (5–20 μM) or corresponding vehicle (DMSO) for 18 h. RNA was analyzed for Prx 6 gene expression by quantitative real-time PCR. The fold difference in mRNA expression, normalized to RPLP0, is indicated. Data are presented as means with SEM (n = 4). (A, B) Comparison of groups was performed using one-way ANOVA followed by Bonferroni’s posttest (**P < 0.01, ***P < 0.001). (C, D) Statistical analysis was performed using Kruskal–Wallis test followed by Dunn’s posttest.

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These data strongly suggest that the induction of Prx 6 gene expression by prostaglandins is exerted by a mechanism dependent on Nrf2 activation.

**Discussion**

Most recently, we demonstrated that stimulation of primary murine macrophages with LPS and IFN-γ leads to COX-2-mediated PGE2 secretion resulting in increased Prx 6 gene expression. Various COX-1 and -2 inhibitors diminished Prx 6 mRNA induction, suggesting that LPS- and IFN-γ-induced gene expression of Prx 6 is regulated by COX enzymes [10]. Further inhibition experiments revealed that, in addition to COX-2, cPLA2 plays an important role in the up-regulation of Prx 6 in response to LPS and IFN-γ (Supplementary Fig. 8). Thus, this study was designed to examine distinct conventional and cyclopentenone prostaglandins for their ability to up-regulate Prx, in particular Prx 6, mRNA expression in BMMs and to determine signaling pathways that mediate this regulation. The oxidative stress protein heme oxygenase-1 (HO-1) has already been reported to be up-regulated by a variety of conventional and cyclopentenone prostaglandins in various cell culture models [13,28–31]. In accordance with Itoh et al. [13], our data indicate that gene expression of Prx 1 is induced in primary macrophages by stimulation with 15d-PGJ2 (Fig. 1). We could show that 15d-PGJ2 also increased Prx 6 mRNA expression in a time- and dose-dependent manner (Figs. 1, 2C, and F). In addition, we found Prx 6 mRNA strongly up-regulated in response to PGD2, PGE2, PGA1, and PGA2 (Figs. 1–3).

Our data also revealed that the increased mRNA expression of Prx 6 was not accompanied by a strongly increased protein expression after treatment with PGD2 or PGE2 (Figs. 3C and F). These differences

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**Fig. 9.** Induction of Prx 6 mRNA expression by prostaglandins is dependent on Nrf2. (A, B) BMMs of C57BL/6 mice were stimulated with PGD2 (50 μM), PGE1 (50 μM), PGE2 (50 μM), PGE2α (50 μM), PGA1 (50 μM, 6 h), PGA2 (50 μM, 6 h), or 15d-PGJ2 (10 μM); Nrf2 activators sulforaphane (Sulf; 20 μM), ibHQ (50 μM), or CAPE (40 μM); or corresponding vehicle (DMSO (D), acetone (Ac; 6 h), methyl acetate (Me)) for 12 h unless otherwise indicated. (A) Expression and subcellular localization of Nrf2 were examined by immunocytochemical staining with anti-Nrf2 and Cy2-conjugated antibodies. Nuclei were counterstained with Hoechst dye. (B) Nuclear extracts were immunoblotted with anti-Nrf2 or anti-histone H3 antibody. Relative band intensity of Nrf2 normalized to histone H3 was determined. (C, D) BMMs of C57B/SV129 Nrf2 KO, C57B/SV129 wild-type, and C57BL/6 mice were stimulated for 18 h with PGs or corresponding vehicle. (E–G) BMMs of C57BL/6 mice were stimulated for 18 h with (E) sulforaphane (5–20 μM), (F) ibHQ (12.5–50 μM), or (G) CAPE (10–40 μM) or corresponding vehicle (DMSO) for 18 h. RNA was analyzed for Prx 6 gene expression by quantitative real-time PCR. The fold difference in mRNA expression, normalized to RPLP0, is indicated. Data are presented as means with SEM (n ≥ 5). (C, D) Comparison of groups was performed using one-way ANOVA followed by Bonferroni’s posttest (*P < 0.05, **P < 0.01, ***P < 0.001). (E–G) Statistical analysis was performed using Kruskal–Wallis test followed by Dunn’s posttest (*P < 0.05, **P < 0.01).
between mRNA and protein levels of Prx 6 are most probably due to translational efficiency, posttranslational regulation, and transcript stability and protein degradation. Furthermore, they may also be due to the secretion of the Prx 6 protein from macrophages. Expression of Prx 6 was previously shown to be inducible by a variety of oxidative stress stimuli, e.g., hydrogen peroxide or paraquat in lung epithelial and hepatocyte cell lines [32,33], and by treatment with growth factors such as keratinocyte growth factor (KGF) in dermal epithelium [25,34,35]. Furthermore, expression of Prx 6 is responsive to hormonal regulation [25]. However, to date specific signal transduction mechanisms regulating Prx 6 induction have rarely been investigated.

Conventional prostaglandins of the D and E classes exert their effects via stimulation of G-protein-coupled receptors, thereby regulating the activity of phospholipase C and AC, respectively. Binding of AC-generated cAMP to the regulatory subunit of PKA leads to dissociation of its catalytic subunit, which then phosphorylates numerous target proteins, including CREB [16]. Using either activators or inhibitors of AC as well as cAMP analogs we observed that PGD$_2$- and PGE$_2$-dependent Prx 6 mRNA induction is a process dependent on AC-derived cAMP (Fig. 4). Surprisingly, our data indicate, however, that cAMP-dependent PKA is not essential (Figs. 5A–E), although CREB is phosphorylated by both PGs (data not shown). Recently, alternative intracellular targets of cAMP, including two isoforms of Epac, have been identified that bind to cAMP with high affinity and activate the Ras superfamily small GTPases Rap1 and Rap2 [36]. However, gene expression of Prx 6 was not up-regulated, neither by a PKA-specific cAMP analog nor by an Epac-specific cAMP analog (Fig. 5F), suggesting that other signal transduction pathways including cyclic nucleotide-gated channels might contribute to the regulation of Prx 6.

Tyrosine kinases and PI3K are downstream targets of G-protein-coupled receptors and have been linked to PG signaling [15]. We previously demonstrated that the LPS-induced up-regulation of Prx 1 in mouse RAW264.7 macrophages [37] as well as the LPS- and IFN-γ-induced up-regulation of Prx 1 in primary mouse macrophages [10] involves PI3K and Src tyrosine kinase, but not JAK2. In line with our recent study showing that PI3K and JAK2 take part in the signaling pathway leading to the up-regulation of Prx 6 mRNA in response to LPS and IFN-γ [10], we report here that both protein kinases also contribute to the PGD$_2$- and PGE$_2$-dependent Prx 6 induction (Figs. 6A–D). PI3K has been reported as a key molecule in the
regulation of Prx 1 and other antioxidant proteins such as HO-1 or thioredoxin 1 by hemin in human neuroblastoma cells [38]. Park et al. [31] observed that PGE2 induces HO-1 expression through PKA and PI3K signaling pathways via EP2 receptors in glia cells. One possible role for PI3K is as a modulator of MAP kinase signaling. The resulting product PIP3 targets PKB, Bruton’s tyrosine kinase, PDKs, integrin-linked kinase, atypical PKCs, phospholipase Cγ, and more [22]. In a previous study, we revealed that all three major subfamilies of MAPKs, most notably p38 MAPK, contribute to increased gene expression of Prx 6 in immunostimulated macrophages [10]. Based on the diverse modes of action of PGs, we hypothesized that MAPKs and/or PKCs might mediate induction of Prx 6. Our present findings identified p38 MAPK (Figs. 6E and F) and PKCs (Fig. 7), in particular Ca2+-independent PKCs, as signal-transducing kinases in response to PGD2 or PGE2. Because Prx 6 gene expression is—similar to results obtained by Wu et al. [39]—not activated by the phorbol ester PMA (data not shown), atypical PKC isoforms, e.g., PKCε and PKCλ, which are independent of Ca2+ and phosphatidyserine, diacylglycerol, or phorbol esters, might participate in Prx 6 induction. In accordance with data from our study, Gallagher and Phelan [25] reported that KGF-dependent Prx 6 induction in a mouse hepatocyte cell line is mediated by both MEK and PKC. PKC is a key signaling molecule involved in redox-dependent signal transduction pathways, and Hess et al. [40] reported that transcriptional up-regulation of Prx 1 in rat liver tissue macrophages by PMA requires a PKC/Ras/MEKK1/p38 MAPK signaling pathway. Furthermore, several studies suggest that HO-1 is markedly increased by 15d-PGJ2 in primary cultured osteoblasts [41], rat vascular smooth muscle cells [42], and murine J774 macrophages [43] via the p38 MAPK pathway. Therefore, our findings provide evidence that regulation of Prx 6 expression might occur through similar cascades.

Numerous studies have suggested that the cyclopentenone prostaglandin 15d-PGJ2 differs from other PGs in several features. There is no specific PG synthase leading to 15d-PGJ2 generation and no specific receptor has been identified so far. 15d-PGJ2 has rather been shown to act via PGD2 receptors (DP1 and DP2) and through interaction with intracellular targets. In particular, 15d-PGJ2 is recognized as an endogenous ligand of PPARγ [11]. This property as well as the inactivation of transcription factors associated with inflammation, such as NF-κB, AP-1, or STAT, are responsible for many of the 15d-PGJ2 anti-inflammatory functions, particularly in the down-regulation of proinflammatory cytokine production or iNOS [44].
and COX-2 expression [12]. However, our data indicate that PPARγ is not involved in the up-regulation of Prx 6 (Fig. 8). In contrast, PPARγ has been shown to play an important role in the induction of CD36 by 15d-PGJ2 or rosiglitazone in both Nrf2+/+ and Nrf2−/− macrophages, suggesting that activation of PPARγ can take place in the absence of Nrf2 [44].

Several studies have shown that Nrf2 plays an important role in regulating antioxidant gene expression through binding to the antioxidant-response element (ARE) in target genes [45]. Keap-1 functions as a negative regulator of Nrf2 by promoting ubiquitination and proteasomal degradation of Nrf2, leading to negative regulation of ARE-driven gene expression. Moreover, various upstream kinases, including PI3K, MAPKs, and PKCs, can modulate the Nrf2–Keap-1–ARE signaling pathway. Our data show that the responses to PGs as well as LPS and IFN-γ were profoundly impaired in Nrf2-deficient macrophages, indicating a crucial role for Nrf2 in the regulation of murine Prx 6 (Fig. 9). In addition, a recent study demonstrated that the ARE within the human Prx 6 promoter is a key regulator of the basal transcription of the Prx 6 gene and of its inducibility under conditions of oxidative stress [24]. In line with these results, preliminary studies by Fisher [46] indicate that ARE and Nrf2 are required for the induction of Prx 6 expression by KGF in dermal epithelium. In addition, Nrf2-deficient mice are not able to induce Prx 1 or HO-1 in response to electrophilic and reactive oxygen species-producing agents [47,48], hemin [38], oxidatively modified LDL, 4-hydroxynonenal [44], and 15d-PGJ2 [13] in several cell culture models. The induction of the Prx 1 gene by 15d-PGJ2, but not other PGs (Fig. 1) in this study argues for a more complex regulation of Prx 1 mRNA compared to Prx 6 mRNA and probably involves both nuclear accumulation of Nrf2 and activation of other signaling pathways that are activated by stimulation with 15d-PGJ2 only.

In light of our findings, we propose that both the iNOS- and the COX-mediated increases in Prx 6 expression in LPS- and IFN-γ-stimulated macrophages are part of the defense system of activated macrophages by contributing to the control of redox homeostasis or by regulating inflammation of host cells. Studies using various cell culture models revealed that overexpression of Prx 6 increases resistance to experimental oxidative stress [49–52], whereas a decline in Prx 6 expression results in oxidant sensitivity [53]. Moreover, transgenic mice overexpressing Prx 6 were more resistant to hyperoxia-induced lung injury [54]. In contrast, Prx 6-deficient mice showed an increased susceptibility to the toxic effects of hyperoxia and to administration of paraquat as demonstrated by lower survival rates, more severe tissue damage in lungs and liver, and higher protein

Fig. 10. Network of identified and proposed signal transduction pathways contributing to the up-regulation of Prx 6 gene expression by PGD2 or PGE2. PGD2 and PGE2 activate gene transcription by binding to corresponding prostaglandin receptors, leading to the activation of adenylate cyclase and several protein kinases. Using either specific activators or inhibitors this study shows that adenylate cyclase-generated cAMP, as well as JAK2, PI3K, atypical PKCs, and p38 MAPK, contributes to the transcriptional induction of Prx 6 in primary macrophages. The usage of stimulated Nrf2 KO macrophages or well-known Nrf2 activators (e.g., sulforaphane, tBHQ, CAPE) suggests that the transcription factor Nrf2 is the terminal component of these signal transduction pathways. Nrf2 is known to bind to an antioxidant response element in the Prx 6 promoter [24]. Continuous and dashed lines refer to identified and proposed signaling pathways, respectively.

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oxidation levels [55–57]. Because there were no differences in the expression of other peroxiredoxins or antioxidant enzymes between Prx6−/− and wild-type mice irrespective of treatment, Wang et al. [55] suggested that Prx6 is a unique nonredundant member of the Prx family that functions independent of other antioxidant proteins.

In summary, our experiments provide evidence that Prx6 gene expression is increased by conventional as well as cyclopentenone prostaglandins. In addition, we have identified an adenylate cyclase–
cAMP-dependent, but PKA- and Epac-independent, signaling pathway in response to PGD2 or PGE2. Moreover, our results provide novel information about the contribution of various protein kinases such as JAK2, PI3K, p38 MAPK, and atypical PKCs as well as the transcription factor Nr2f to the Prx6 gene expression in PGD2- and PGE2-stimulated primary macrophages (Fig. 10). It has been proposed that the cytoprotective function of genes targeted by Nr2f might regulate the immune response and also repress the induction of proinflammatory genes including cytokines, chemokines, COX-2, or iNOS [58]. In this context it will be of interest to elucidate whether up-regulation of Prx6 is only a protective mechanism against LPS/IFN-γ or PG-induced oxidative stress or whether it plays a role in modulating inflammatory pathways.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.freeradbiomed.2011.05.022.

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