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Role of sestrin2 in peroxide signaling in macrophages

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ABSTRACT

Reactive oxygen species not only serve as signaling molecules, they also contribute to oxidative stress and cell damage. The thioredoxin and glutaredoxin systems form along with peroxiredoxins a precisely regulated defense system to maintain the cellular redox homeostasis. There is evidence that nitric oxide (NO) protects cells from oxidative stress by preventing inactivation of peroxiredoxins by sulfinylation. Here we demonstrate that NO and hypoxia upregulate Sestrin2 by HIF-1-dependent and additional mechanisms and that Sestrin2 contributes to preventing peroxiredoxins from sulfinylation. We conclude that Sestrin2 plays a role in peroxide defense as a reductase for peroxiredoxins.

1. Introduction

Macrophages are immune effector cells that express several pattern recognition receptors and upon activation produce cytokines, reactive oxygen species (ROS) and NO [1]. ROS are commonly viewed as cell toxic compounds inducing oxidative stress, damaging proteins, DNA or lipids, which may cause apoptosis or necrosis [2]. However, they are also integrated into several signal transduction pathways, mediating responses from transmembrane receptors such as transforming growth factor-β1 or platelet derived growth factor (PDGF) receptor [3–5]. Antioxidant enzymes including glutathione-S-transferase-μ (GST-μ) or superoxide dismutase (SOD) and small molecule antioxidants such as glutathione are tightly controlled to maintain a cellular redox homeostasis, thereby protecting against ROS-induced cell damage and allowing ROS-mediated signaling [6]. Peroxiredoxins represent a superfamily of antioxidant enzymes containing active cysteine (Cys) residues to reduce H_2O_2 and other peroxides. They are grouped in typical (group I–IV), atypical double-cysteine (type V) and single-cysteine type VI peroxiredoxins [1,3]. While decomposing H_2O_2 peroxiredoxins are subjected to inactivation due to formation of an intramolecular disulfide bond. Thioredoxins reduce peroxiredoxins to the active form. The disulfide intermediate of peroxiredoxins occasionally undergoes overoxidation to sulfinic acid, which no longer is reduced by the thioredoxin system [7]. Recently the repair proteins sulfiredoxin and Sestrin2 (Sesn2) were discovered to catalyze the reduction of sulfinic acid in an ATP-dependent manner [8–11]. In addition, NO preconditioning protects peroxiredoxins towards H_2O_2-induced overoxidation but mechanisms remain elusive as the role of sulfiredoxin was excluded and the role of Sesn2 remained questionable [1].

In the past transcription factors were shown to be activated by hypoxia or NO. Hypoxia-inducible factor-1 (HIF-1) emerged as a target for both stimuli. The loss of HIF-1α lowers the expression of some antioxidant proteins such as GST-μ, SOD1 or SOD2, which explains the increased vulnerability of these cells towards oxidative stress [12]. HIF-1 is a dimer consisting of α- and β-subunit. While the β-subunit is constitutively expressed the α-subunit is oxygen-dependently degraded. Prolyl hydroxylases (PHDs) are oxygen sensors that hydroxylate specific prolyl residues within the HIF-1α protein, thereby marking it for proteasomal degradation. A lack of oxygen inactivates PHDs and subsequently stabilizes HIF-1α protein, provokes its translocation to the nucleus and HIF-1 target gene expression. NO attenuates PHD activity by coordinating the catalytic iron of PHDs [13,14].

This study determines the effect of hypoxia and NO on the transcriptional regulation of genes involved in peroxide signaling in macrophages. Sesn2 was strongly upregulated by hypoxia and DETA-NO in a HIF-1-dependent manner. Expression of Sesn2 accounts for NO-induced protection towards H_2O_2-induced peroxiredoxin overoxidation.

Abbreviations: ROS, reactive oxygen species; Sesn2, Sestrin2; SOD, superoxide dismutase; HIF-1, hypoxia-inducible factor-1; PHD, prolyl hydroxylase; DETA-NO, 2,2′-(hydroxynitrosohydrazono)bis-ethanimine; Nrf2, NF-E2-related factor-2

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2. Materials and methods

2.1. Materials

Medium and supplements were purchased from PAA (Linz, Austria) and fetal calf serum (FCS) from Biochrom (Berlin, Germany). DETA-NO (2,2′-(Hydroxynitrosohydrazono)bis-ethanimine) was from Sigma-Aldrich (Steinheim, Germany). IScript™ cDNA Synthesis Kit came from Bio-Rad Laboratories (Munich, Germany) and SYBR Green real time PCR reaction MIX was from ABgene (Hamburg, Germany). Horseradish peroxidase-labeled anti-rabbit and anti-mouse secondary antibodies and the ECL™ detection system were from (GE Biosciences, Freiburg, Germany).

2.2. Cell culture

RAW 264.7 cells and primary peritoneal mouse macrophages were cultured in RPMI 1640. NIH 3T3 cells were cultured in DMEM high glucose. Media were supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% FCS. Primary mouse macrophages were isolated by peritoneal lavage with 15 ml PBS, containing 10% FCS. Mice with a conditional knockout of HIF-1α in the myeloid lineage were provided by Dr. R. Johnson (Division of Biological Sciences, University of California, San Diego, USA) and bred as described [15]. Cells were kept at 37 °C in a humidified atmosphere with 5% CO_2.

2.3. Western analysis

Western analysis was performed as previously described using lysis buffer composed of 10 mM Tris/HCl, 6.65 M urea, 10% glycerol, 1% SDS, protease inhibitor cocktail, pH 7.4 [16]. Samples were analyzed for HIF-1α (Novus Biologicals, Littleton, USA), Sesn2 (ProteinTech Group, Inc., Chicago, USA), actin (Sigma–Aldrich, Steinheim, Germany), 2-Cys peroxiredoxin and 2-Cys-peroxiredoxin-SO_2H (Abfrontier, Seoul, Korea).

2.4. Quantitative real time RT-PCR

Isolation of whole cell RNA and quantitative real time PCR was performed as described [17]. Expression of Sesn2 was normalized to 16S ribosomal protein RNA. Primers were obtained from biomers.net (Ulm, Germany).

Sesn2 forward: 5′-GCATTACCTGCTGCTGCATA-3′;
Sesn2 reverse: 5′-AAGGCCTGGATATGCTCCTT-3′;
16S ribosomal protein forward: 5′-AGATGATCGAGCCGCGC-3′;
16S ribosomal protein reverse: 5′-GCTACCAGGGCCTTTGAGATGGA-3′.

2.6. Cell transfection for Sesn2 knockdown

2.5×10^5 NIH 3T3 cells per well were seeded in six-well plates one day prior to transfection. Cells were transfected with ON-TARGETplus SMARTpool mouse Sesn2 siRNA (L-052642-01-0020) and ON-TARGETplus non-targeting pool siRNA (D-001810-10-05) as a control, using the Dharmacon transfection reagent DharmaFECT 3 as suggested by the manufacturer (Dharmacon/Thermo Fisher Scientific, Schwerte, Germany). After 48 h medium was replaced and cells were treated as indicated.

2.7. Statistical analysis

Data were obtained from at least three independent experiments and representative data (Western blots) are shown. Data in bar graphs are given as the mean ± standard error of mean (S.E.M.). Means were analyzed for statistical significance using one-way analysis of variance (ANOVA), followed by Tukey tests.

3. Results

3.1. HIF-1α-dependent Sesn2 regulation by hypoxia and DETA-NO

cDNA Microarray analysis performed with RNA isolated from nuclear run-on reactions with nuclei isolated from RAW 264.7 cells treated with DETA-NO or hypoxia or a combination of both showed 14 commonly regulated genes, one of them being Sesn2 [18]. Sesn2 was significantly induced by DETA-NO (0.5 mM) and hypoxia (1% O_2) (Fig. 1A). Microarray data of Sesn2 were verified by quantitative real time RT-PCR using whole cell mRNA from RAW 267.4 cells. Data showed a significantly higher induction of Sesn2 by DETA-NO compared to hypoxia (Fig. 1B). In addition Sesn2 expression in primary peritoneal mouse macrophages from either wild type or HIF-$1\alpha^{-/-}$ knockout mice exposed to hypoxia or DETA-NO for 12 h was analyzed. Again Sesn2 was significantly stronger upregulated by DETA-NO compared to hypoxia in wild type macrophages. Experiments with HIF-$1\alpha^{-/-}$ knockout peritoneal mouse macrophages showed no induction of Sesn2 mRNA expression, neither by hypoxia nor DETA-NO (Fig. 1C) thus, implying that Sesn2 is a HIF-1 target gene.

3.2. Effect of hypoxia and DETA-NO on Sesn2 protein expression

Western analysis confirmed regulation of Sesn2 by hypoxia and DETA-NO at the protein level. Sesn2 expression was induced by hypoxia and more pronounced by DETA-NO thus, reflecting mRNA data. HIF-1α protein was stabilized in response to DETA-NO and showed an even stronger expression after hypoxia (Fig. 2A). Additionally protein expression of HIF-1α and Sesn2 in NIH 3T3 cells was examined (Fig. 2B). HIF-1α and Sesn2 expression was more pronounced after the treatment with DETA-NO compared to hypoxia. Irrespective to the cell type being used Sesn2 protein expression responded stronger to DETA-NO compared to hypoxia.

3.3. Effect of DETA-NO and hypoxia on peroxiredoxin overoxidation

Considering that Sesn2 is discussed as a reductase for sulfinic peroxiredoxins we analyzed H_2O_2-induced overoxidation of peroxiredoxins using a specific antibody. RAW 264.7 and NIH 3T3 cells were exposed to hypoxia or DETA-NO for 6 h, media were changed and cells were stimulated for additional 30 min with H_2O_2. Treatment of RAW 264.7 cells with 50 μM H_2O_2 caused overoxidation of 2-Cys peroxiredoxins to the sulfinic acid form, which was absent under control conditions (Fig. 3A). Preincubation of RAW 264.7 cells with DETA-NO reduced sulfinic acid formation, while the preincubation with hypoxia had no effect. Total peroxiredoxin expression remained unaltered. This accounts for the upper band detected in RAW 264.7 lysates corresponding to peroxiredoxin III, as well as the lower band showing peroxiredoxin I and II [10]. The protective effect of DETA-NO preincubations on sulfinic acid formation in 2-Cys peroxiredoxins after H_2O_2-treatment was also observed in NIH 3T3 cells (Fig 3B). A hypoxic preincubation failed to prevent peroxiredoxin overoxidation (data not shown).

3.4. Sesn2 reduces sulfinic acid formation

Considering expression of Sesn2 mRNA and protein as well as the protective effect elicited by DETA-NO preincubation provoked the question whether Sesn2 acts as a peroxiredoxin reductase. As a proof of concept a Sesn2 knockdown was performed in NIH3T3

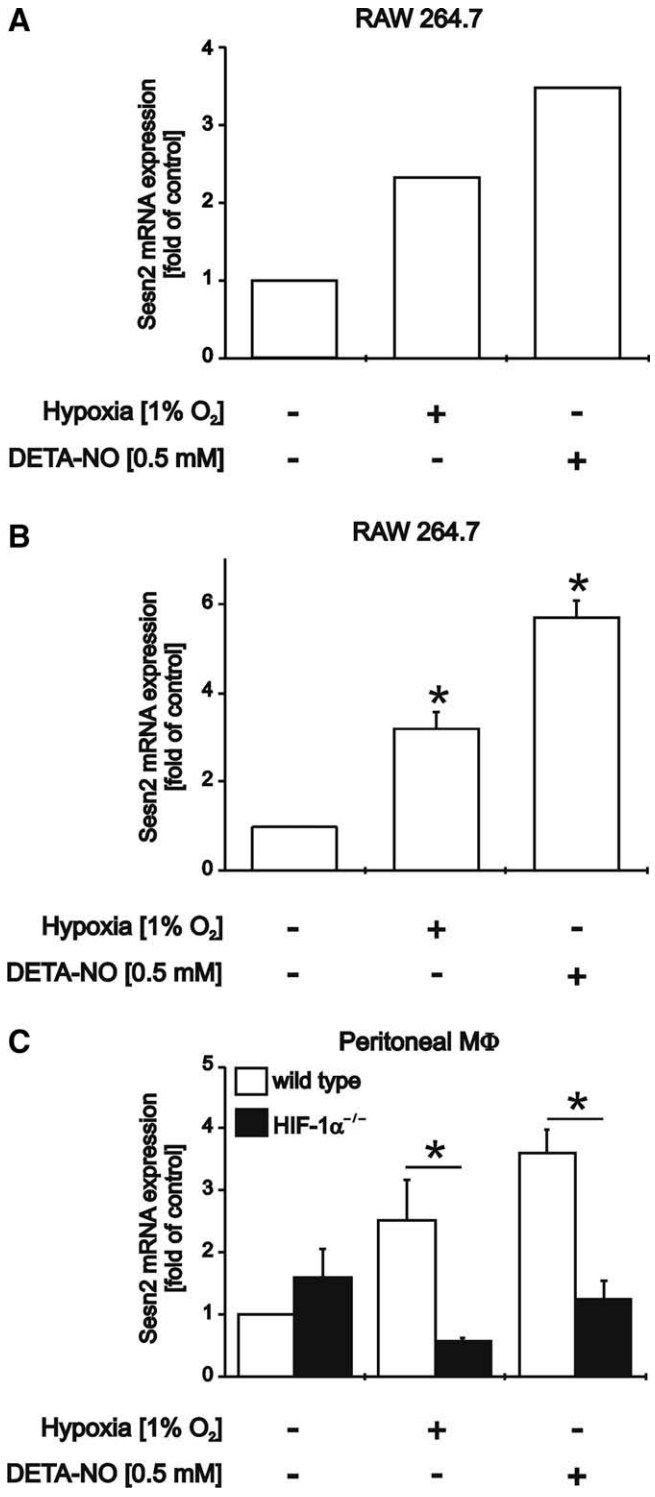

Fig. 1. Sesn2 mRNA regulation in macrophages by hypoxia and NO. RAW 264.7 cells were treated with hypoxia (1% O_2) or DETA-NO (0.5 mM) for 6 h. (A) Microarray samples were obtained from RNA isolated from nuclear run-on reactions. (B) Whole cell mRNA was isolated and analyzed by qRT-PCR. (C) Primary peritoneal wild type macrophages and HIF-1$\alpha^{-/-}$ knockout macrophages were exposed to hypoxia (1% O_2) or DETA-NO (0.5 mM) for 12 h. Whole cell mRNA was isolated and analyzed by qRT-PCR. *Significant ($P < 0.05$).

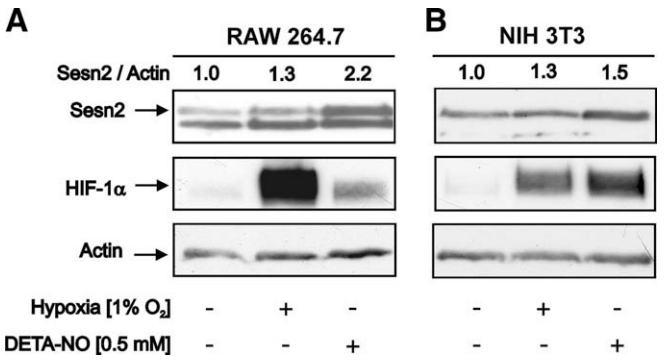

Fig. 2. HIF-1α and Sesn2 expression in RAW 264.7 and NIH 3T3 cells. (A) RAW 264.7 and (B) NIH 3T3 cells were exposed to hypoxia (1% O_2) or DETA-NO (0.5 mM) for 6 h. Sesn2 and HIF-1α expression were detected by Western analysis. Quantification of Western blots (Sesn2/Actin) were performed with the ImageJ software.

cells using siRNA technology. Transfection with Sesn2 siRNA significantly reduced Sesn2 protein expression compared to the non-targeting siRNA (Fig. 4A). Transfected NIH 3T3 cells were preincubated with DETA-NO and after medium replacement exposed to 10 μM H_2O_2 for 30 min. Cells transfected with non-targeting siRNA showed a significant reduction of peroxiredoxin overoxidation after preincubation with DETA-NO as seen with wild type cells. Sesn2 siRNA transfected cells showed an impaired ability to prevent overoxidation of peroxiredoxins after preincubations with DETA-NO. Densitometry of three independent experiments showed that this effect was statistically significant (Fig. 4B). These results show that pretreatment of RAW 264.7 or NIH 3T3 cells with NO induced a HIF-1-dependent upregulation of Sesn2, which protected peroxiredoxins against H_2O_2-induced overoxidation.

4. Discussion

To prevent oxidative damage but at the same time to facilitate peroxide signaling, the concentration of ROS needs to be tightly controlled. Peroxiredoxins are a recently discovered family of thiol peroxidases linked to H_2O_2 signaling [19]. Increased H_2O_2 concentrations provoke inactivation of peroxiredoxins by overoxidation of cysteine residues to a sulfinic acid. Repair cycles are operating to recover inactivated peroxiredoxins to their fully active form [8,11,20]. Analyzing microarray data obtained from hypoxic and DETA-NO-treated macrophages highlighted Sesn2, known as the p53 target hypoxia-inducible gene 95, to be upregulated by both treatments [8,21]. Hypoxia and NO stabilize the HIF-1α protein and activate HIF transcriptional activity [13]. Using HIF-1$\alpha^{-/-}$ knockout primary peritoneal mouse macrophages we underscore that NO-induced Sesn2 regulation is clearly HIF-1-dependent. However, upregulation of Sesn2 mRNA and protein after DETA-NO treatment in RAW 254.7 cells was significantly higher, while the accumulation of the HIF-1α protein by DETA-NO was lower compared to hypoxia. NF-E2-related factor-2 (Nrf2) is a major activator of the cellular antioxidant defense and is activated by NO but not hypoxia [22,23]. The Sesn2 promoter contains a putative Nrf2 binding site. It might be that Nrf2 is more important in affecting Sesn2 expression in response to NO compared to hypoxia, although the promoter contains a functional hypoxia-responsive element (HRE) as suggested by our experiments. Considering Sesn2 as a repair protein for overoxidized peroxiredoxins this study analyzes the impact of hypoxic and DETA-NO preincubation on the peroxiredoxin inactivation-reactivation cycle. Sulfiredoxin, an ATP-dependent peroxiredoxin reductase, is a well characterized component of this cycle [8]. There is evidence that sulfiredoxin is largely responsible for the reduction of the sulfinic acid form of peroxiredoxins [20]. In addition it is known that macrophages stimulated with lipopolysaccharide and interferon-γ show an NO-dependent increase in the expression of antioxidant enzymes that renders these macrophages less susceptible to H_2O_2-induced peroxiredoxin

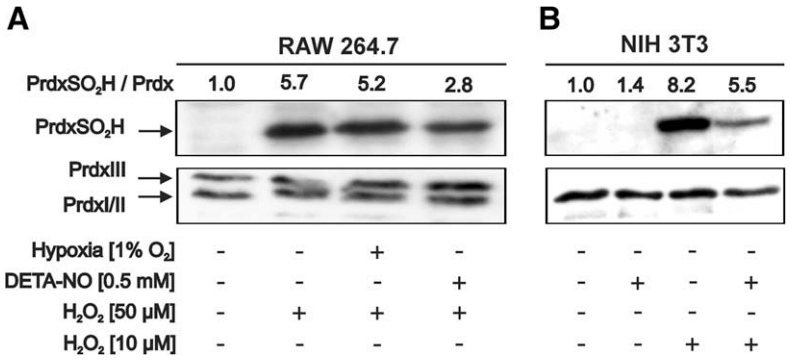

Fig. 3. Overoxidation of peroxiredoxin. Cells were pretreated with hypoxia (1%) or DETA-NO (0.5 mM) for 6 h and afterwards (A) RAW 264.7 were exposed to H_2O_2 (50 μM) and (B) NIH 3T3 cells to H_2O_2 (10 μM) for 30 min. Protein expression of 2-Cys peroxiredoxin and 2-Cys-peroxiredoxin-SO_2H were analyzed by Western analysis. Quantification of Western blots ($PrdxSO_2H$/Prdx) were performed with the ImageJ software.

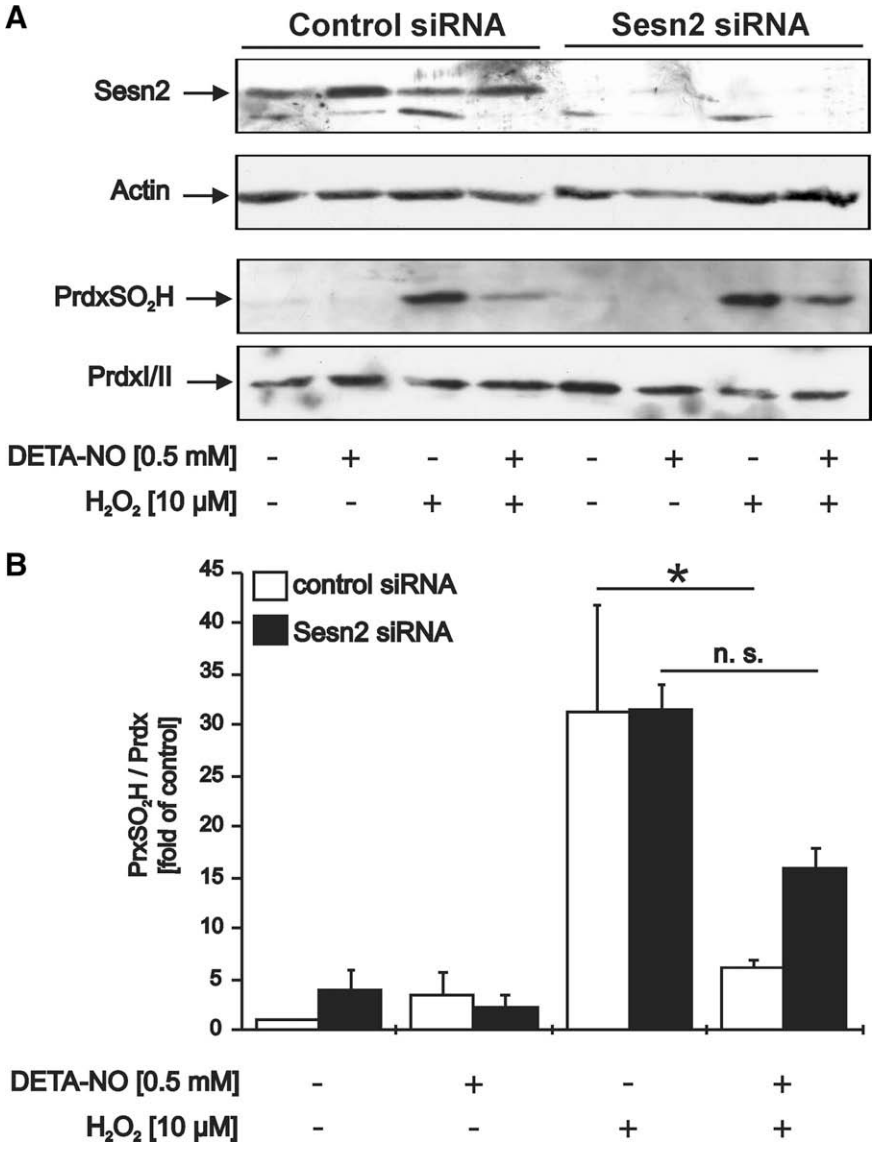

Fig. 4. Sesn2-dependent modulation of peroxiredoxin overoxidation. NIH 3T3 cells transfected with non-targeting siRNA or Sesn2 siRNA were pretreated with DETA-NO (0.5 mM) for 6 h and then exposed to 10 μM H_2O_2 for 30 min. (A) Expression of Sesn2, 2-Cys peroxiredoxin and 2-Cys peroxiredoxin-SO_2H were analyzed by Western analysis. (B) Quantification of Western blots ($PrdxSO_2H$/Prdx) of three independent experiments were performed with the ImageJ software. *Significant ($P < 0.05$), n.s. = not significant.

overoxidation. Interestingly, preventing peroxiredoxin overoxidation by NO is not mediated by sulfiredoxin [1]. Our work revealed Sesn2 to be strongly NO-inducible in macrophages, which provokes the question whether Sesn2 is involved in protection against

oxidative stress in immunostimulated macrophages. NO-preincubation prevented peroxiredoxin overoxidation in macrophages, whereas hypoxic preincubation did not. This is reflected by the significant higher Sesn2 protein accumulation after NO compared to hypoxic incubations, indicating that a critical amount of Sesn2 is necessary for protection. Nevertheless a knockdown of Sesn2 revealed that Sesn2 significantly but not completely facilitates NO-induced protection against peroxiredoxin overoxidation and therefore corrobates Sesn2 as a peroxiredoxin sulfinic acid reductase [11]. Besides induction of Sens2 via HIF-1, S-nitrosylation of peroxiredoxins and/or Nrf2-dependent upregulation of antioxidants such as heme oxygenase-1 (HO-1) are established defense mechanisms that may contribute to attenuate peroxiredoxin overoxidation by NO [19,24]. Sesn2 seems to be important in preventing acute sulfinic acid formation, while sulfiredoxin promote long-term effects to recover overoxidized peroxiredoxins, thus compensating oxidative stress [1,9,20].

Studying the peroxiredoxin inactivation-reactivation cycle, we propose that Sesn2 is involved in reactivation of overoxidized peroxiredoxins. NO as a molecule with abilities to protect from endogenous and exogenous oxidative stress is able to activate the antioxidative defense system at least in part by HIF-1α-induced Sesn2 upregulation.

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