Original Contribution

Nitric oxide activates an Nrf2/sulfiredoxin antioxidant pathway in macrophages

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A B S T R A C T

Peroxiredoxins (Prx's) are a family of peroxidases that maintain thiol homeostasis by catalyzing the reduction of organic hydroperoxides, H_2O_2, and peroxynitrite. Under conditions of oxidative stress, eukaryotic Prx's can be inactivated by the substrate-dependent oxidation of the catalytic cysteine to sulfinic acid, which may regulate the intracellular messenger function of H_2O_2. A small redox protein, sulfiredoxin (Srx), conserved only in eukaryotes, has been shown to reduce sulfinylated 2-Cys Prx's, adding to the complexity of the H_2O_2 signaling network. In this study, we addressed the regulation of Srx expression in immunostimulated primary macrophages that produce both reactive oxygen species (ROS) and nitric oxide (NO•). We present genetic evidence that NO-mediated Srx up-regulation is mediated by the transcription factor nuclear factor erythroid 2-related factor (Nrf2). We also show that the NO•/Srx pathway inhibits generation of ROS. These results reveal a link between innate immunity and H_2O_2 signaling. We propose that an NO•/Nrf2/Srx pathway participates in the maintenance of redox homeostasis in cytokine-activated macrophages and other inflammatory settings.

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Peroxiredoxins (Prx's) are a recently described family of peroxidases, which plays an important role in peroxide detoxification and regulation of H_2O_2 signaling [1–3]. Loss- and gain-of-function studies have revealed that deregulation of mammalian Prx's is involved in many biological functions and pathological processes including cancer and cardiovascular and neurodegenerative diseases [4–6]. Prx's are regulated at the transcriptional [7,8] and posttranslational levels. Posttranslational modifications include phosphorylation, oligomerization, acetylation, and oxidation [9]. In the normal catalytic cycle, the Prx catalytic ("peroxidatic") cysteine is oxidized to sulfenic acid, and after formation of an intra- or intersubunit bond, 2-Cys Prx's are converted back to a reduced active state by thioredoxin.

In eukaryotic cells, increasing H_2O_2 flux leads to overoxidation of the peroxidatic cysteine of several Prx's to sulfinic acid, which cannot be reduced by the Trx system. Under these conditions, inactive Prx's allow H_2O_2 to fulfill essential functions such as control of kinase/phosphatase signaling pathways [2,10]. As first shown by 2-D PAGE and mass spectrometry, Prx sulfinylation is reversible [11,12], and ATP-dependent enzymes have recently been identified in eukaryotic cells as sulfinyl reductases, namely sulfiredoxin (Srx) [13–15] and possibly sestrins. The

role of sestrin 2 as a sulfinyl reductase is a controversial issue [16,17], but sestrin 2 has been shown to partially mediate protection by NO• against Prx overoxidation [18]. Nonetheless, Srx is largely acknowledged as the "Prx repair enzyme" that helps keep 2-Cys Prx's active by reducing the overoxidized (inactive) forms [19,20]. Interestingly, Srx reduces sulfinic cytosolic Prx's 1 and 2 as well as mitochondrial Prx 3, with subsequent cellular resistance to apoptosis [21]. By favoring reduction of H_2O_2 by Prx's, Srx also modulates the activity of redox-sensitive phosphatases, including PTEN and PTP1B [22], and therefore key kinase-dependent signaling pathways. In addition, Srx overexpression was shown to enhance c-Jun phosphorylation [23] and to alter phosphorylation and expression of cell cycle regulators p21 and p27 [22]. Srx also has a general role in protein deglutathionylation of proteins modified by nitrosative stress [24,25].

As regards human diseases, it has been reported that Srx is high in skin tumors [23] and in alveolar macrophages of patients suffering from interstitial pneumonia [26], making it a potential therapeutic target. It was therefore important to address the question of the regulation of Srx. Srx expression is increased by chemical electrophiles [27], hyperoxia [28,29], exposure to cigarette smoke [30], and TPA [23]. However, little is known about the regulation of Srx by physiological molecules. Glucose and cAMP were shown to induce Srx in insulin-secreting cells [31], and in a previous paper, we showed that NO• shortens recovery of 2-Cys Prx's after inactivation by overoxidation, by an Srx-dependent process [32]. NO• by itself is not very toxic, and there are many reports claiming that NO• is cytoprotective in situations in which the inducible NO synthase (NOS2) is expressed, i.e., immune response or ischemia–

reperfusion. However, the molecular mechanisms involved in this protection are not clear. In this study, we report that NO elicits an adaptive response to oxidative stress by inducing Srx in primary immunostimulated macrophages. We also present genetic evidence that NO-mediated Srx up-regulation relies on the transcription factor Nrf2.

Materials and methods

Reagents

Recombinant mouse interferon-γ (IFN-γ; sp act 8.4×10^6 U/mg) was from R&D Systems (Abingdon, UK). Escherichia coli lipopolysaccharide (LPS) was from Alexis Biochemicals, DETA-NONOate was from Cayman Chemical (Ann Arbor, MI, USA). Culture media and endotoxin-low fetal calf serum were from Gibco–Invitrogen. Hydrogen peroxide and tert-butylhydroperoxide were from Sigma, tert-butylhydroquinone was from Fluka.

Cell culture and treatments

Protocols involving animal experimentation were approved by the CNRS Animal Care Committee (Gif-sur-Yvette). Bone marrow cells were obtained by flushing femurs from $Nrf2^{-/-}$ [33], $Srx^{-/-}$ [32], and $NOS2^{-/-}$ [34] mice on a C57BL/6 background and from wild-type (WT) C57BL/6 as controls. Bone-marrow-derived macrophages were differentiated by culture in RPMI 1640 (Gibco–Invitrogen) supplemented with 10% fetal bovine serum (from Lonza, France) and 10% L929 cell-conditioned medium as a source of macrophage colony-stimulating factor.

Preparation of cell extracts

Macrophages were washed two times with cold PBS and lysed in 0.5% Triton X-100 in 100 mM Tris, pH 7.4, containing protease inhibitor cocktail set III (Calbiochem, Merck). Cell lysates were then centrifuged at $10,000 \times g$ at 4 °C for 10 min, and the protein content of the supernatant was determined spectrophotometrically at 595 nm using the Bio-Rad protein assay.

### Antibodies and immunoblot analyses

Anti-Srx monoclonal antibody and anti-Nrf2 were from Santa Cruz. Anti-vinculin and anti-β-actin antibodies were from Sigma–Aldrich, and anti-Prx $(1–4)$–$SO_{2/3}$ and anti-2-Cys-Prx antibodies were from Lab-Frontier (Seoul, South Korea). Cell lysates were fractionated by SDS–PAGE in 16% polyacrylamide gels under reducing conditions. After electrophoresis and protein immobilization, polyvinylidene difluoride membranes (Amersham–GE Healthcare) were blocked with nonfat milk and incubated with primary antibodies. Proteins were visualized with horseradish peroxidase secondary antibody (Dako, Denmark) using enhanced chemiluminescence assay (Millipore) or using the Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE, USA) with fluorescent secondary antibodies coupled to either Li-Cor IRDye 700 or IRDye 800.

RNA extraction and real-time quantitative PCR

Total RNA was extracted from macrophages using Trizol (Invitrogen) according to the manufacturer's protocol. Transcription of total RNA was performed using MMLV reverse transcriptase (Promega) and random primers. Quantitative real-time PCR was performed using a LightCycler (Roche Diagnostics, Meylan, France), and amplification products were detected using the LightCycler DNA Master SYBR Green I kit. Data were analyzed with LightCycler 3.5 software. Quantification was performed relative to the 18S rRNA. Values represent the fold change in Srx gene expression normalized to 18S rRNA with an arbitrary value of 1 assigned to control. All assays were performed in triplicate. The sequences of the specific primers used in this study were Srx forward, 5′-AACCGGGAAA-GAAGGTGAA-3′, reverse, 5′-CTCCAGCTTGCTCCACAAA-3′; 18S rRNA forward, 5′-CTGAGAAACGGCTACCACATC-3′, reverse, 5′-CGCTCCCAA-GATCCAACTAC-3′; NQO1 forward, 5′-TTCTCTGGCCGATTCAGAGT-3′, reverse, 5′-GGCTGCTTGGAGCAAAATAG-3′; HO-1 forward, 5′-CACGCA-TATACCCGCTACCT-3′, reverse, 5′-CCAGAGTGTTCATTCGAGCA-3′.

Measurement of intracellular ROS levels

After 16-h treatment with DETA-NO, cells were washed exhaustively with PBS and then incubated with or without 100 mM tert-BHP for 30 min at 37 °C. The cells were then washed in PBS and detached
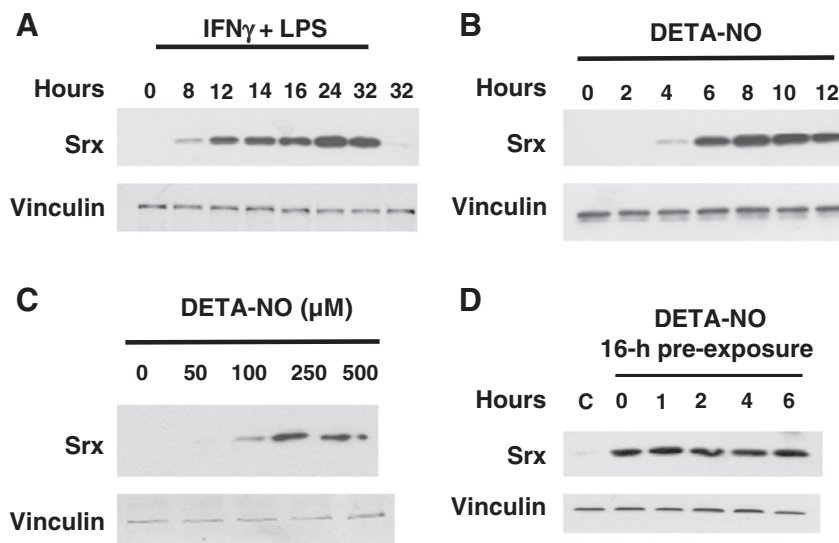
Fig. 1. Srx protein expression is strongly induced in primary macrophages. Bone-marrow-derived macrophages were exposed for the indicated time periods to (A) 50 U/ml IFN-γ and 100 ng/ml LPS or (B) 500 mM DETA-NO. Cell lysates were collected, and Srx protein expression was assessed by immunoblotting using anti-Srx and anti-vinculin as a loading control. One experiment representative of three is shown. (C) Macrophages were cultured in the presence of increasing concentrations of DETA-NO, and after 16 h Srx protein expression was analyzed by immunoblotting as in (B). (D) Macrophages were exposed to 500 mM DETA-NO for 16 h and after exhaustive washings, they were reincubated in fresh medium for the indicated times. Lane C, macrophage lysates prepared before the 16-h incubation with DETA-NO. Total lysates were analyzed by immunoblotting with the indicated antibodies.

using trypsin. The cells were washed with PBS, incubated at 37 °C in 10 μM DCFDA (ex/em = 495/529 nm) for 15 min and analyzed for intracellular ROS by fluorescence-activated cell sorting (Beckman Coulter Epics Elite) using WinMDI software for acquisition and analysis.

Nitrite measurement

Nitrite, the stable end product of NO•, was quantified in culture medium using the Griess reagent. Briefly, 200 μl of medium was reacted with 800 μl of Griess reagent (0.5% sulfanilamide and 0.05% *N*-(1-naphthyl) ethylenediamine from Sigma in 45% acetic acid), and the absorbance was measured at 543 nm. The nitrite concentration was determined from a sodium nitrite standard curve.

Data analysis

For statistical determinations, a nonparametric Wilcoxon test for paired differences was used.

Results

Characterization of Srx up-regulation in immunostimulated macrophages

In a previous study, we showed that macrophages stimulated with a combination of IFN-γ and LPS display high Srx expression [32]. Results in Supplementary Fig. 1A show the effect of each single stimulus applied alone on Srx gene expression (≈3-fold increase) and the synergy provided by the combination of both stimuli (≈10-fold increase). We also performed mRNA-decay experiments using the transcriptional inhibitor actinomycin D, showing that mRNA stability was roughly similar in control and stimulated macrophages, with a half-life of approximately 5 h (Supplementary Fig. 1B), which strongly suggests that Srx is regulated at the transcriptional level in macrophages after immunostimulation. The level of Srx protein in immunostimulated macrophages was also assessed by immunoblot analysis. In agreement with our previous data showing an NO•-dependent increase in Srx mRNA level [32], macrophages from NOS2-deficient mice were unable to express Srx protein. Yet they were able to respond to exogenous NO• provided by the NO• donor DETA-NO (Supplementary Fig. 2), showing that NO is the only signaling molecule that mediates Srx up-regulation in stimulated macrophages.

Time-course experiments indicated that Srx protein expression can be detected in IFN-γ/LPS-stimulated macrophages as early as 8 h after stimulation (Fig. 1A), whereas macrophages exposed to 500 μM DETA-NO exhibited significant Srx protein expression after 4 h and displayed maximal expression after 8 h (Fig. 1B). The inactive DETA moiety had no marked effect on Srx protein expression (Supplementary Fig. 3). After 16 h, a significant effect was observed at a concentration of DETA-NO as low as 100 μM, which corresponds to a steady-state concentration of NO• of only 50–100 nM [35–37] (Fig. 1C). After the macrophage monolayer previously exposed to DETA-NO was washed and reincubated in fresh medium, Srx protein levels remained high for at least 6 h (Fig. 1D). Altogether, these results indicate that NO• induces high and sustained Srx protein expression. Peroxynitrite is a reactive NO-derived species produced by immunostimulated macrophages [38,39], and certain peroxiredoxins are also peroxynitrite reductases [40,41]. To determine whether peroxynitrite could induce Srx expression, macrophages were exposed to 1 mM 3-morpholinosydnonimine (SIN-1), which generates nitric oxide and superoxide ion and thus can be considered a peroxynitrite donor in normoxia. The results of Western blot assays shown in Supplementary Fig. 3 indicate that SIN-1 alone had only a modest effect on Srx protein expression. However, when SOD (3000 U/ml) was added simultaneously with SIN-1, the signal was

markedly increased, implying that peroxynitrite does not play a major role in Srx expression.

NO• activates soluble guanylate cyclase and cGMP-mediated signaling cascades. However, macrophages exposed to the cell-permeative analog 8-bromo-cGMP (200 μM) for 16 h did not show any change in Srx protein expression (Supplementary Fig. 3). These data suggest that soluble guanylate cyclase is not involved in NO•-mediated induction of Srx.

NO•-mediated up-regulation of Srx depends on Nrf2 activation

The Srx gene (formerly named npn3) is regulated by the transcription factor Nrf2 in response to oxidants and electrophiles [28,29,42]. Moreover, induction of Nrf2 by the anticarcinogen 3*H*-1,2-dithiole-3-thione leads to a decrease in H_2O_2-mediated overoxidation of Prx's [29]. Nrf2 is a transcription factor that controls one of the most important cellular defense mechanisms against oxidative stress and electrophiles. Under basal conditions, Nrf2 is sequestered in the cytosol by its repressor Keap1 and undergoes ubiquitin-dependent proteasomal degradation [43,44]. Keap1 is a sensor of stress signals (electrophiles, ROS, NO•, etc.), which, by alkylating or oxidizing reactive cysteine residues, triggers a change in conformation that inhibits ubiquitination and in turn stabilizes Nrf2 [45–47]. Therefore, we investigated whether NO•-dependent up-regulation of Srx is mediated by the Nrf2/Keap1 system. After challenging macrophages with DETA-NO or the electrophile stressor *tert*-butylhydroquinone (*tert*-BHQ), we examined the expression levels of Nrf2 and Srx by immunoblot analyses. As shown in Fig. 2A, DETA-NO, like *tert*-BHQ, increased Nrf2 and Srx protein levels. In
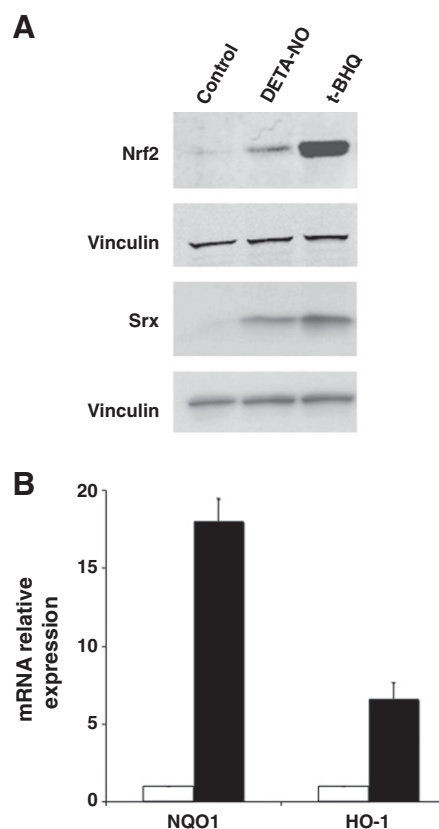
Fig. 2. NO-induced expression and activation of Nrf2 in macrophages. (A) Macrophages were treated with DETA-NO (500 μM, 16 h) and with the Nrf2 inducer *tert*-BHQ (10 μM, 16 h), and expression of both Nrf2 and Srx was analyzed by immunoblotting using anti-Srx antibody, anti-Nrf2 antibody, and anti-vinculin antibody as a loading control. (B) Macrophages were exposed to 500 μM DETA-NO, and transcript levels of the Nrf2-dependent genes NQO1 and HO-1 were measured by real-time RT-PCR. Means ± SD of at least three independent experiments are shown.

addition, mRNA levels of NQO1 and HO-1, two typical Nrf2 target genes, were markedly increased, providing evidence that Nrf2 is expressed and active under our experimental conditions (Fig. 2B). Moreover, to investigate whether Nrf2 activation by NO$^{\bullet}$ is sufficient to induce Srx expression, macrophages from Nrf2-deficient mice were exposed to NO. Macrophages were exposed to exogenous NO$^{\bullet}$ released from DETA-NO or stimulated with a combination of IFN-γ and LPS to induce NOS2 expression and production of endogenous NO$^{\bullet}$. In parallel, macrophages were also stimulated in the presence of the NOS2 inhibitor 1400W. Real-time RT-PCR and Western blot analyses showed that upon exposure to DETA-NO, Srx was induced in WT macrophages but not in Nrf2$^{-/-}$ macrophages (Figs. 3A and B, left). Furthermore, Srx protein level was markedly increased in Nrf2$^{+/+}$ macrophages upon stimulation with IFN-γ/LPS, whereas no significant signal corresponding to Srx protein was detected in either macrophages stimulated in the presence of 1400W or Nrf2$^{-/-}$ macrophages (Fig. 3B, right). These results indicate that basal and NO$^{\bullet}$-induced Srx expression is dependent on Nrf2 activation in macrophages. The samples were then analyzed by Western blot using an anti-2-Cys Prx–SO$_{2/3}$ antibody (Fig. 3C). In lysates of both WT and Nrf2$^{-/-}$ macrophages exposed to NO$^{\bullet}$, the level of sulfinylated Prx's was lower than in controls. Overall, these data show that Nrf2 does not mediate the NO$^{\bullet}$-dependent decrease in 2-Cys Prx sulfinylation. This is consistent with previous data showing that that

NO$^{\bullet}$ directly targets 2-Cys Prx and promotes posttranslational modification [32,48]. As regards Prx 2 of dopaminergic neurons, it has been reported that S-nitrosation of redox-active catalytic cysteines confers protection against overoxidation [48].

As Srx regenerates active 2-Cys Prx by reducing the overoxidized forms, we investigated whether Nrf2 is limiting in 2-Cys Prx recovery. To this end, we monitored sulfinic 2-Cys Prx protein level for 6 h in WT and Nrf2$^{-/-}$ macrophages after removal of H$_2$O$_2$ and reincubation in fresh medium (Fig. 4). Quantitative analysis of sulfinylated Prx levels normalized against β-actin, which appears here as a doublet, revealed that the amount of overoxidized Prx's decreased vs time, reaching about 20% of control level after 5 h in WT macrophages, whereas it remained high in Nrf2$^{-/-}$ macrophages.

NO prevents oxidative stress via Srx

Macrophages display high oxidative activity and are therefore exposed to their own reactive metabolites. Various types of cellular damage are caused by ROS, especially the hydroxyl radical, and understanding the mechanisms of macrophage protection against ROS toxicity is a challenging issue. In a previous report, we showed that bone-marrow-derived macrophages exhibit basal overoxidation of 2-Cys Prx's that can be markedly enhanced upon cell exposure to
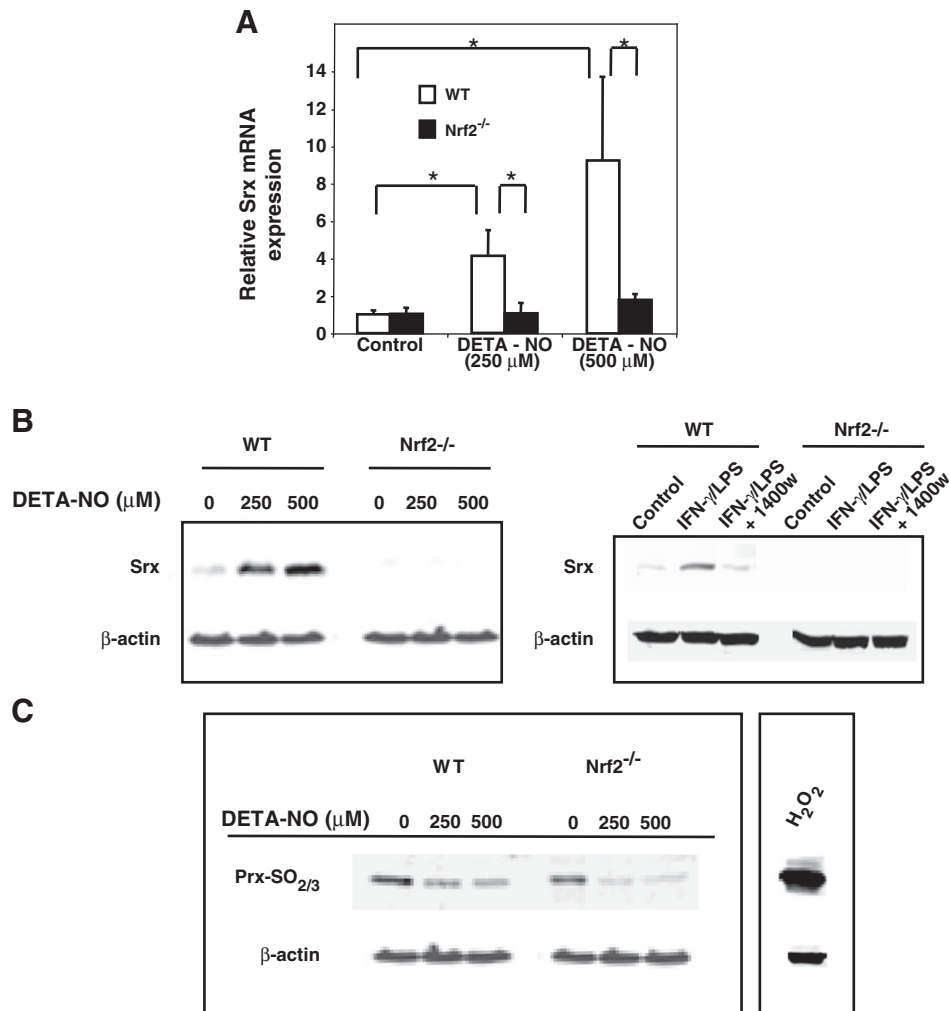
Fig. 3. Effects of NO$^{\bullet}$ on Srx expression in macrophages from WT and Nrf2$^{-/-}$ mice. Macrophages from the indicated strains were exposed for 16 h to DETA-NO and were analyzed (A) by real-time RT-PCR and (B, left) by immunoblotting using an anti-Srx antibody. (B, right) Macrophages were also stimulated with 50 U/ml IFN-γ and 100 ng/ml LPS with or without 500 mM 1400 W, a potent NOS2 inhibitor. (C) Immunoblots analyzed in (B) were probed for peroxiredoxin overoxidation with an anti-2-Cys Prx–SO$_{2/3}$ antibody and an antibody against β-actin used as a loading control. The data shown are representative of at least three independent experiments. As a positive control for the detection of 2-Cys-Prx overoxidation, lysate of H$_2$O$_2$-challenged macrophages (100 mM, 20 min) was also analyzed by immunoblotting (right). $^*p \leq 0.1$, Wilcoxon paired test.
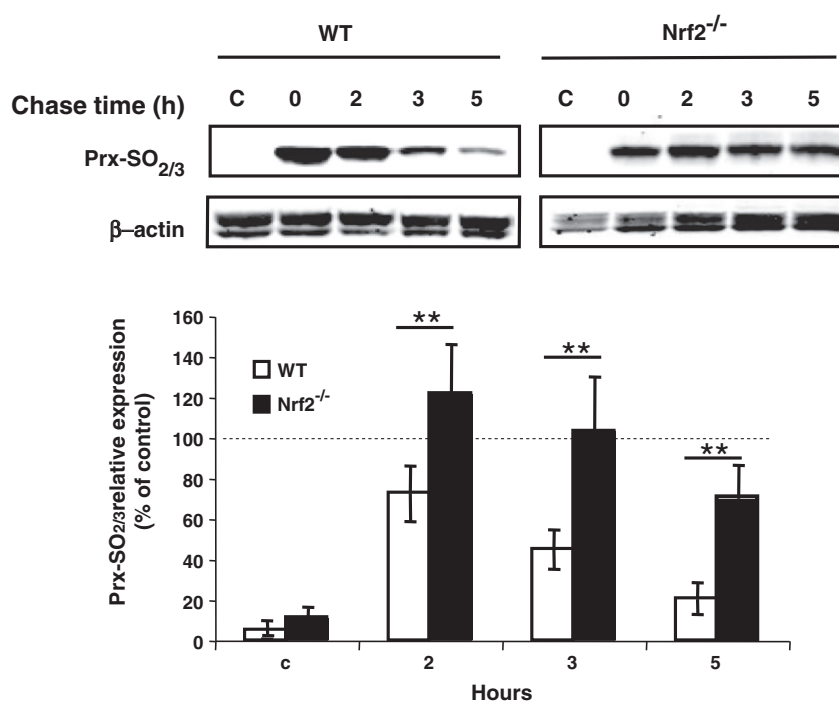
Fig. 4. Effect of Nrf2 on Prx regeneration. Macrophages from WT and Nrf2$^{-/-}$ mice were washed exhaustively and exposed to 250 μM H$_2$O$_2$ for 30 min (time 0), washed again with HBSS, and incubated for 5 more hours in fresh medium. Lane C, macrophage lysates prepared before the 30-min exposure to H$_2$O$_2$. Overoxidation of 2-Cys Prx's was assessed using an anti-2-Cys Prx–SO$_{2/3}$ antibody and an antibody against β-actin used as a loading control. A representative Western blot is shown and densitometric analysis was performed to quantify the immunoblots based on three independent experiments. Prx–SO$_{2/3}$ amount at each time point is expressed as a percentage of the maximum level (time 0) taken as control. Means ± SD of three independent experiments are shown. **p ≤ 0.05, Wilcoxon paired test.

oxidative stress inducers such as *tert*-BHP [32]. We therefore addressed the consequences of NO$^•$-mediated Srx induction on basal and induced ROS production. Macrophages were sequentially exposed or not to NO$^•$ and, after exhaustive washings, to *tert*-BHP. Cells were harvested, washed, resuspended in PBS, and incubated at 37 °C in the presence of the cell-permeative probe DCFDA. The level of intracellular ROS was then monitored by FACS analysis. As shown in Fig. 5, preconditioning WT macrophages with DETA-NO for 18 h markedly reduced *tert*-BHP-induced ROS production. The DCF probe is sensitive to several types of ROS but it is particularly reactive with the hydroxyl radical [49], which is produced by H$_2$O$_2$ in the presence of redox-active transition metals (Fenton reaction) or quinones [50] and by peroxynitrite. As Srx regenerates Prx's that display peroxidase and peroxynitrite reductase activities, we hypothesized that induction of Srx restrains production of ROS including hydroxyl radicals. Therefore, we also measured DCF fluorescence within Srx$^{-/-}$ macrophages. Interestingly, the basal ROS level in Srx$^{-/-}$ macrophages was higher than that in WT macrophages. Upon challenge with *tert*-BHP, DCF fluorescence in Srx$^{-/-}$ macrophages was barely higher than in unchallenged macrophages, suggesting that Srx deficiency strongly deregulates macrophage ROS homeostasis. In contrast to what happened with WT macrophages, pretreatment of Srx$^{-/-}$ macrophages with DETA-NO did not reduce ROS production. DETA-NO, when applied alone, had no effect on ROS production in macrophages from either WT or Srx$^{-/-}$ mice. Overall, these results imply that Srx is a major component responsible for NO$^•$-dependent decrease in ROS production in macrophages.

Discussion

The importance of Prx's in protecting against ROS-associated damage and in regulating H$_2$O$_2$ signaling has recently been emphasized. Regulation of ROS homeostasis in macrophages has particular relevance because high levels of ROS produced by phagocytic Nox2

NADPH oxidase participate in bacterial killing and are also involved in inflammation and tissue injury. When adequately stimulated, macrophages also produce large amounts of NO$^•$ upon induction of NO synthase 2 [51,52]. Macrophages, key players in the immune response, therefore have to cope with potentially harmful oxygen-derived species to fight foreign invaders [53] and to maintain a delicate balance between the production of reactive oxygen and nitrogen species and redox homeostasis.

Others and we have previously shown that NO$^•$ produced upon physiological stimulation prevents 2-Cys Prx overoxidation in macrophages [32] and neurons [48]. We have also shown that expression levels of Srx mRNA and protein by macrophages are dramatically increased in response to a combination of IFN-γ and LPS via a nitric oxide mechanism [32]. A recent report confirmed that stimulation of macrophages by LPS induces Srx expression [54]. In this study, we have delineated the conditions for inducing Srx, the 2-Cys Prx repair enzyme, and have assessed the impact of Srx induction on macrophage ROS production. Induction of Srx by NO$^•$ was further characterized, and we report for the first time that NO$^•$-dependent Srx up-regulation relies on the transcription factor Nrf2. These results support the argument that NOS2-derived NO$^•$, by activating the Nrf2/Srx pathway, bridges innate immunity signals to redox signaling. Several Prx's have proven efficacy in reducing peroxynitrite, the coupling product of NO$^•$, and superoxide that may kill foreign invaders [38] by damaging proteins, DNA, and lipids, but also represents a threat to the producing cells and nearby tissues [55]. It is therefore conceivable that induction of Srx by NO$^•$ is a feedback loop to prevent sustained and harmful NOS-dependent peroxynitrite production.

ROS are also signaling molecules, and Prx's control the flux of H$_2$O$_2$ whose role in signal transduction has been increasingly acknowledged [2]. It is therefore remarkable that immunoinflammatory settings that feature NO$^•$ production can affect Srx- and Prx-controlled redox homeostasis. Kinase cascades are crucial transduction pathways in the host cell response, and the balance between kinases and
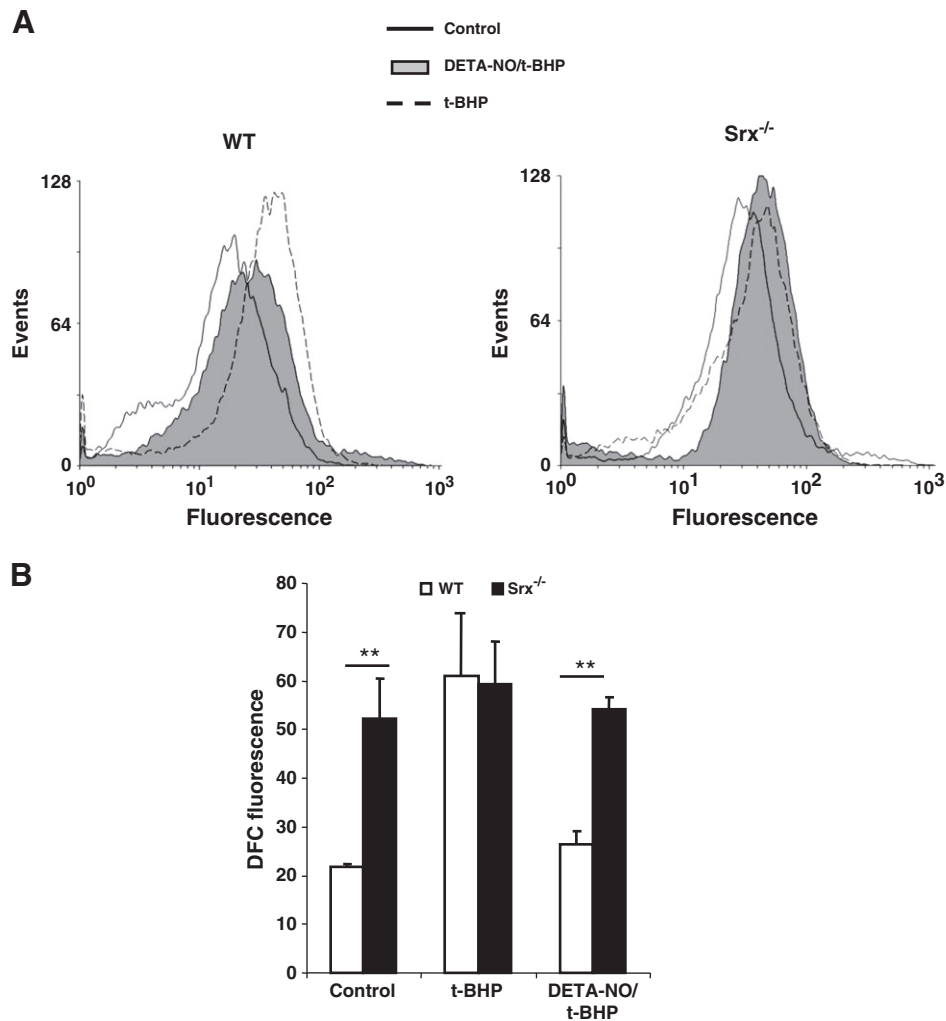
Fig. 5. Effects of Srx deficiency on intracellular ROS level in WT and Srx$^{-/-}$ macrophages. Macrophages were preexposed or not to 250 μM DETA-NO for 16 h, and after exhaustive washings, the cells were challenged for 30 min with 100 μM *tert*-BHP. After being washed and treated with trypsin, the cells were incubated in PBS containing the cell-permeative CM-H$_2$DCFDA (10 mM) at 37 °C in the dark for 15 min. Samples were then analyzed for intracellular ROS production by flow cytometry. (A) Representative histograms and (B) mean fluorescence intensity ± SD from three independent experiments. **$p \le 0.05$, Wilcoxon paired test.

phosphatases finely regulates cytokine and LPS signaling. Many redox thiol-containing proteins are targets of H$_2$O$_2$, including phosphatases such as PTEN and PTB-1B [22,56], which have been associated with components of immune defense [57,58]. It is therefore plausible that macrophage-derived NO$^\bullet$ modulates phosphatase oxidation by H$_2$O$_2$ by acting on both Srx expression and Prx redox status.

Because regulation of Srx by NO$^\bullet$ occurs at the transcriptional level, we addressed the question of which NO$^\bullet$-responsive redox transcription factor can account for an increase in Srx gene expression. Srx is regulated by Nrf2, a master regulator that activates antioxidant response element (ARE)-dependent genes and protects cells against oxidative stress and electrophiles [43,45], and there is a functional ARE in the mouse and human Srx promoters [59]. As it is common in Nrf2 target genes, an AP-1 site is embedded in the Srx ARE [59], and Srx has recently been described as an AP-1- and/or Nrf2-dependent gene [23,28–31,60]. Very recently, it was reported that LPS-mediated Srx gene expression depends on both AP-1 and Nrf2 [54]. Our experiments showing that macrophages from Nrf2$^{-/-}$ mice exposed to exogenous or endogenous NO$^\bullet$ are unable to up-regulate Srx suggest that the Nrf2/ARE pathway is the predominant sensing system for inducing high levels of Srx protein by NO$^\bullet$ in macrophages. We also showed that Nrf2-deficient macrophages failed to rapidly regenerate the reduced form of the 2-Cys Prx's, strengthening the proposal that Nrf2 is required for the desulfinylation of 2-Cys Prx's by

Srx. The most likely sensor of NO$^\bullet$ that regulates Nrf2 concentrations is Keap1, an adaptor that bridges Nrf2 to a Cul3-based E3 ligase under basal conditions and mediates Nrf2 degradation by a ubiquitin-dependent process. Upon exposure to ROS and electrophiles, modification of Keap1 redox-sensitive cysteines induces a conformational change that results in a lower level of ubiquitin conjugates on Nrf2 and a subsequent increase in protein level [61,62]. NO$^\bullet$ has been shown to activate Nrf2 translocation in the nucleus [47,63], and it was recently reported that NO$^\bullet$ activates Nrf2 by promoting an intermolecular disulfide bridge between two Keap1 molecules [64]. Nrf2$^{-/-}$ mice are more sensitive to LPS-induced septic shock and to an upsurge in proinflammatory genes induced by the innate immune response [65]. The ARE/Nrf2 pathway is therefore crucial in controlling immune dysregulation and protecting against deleterious effects of uncontrolled inflammation. Cells such as macrophages, which exhibit high oxidative metabolism when stimulated by proinflammatory cytokines and microbial products, release a plethora of reactive molecules that may initiate persistent inflammation [66]. In many inflammatory and infectious settings involving cytokines or endotoxins, excessive ROS production is harmful to cell viability [53,65]. Our results showing that physiological NO$^\bullet$ levels induce the Nrf2/Srx/Prx system allows us to suggest that the protective pathway described here may relieve the burden of an excessive and/or persistent inflammatory response (Fig. 6). In brief, NO appears to exert a two-level control over 2-Cys Prx's: a fast-
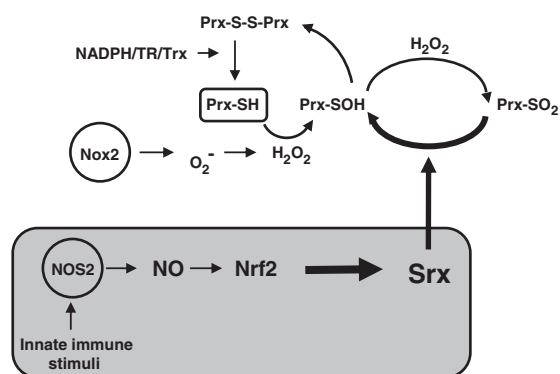
Fig. 6. Scheme showing the Nrf2/Srx pathway through which NO$^\bullet$ favors 2-Cys-Prx regeneration. In primary macrophages stimulated by immune stimuli, Srx expression is strongly enhanced via NO$^\bullet$ and activation of the Keap1/Nrf2 system. This protective pathway may help prevent impairment of macrophage function during host response to pathogens.

acting modification that limits inactivation by overoxidation [32,48] and, as shown here, an Nrf2-mediated transcriptional up-regulation of the Prx repair enzyme, Srx. In this scheme, both effects would be complementary: the former, by anticipating overwhelming of Prx's by sudden bursts of H_2O_2 (feedforward mechanism) and the latter, by regenerating Prx's.

Many studies have shown that uncontrolled ROS accumulation can induce cellular dysfunctions such as genomic instability via oxidation and generation of DNA breaks [67] and is involved in many diseases, including neurodegenerative disorders, cancer, and cardiovascular dysfunction [68,69]. Meanwhile, NO$^\bullet$ protects against H_2O_2-induced cytotoxicity in macrophages [70] and many other cell types, including astrocytes [71], fibroblasts, mesencephalic cells [72], and endothelial cells [73,74]. Our results showing that NO$^\bullet$, via Srx up-regulation, helps keep ROS levels low thus provide a novel molecular mechanism supporting the long-held view that NO$^\bullet$ protects against ROS-induced injury [72,75]. As regards macrophages, it is likely that NO$^\bullet$-dependent induction of Srx is an adaptive mechanism to avoid sustained excessive ROS production to keep classically activated macrophages alive and potent. Srx is a ubiquitous enzyme that also has cytoprotective effects in other cell types. In neurons and glial cells, Srx induction by Nrf2 activators correlates with neuroprotection [29], and in lungs from patients with chronic obstructive pulmonary disease, Srx participates in protection against oxidative insults [30]. In vivo, the protective role of Srx is emphasized by the fact that Srx-deficient mice are more sensitive to LPS-induced lethality than their wild-type littermates [76].

In summary, our results unveil a link between innate immunity and the Nrf2/Srx pathway whose implications may be far-reaching with respect to both protection against ROS toxicity and Prx-controlled H_2O_2 signaling. Further, as most mammalian cells can induce NOS2 when appropriately stimulated [51], it is reasonable to consider that the NO$^\bullet$/Nrf2/Srx axis is operational under many pathophysiological conditions.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.freeradbiomed.2011.03.039.

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