Low doses of reactive oxygen species protect endothelial cells from apoptosis by increasing thioredoxin-1 expression

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Abstract The redox regulator thioredoxin-1 (Trx-1) is required for the redox potential of the cell and exerts important functions in cell growth and apoptosis. Severe oxidative stress has been implicated in the oxidation of proteins and cell death. However, the role of low doses of reactive oxygen species (ROS) is poorly understood. Here, we show that 10 and 50 μM H_2O_2 and short-term exposure to shear stress significantly increased Trx-1 mRNA and protein levels in endothelial cells. Since it is known that Trx-1 exerts anti-apoptotic functions, we next investigated whether low doses of ROS can inhibit basal and serum-depletion induced endothelial cell apoptosis. Indeed, treatment of endothelial cells with 10 and 50 μM H_2O_2 significantly reduced apoptosis induction. Reduction of Trx-1 expression using an antisense oligonucleotide approach resulted in the induction of apoptosis and abolished the inhibitory effect of low doses of H_2O_2. Taken together, our results demonstrate that low doses of ROS act as signaling molecules and exert anti-apoptotic functions in endothelial cells via upregulation of the redox-regulator Trx-1.

Keywords: Apoptosis; Endothelial cell; ROS; Thioredoxin-1

1. Introduction

Reactive oxygen species (ROS) such as H_2O_2 induce different cellular effects depending on concentration and cell type. Several studies documented the involvement of oxidative stress processes in the determination of apoptosis. Upon production of high levels of ROS from exogenous or endogenous sources, the redox balance is perturbed and cells are shifted into a state of oxidative stress [1]. These excessive high concentrations of ROS directly cause oxidative damage of DNA, lipids, and proteins and may impair cellular functions, which can result in apoptosis induction in severely damaged cells [2–5]. In contrast, several studies have demonstrated that ROS at subtoxic concentrations may act as signal transduction messengers and modulate gene expression [6–10]. Cells possess antioxidative enzymes that protect them from oxidative damage by maintaining the intracellular ROS at appropriate levels. Among these antioxidative enzymes, superoxide dismutase and catalase play key roles in converting superoxide to H_2O [11,12]. On the other hand, two other major intracellular redox systems are the thioredoxin (Trx) and glutathione systems [13,14]. The Trx family includes three proteins, Trx-1, mitochondrial thioredoxin/thioredoxin-2, and SpTrx [15–17]. They contain a conserved –Cys–Gly–Pro–Cys– active site (cysteine 32 and cysteine 35 within Trx-1), which is essential for the redox regulatory function of the Trxs [18]. Trx-1 is ubiquitously expressed in mammalian cells and knocking out Trx-1 leads to a lethal phenotype [18,19]. Besides its enzymatic activity as an oxidoreductase, Trx-1 directly interacts with other proteins such as the ribonucleotide reductase, protein disulfide isomerase (PDI), and several transcription factors (including p53, NFκB, and AP-1 via Ref-1) by forming disulfide bridges [20–25]. Several studies supported that oxidative stress inhibited Trx-1 ROS-scavenging function and expression [26,27], which suggests a regulation of Trx-1 by ROS. Moreover, overexpression of Trx-1 or recombinant Trx-1 inhibited oxidative stress-induced apoptosis in different cell types [22,28–32]. Recently, one mechanism was described that Trx-1 exerts its anti-apoptotic effects via binding to an inhibitory site on the apoptosis signal-regulating kinase 1 [33].

Therefore, we investigated the effect of different concentrations of H_2O_2 on Trx-1 expression and their role in apoptosis induction of endothelial cells. Our results demonstrate that low doses of H_2O_2 (10 and 50 μM) induce Trx-1 mRNA and protein levels and increased intracellular ROS formation. Moreover, simulation of the laminar blood flow by exposure of endothelial cells to shear stress increases intracellular ROS to a similar extent as low doses of H_2O_2 and also leads to an induction of Trx-1 mRNA. Low doses of H_2O_2 inhibited apoptosis in endothelial cells. However, 10 μM H_2O_2 did not inhibit Trx-1 antisense-induced apoptosis in endothelial cells, demonstrating a key role of the Trx redox system for redox status and apoptosis protection of endothelial cells.

2. Materials and methods

2.1. Cell culture

Endothelial cells (human umbilical vein endothelial cells) were cultured in endothelial basal medium supplemented with hydrocortisone (1 μg/ml), bovine brain extract (12 μg/ml), gentamicin (50 μg/ml), amphotericin B (50 ng/ml), epidermal growth factor (10 ng/ml), and 10% fetal calf serum. After detachment with trypsin, cells were grown for at least 18 h [34,35]. Endothelial cells were grown onto 6-cm dishes

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Abbreviations: Trx-1, thioredoxin-1; ROS, reactive oxygen species; DCF, (dichloro)fluorescein; NAC, *N*-acetylcysteine; eNOS, endothelial nitric oxide synthase; L-NMMA, NG-monomethyl-L-arginine; EGF, epidermal growth factor; PDGF, platelet-derived growth factor

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and exposed to laminar flow in a cone-and-plate apparatus as described [36].

2.2. Transfection

Phosphorothiolated sense or anti-sense oligonucleotides (5 μg) corresponding to the Trx-1 sequence (sense: 5′-ATGGTGAAGCAGATCGAGAGCAAGACT and anti-sense: 5′-AGTCTTGCTCTCGATCTGCTTCACCAT) were transfected into endothelial cells using 5 μl lipofectamine (Invitrogen) and cells were incubated with the oligonucleotides for 24 h as described [31].

2.3. Isolation of RNA and quantitative RT-PCR

RNA was isolated using the triazole reagent (Gibco). Trx-1 mRNA and GAPDH mRNA were quantitated using the Qiagen SYBR Green kit according to the manufacturer's instructions in a light cycler (Roche). After light cycler analysis, the reaction was analyzed on an agarose gel.

2.4. Immunoblot

After stimulation for the indicated times, endothelial cells were scraped off the plates and lysed in RIPA buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% Nonidet-P40, 0.5% deoxycholic acid, and 0.1% sodium dodecyl sulfate). After removing cell debris (15 min, 4 °C, 20 000 × *g*), 60 μg protein/slot was resolved on SDS–polyacrylamide gels and was blotted on PVDF membranes. For detection of protein expression, membranes were incubated with antibodies against Trx-1 (1:500, overnight, Pharmingen) or tubulin-β (clone DM1B, 1:1000, 2 h, Neomarkers). After incubation for 2 h with the corresponding secondary antibody tagged with horseradish peroxidase, signals were detected by the enhanced chemiluminescence system (Amersham).

2.5. Detection of ROS formation

Living cells were incubated for dye uptake with 20 μM 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) for 30 min (Molecular Probes). The acetates of H_2DCF-DA are cleaved by intracellular esterases releasing the reduced fluorescein (H_2DCF), which is converted to (dichloro)fluorescein (DCF) in the presence of reactive oxygen species and can be detected by FACS or fluorescence microscopy. In brief, dye-loaded cells were trypsinized for 2 min and reaction was stopped with PBS containing FCS. Living cells were pelleted by centrifugation (800 × *g*, 10 min). After washing with PBS, cells were resuspended in PBS and measured using FACS analysis. The area under the curve was calculated as DCF fluorescence.

2.6. Detection of cell death by FACS

Detection of cell death was performed by FACS analysis using annexin V-PE and 7-Amino-actinomycin (7AAD)-FITC staining (Pharmingen). Annexin V is a Ca^{2+}-dependent phospholipid-binding protein that has a high affinity to phosphatidylserine, which is exposed on the cell surface of apoptotic cells. In contrast, 7AAD is only cell permeable, when the cell membranes are damaged indicative of necrotic cell death. Therefore, apoptotic cells were defined as annexin V-positive, 7AAD-negative cells. In brief, cells were trypsinized off the dish and pelleted. After washing twice with annexin binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$, pH 7.4), cell pellets were resuspended in 50 μl of annexin binding buffer and incubated with 2.5 ng/ml annexin V-PE and 2.5 ng/ml 7AAD-FITC for 20 min. The reaction was terminated by adding 250 μl of annexin binding buffer and analyzed using FACS.

2.7. Detection of caspase-3 like activity

Caspase-3 like activity was measured using a commercially available ELISA according to the manufacturer's instructions (Roche).

2.8. Statistics

Statistical analysis was performed with Student's *t*-test or ANOVA followed by modified LSD (Bonferroni) test (SPSS-Software).

3. Results

3.1. Low doses of H_2O_2 induce Trx-1 mRNA in endothelial cells

To address whether low doses of ROS induce Trx-1 mRNA, endothelial cells were incubated with 10, 50 and 100 μM H_2O_2 for 3 h. Quantitative RT-PCR using a light cycler revealed that 10 μM H_2O_2 and 50 μM H_2O_2 significantly increased Trx-1

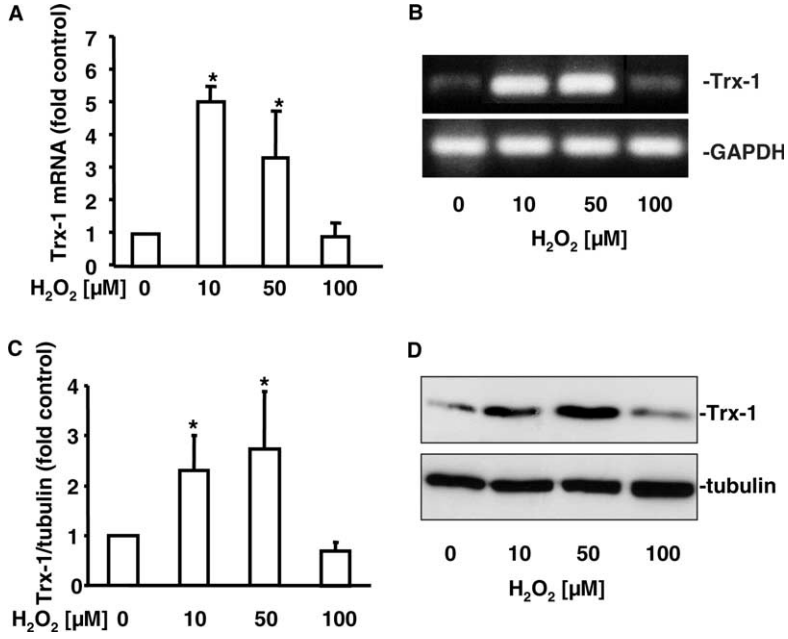

Fig. 1. (A) Low doses of H_2O_2 induce Trx-1 mRNA expression. Endothelial cells were incubated with 10, 50 or 100 μM H_2O_2 for 3 h. Quantitative RT-PCR was performed as described under Section 2. (Data are means ± S.E.M., $n = 7$, $^*P < 0.05$ versus control). (B) Low doses of H_2O_2 induce Trx-1 mRNA expression. Quantitative RT-PCR was performed as described under Section 2. The resulting products were loaded on a 2% agarose gel. A representative figure is shown ($n = 7$). (C/D) Low doses of H_2O_2 increase Trx-1 protein levels. Endothelial cells were incubated with 10, 50 or 100 μM H_2O_2 for 6 h. Immunoblotting with an anti-Trx-1 antibody was performed. Membranes were reprobed with anti-tubulin antibody. Trx-1/tubulin ratio was quantified by scanning densitometry using the Scion Image program ($n = 4$, $^*P < 0.05$ versus control). A representative immunoblot is shown in panel D.

mRNA (Fig. 1A), which was confirmed on an agarose gel (Fig. 1B). The results obtained by RT-PCR were confirmed on protein levels by an immunoblot approach. As shown in Fig. 1C and D, incubation with 10 and 50 μM H_2O_2 for 6 h profoundly increased Trx-1 protein levels.

3.2. Exposure to shear stress increases Trx-1 mRNA in endothelial cells

Recent studies have indicated that short-term exposure to shear stress may result in an increase of intracellular ROS [37]. Since low doses of H_2O_2 induced Trx-1 mRNA, we hypothesized that short-term exposure to shear stress may also increase Trx-1 mRNA. Therefore, endothelial cells were exposed to shear stress for 1 and 3 h. As demonstrated in Fig. 2A, Trx-1 mRNA was significantly increased by exposure to shear stress. The results by RT-PCR were confirmed by immunoblot as shown in Fig. 2B. To further elucidate whether this increase in Trx-1 mRNA is indeed due to formation of ROS, endothelial cells were coincubated with the antioxidant *N*-acetylcysteine (NAC). 10 mM NAC completely inhibited shear stress-induced increase in Trx-1 mRNA (Fig. 2C). Of note, incubation with NAC already significantly reduced Trx-1 mRNA under basal conditions (Fig. 2C). Next, we determined whether blockade of the endothelial nitric oxide synthase (eNOS) also influences shear stress-induced increase in Trx-1 mRNA similar to NAC. However, coincubation with the NO-synthase inhibitor NG-monomethyl-L-arginine (L-NMMA) did not influence the induction in Trx-1 mRNA by shear stress (Fig. 2D), suggesting that the increase of Trx-1 mRNA-induced by short-term exposure to shear stress is independent of eNOS activation.

3.3. ROS formation-induced by low doses of H_2O_2 is comparable to short-term exposure of shear stress

To examine whether the induction of Trx-1 mRNA by H_2O_2 or by the physiological stimulus of shear stress is due to comparable ROS formation, we loaded endothelial cells with H_2DCF-DA to detect endogenous ROS. FACS analysis

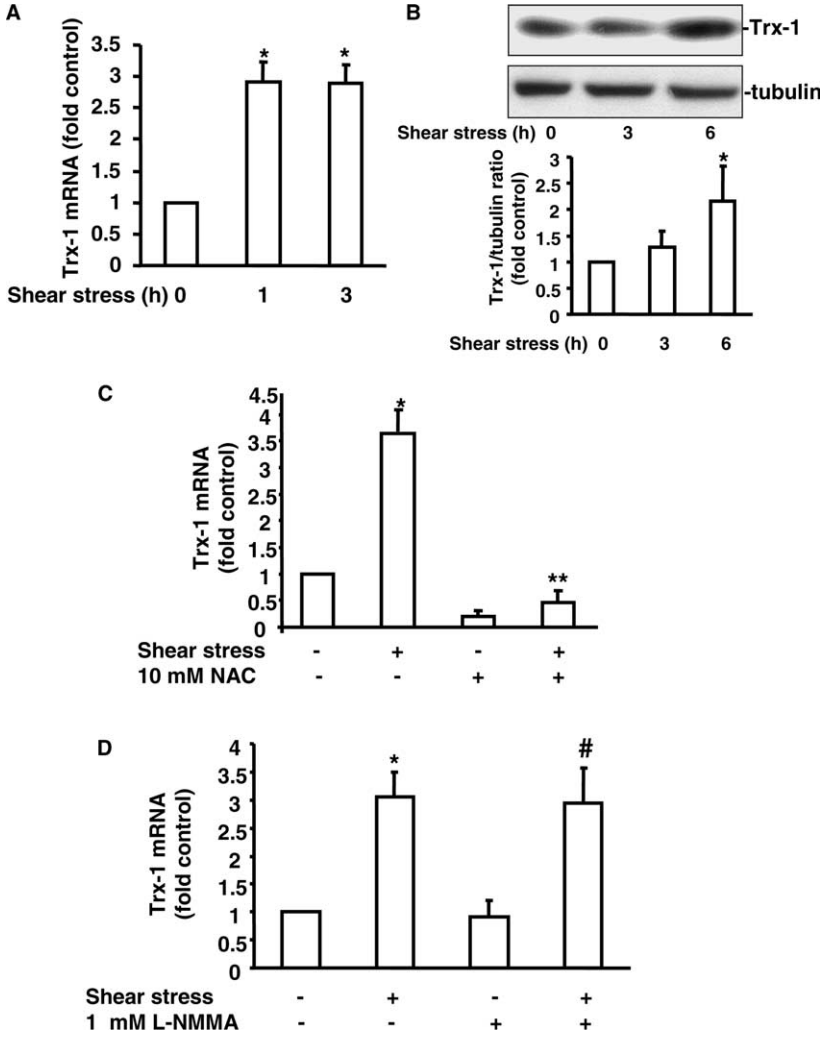

Fig. 2. (A/B) Shear stress increased Trx-1 mRNA and protein. Endothelial cells were exposed to shear stress (15 dynes/cm^2) for the times indicated. (Data are means ± S.E.M., $n = 5$, $^*P < 0.05$ versus static control). (C) NAC inhibited shear stress-induced increase in Trx-1 mRNA. Endothelial cells were incubated with 10 mM NAC and exposed to shear stress (15 dynes/cm^2) for 1 h. Quantitative RT-PCR was performed as described under Section 2. Data are means ± S.E.M. ($n = 4$, $^*P < 0.05$ versus static control, $^{**}P < 0.05$ versus 1 h of shear stress). (D) Inhibition of eNOS has no effect on shear stress-induced increase in Trx-1 mRNA. Endothelial cells were incubated with 1 mM L-NMMA and exposed to shear stress (15 dynes/cm^2) for 1 h. Quantitative RT-PCR was performed as described under Section 2. (Data are means ± S.E.M., $n = 4$, $^*P < 0.05$ versus static control, $^{\#}P < 0.05$ versus static control + 1 mM L-NMMA).

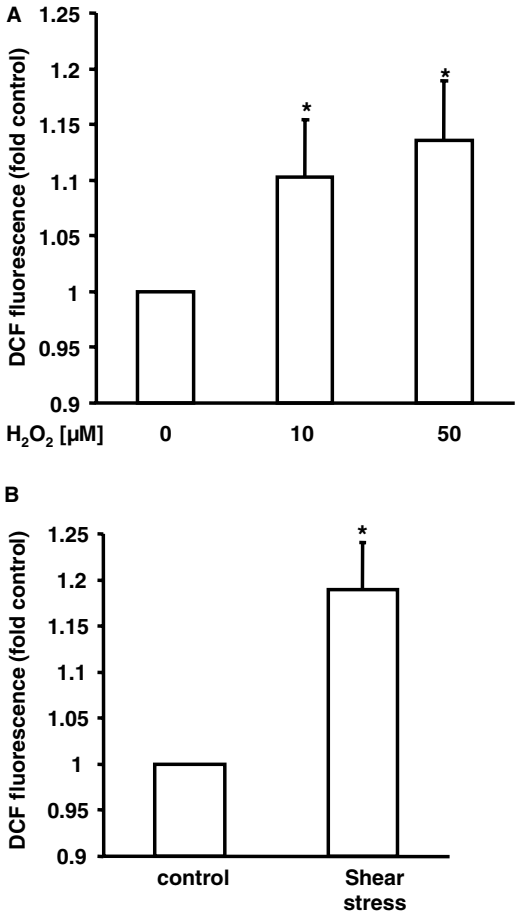

Fig. 3. (A) Low doses of H_2O_2 increase intracellular ROS formation. Endothelial cells were treated with 10 and 50 μM H_2O_2 for 6 h and intracellular ROS were measured after loading the cells with H_2DCF-DA by FACS analysis. ($^*P < 0.05$ versus control, $n = 4$) Data are means ± S.E.M. (B) Shear stress exposure increases intracellular ROS formation. Endothelial cells were exposed to shear stress (15 dynes/cm^2) for 1 h and intracellular ROS were measured after loading the cells with H_2DCF-DA by FACS analysis. ($^*P < 0.05$ versus control, $n = 4$) Data are means ± S.E.M.

revealed that 10 and 50 μM H_2O_2 showed similar intracellular ROS formation as compared to 1 h of shear stress exposure (Fig. 3A and B). These results demonstrate that low doses of exogenous H_2O_2 and short-term exposure to shear stress increase endogenous ROS, Trx-1 mRNA and protein to a similar extent.

3.4. Low doses of H_2O_2 inhibit apoptosis in endothelial cells

Several studies have documented the involvement of oxidative stress in the determination of apoptosis in various experimental conditions. In contrast, exposure to low levels of ROS can exert a stimulatory effect on proliferation of several cell types [8,38] and can activate vascular endothelial growth factor receptor-3 signaling to promote cell survival [39]. To investigate which levels of intracellular ROS act anti- or pro-apoptotic in endothelial cells, we measured apoptosis in endothelial cells treated with concentrations of H_2O_2 ranging from 10 to 100 μM for 12 h. 10 and 50 μM H_2O_2 reduced basal apoptosis induction up to 50% (Fig. 4A), whereas necrotic cell death was not affected (control: 0.74 ± 0.25 7-AAD positive cells, 10 μM H_2O_2: 0.75 ± 0.25 7-AAD positive cells, 50 μM H_2O_2: 0.85 ± 0.27 7-AAD positive cells ($n = 7$)). In contrast, incubation with 100 μM H_2O_2 resulted in a profound 2-fold increase in apoptosis (Fig. 4A). Of note, only 10 and 50 μM of H_2O_2 significantly enhanced Trx-1 protein levels, whereas 100 μM H_2O_2 even reduced Trx-1 protein (Figs. 1C, D and 4A, inset). Next, we investigated the ability of low doses of H_2O_2 to inhibit serum depletion-induced apoptosis. 10 μM H_2O_2 completely prevented serum depletion-induced apoptosis of endothelial cells (Fig. 4B). The reduction of apoptosis by low doses of H_2O_2 under serum-containing and under serum-depletion conditions obtained by FACS analysis using annexin V-PE was confirmed by caspase-3 like activity measurements (Fig. 4C and data not shown).

To finally determine whether the increase in Trx-1 expression is involved in the anti-apoptotic activity of low doses of H_2O_2, we used an antisense approach to reduce Trx-1 protein expression. Whereas low doses of H_2O_2 reduced apoptosis in sense transfected cells, the anti-apoptotic effect was completely abolished in antisense transfected cells (Fig. 4D). Thus, these data demonstrate that enhancing Trx expression by low doses of H_2O_2 importantly contributes to the anti-apoptotic effects in endothelial cells.

4. Discussion

The results of the present study demonstrate that low doses of exogenous H_2O_2 or endogenous stimulation of low doses of ROS by shear stress increase expression of Trx-1. Moreover, increased Trx-1 expression importantly contributes to the anti-apoptotic effect of low doses of ROS. In contrast, higher doses of H_2O_2 induced apoptosis in endothelial cells and did not increase Trx-1 expression.

ROS are formed and degraded by all aerobic organisms, leading to either physiological concentrations required for normal cell function or excessive quantities leading to oxidative stress. Ideally, a metabolically active cell should strike a balance between ROS production and the cellular antioxidant defense system. ROS are endogenously synthesized by tumor necrosis factor α, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and several hormones and neurotransmitters (for review, see [14]). Different studies predominantly in smooth muscle cells show that proliferation is induced in a ROS-dependent manner upon stimulation with EGF or PDGF [40,41]. Recently, Wang et al. provided evidence that activation of vascular endothelial growth factor receptor-3 signaling in response to H_2O_2 promoted endothelial cell survival [39]. Therefore, intracellular ROS is regarded as a signal transduction messenger involved in several intracellular mechanisms. Here, we show that exposure of endothelial cells to shear stress also releases endogenous ROS, which act as a signal transduction messenger by increasing Trx-1 expression. Moreover, we demonstrate that low concentrations of ROS in the range from 10 to 50 μM of H_2O_2 are protective for endothelial cells, whereas 100 μM of exogenous H_2O_2 already exerts cytotoxic effects and induces apoptosis. These data suggest that intracellular ROS act as a double-edged sword depending on their concentration. One should keep in mind that the concentration dependence of ROS may differ with cell type. The effects observed for ROS are comparable with those of nitric oxide, since several studies demonstrated that for

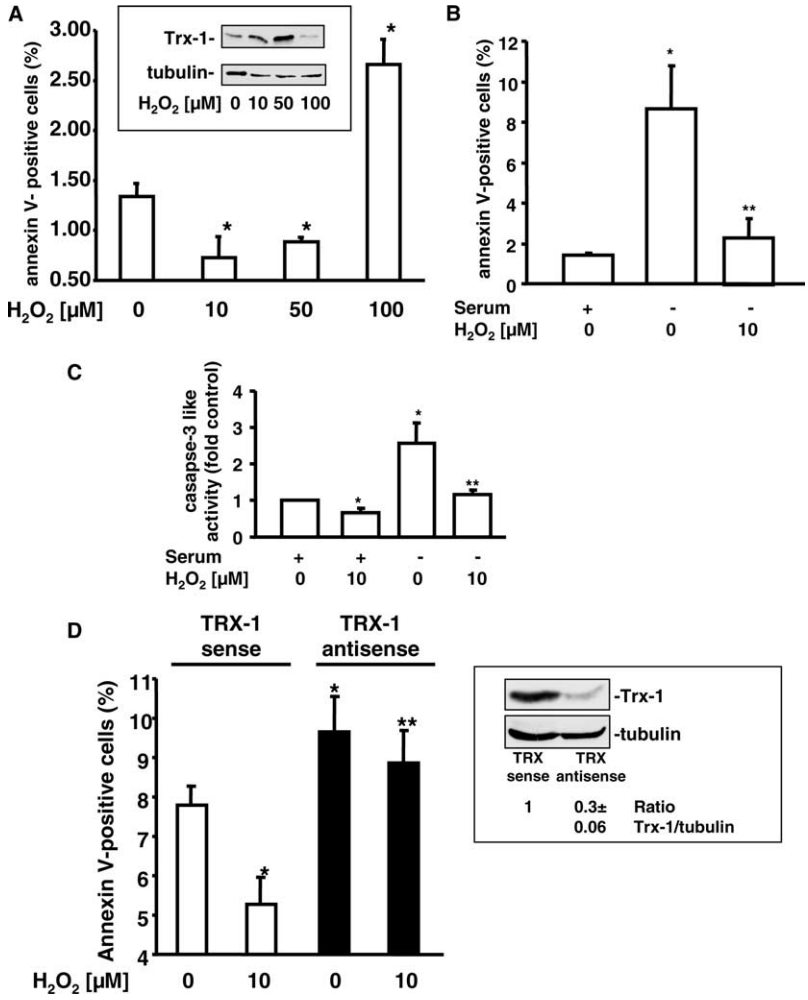

Fig. 4. (A) Low doses of H_2O_2 inhibit endothelial cell apoptosis. Endothelial cells were incubated for 12 h with H_2O_2 concentrations as indicated. Apoptosis was measured using Annexin-PE and analyzed by FACS ($^{*}P < 0.05$ versus control, $n = 4$). Data are means ± S.E.M.. The inset shows regulation of Trx-1 protein levels by different H_2O_2 concentrations as indicated. (B) Low dose of H_2O_2 reduced serum depletion-induced apoptosis. Endothelial cells were incubated for 12 h with H_2O_2 concentrations as indicated. Apoptosis was measured using Annexin-V PE and analyzed by FACS ($^{*}P < 0.05$ versus control, $^{**}P < 0.05$ versus serum deprivation, $n = 4$). Data are means ± S.E.M. (C) Low doses of H_2O_2 reduced caspase-3 like activity. Endothelial cells were incubated as indicated. Caspase-3 like activity was measured with an ELISA according to the manufacturer's instructions ($^{*}P < 0.05$ versus control, $^{**}P < 0.05$ versus serum deprivation, $n = 3$). (D) Inhibition of Trx-1 protein expression completely inhibited the protective effect of low doses of H_2O_2. Endothelial cells were transfected with oligonucleotides against Trx-1 and incubated with 10 μM H_2O_2 for 12 h. Apoptosis was measured using Annexin-PE and analyzed by FACS ($^{*}P < 0.05$ versus Trx-1 sense transfected cells; $^{**}P < 0.05$ versus Trx-1 sense transfected cells + 10 μM H_2O_2, $n = 4$). Data are means ± S.E.M. The inset shows Trx-1 protein expression and the calculated ratio of Trx-1/tubulin.

endothelial cells nitric oxide can act cytotoxic and pro-apoptotic in high concentrations, whereas nitric oxide at low concentrations was identified as an important anti-apoptotic signaling molecule [35,42].

The pathways, by which physiological concentrations of ROS act as a signal transduction messenger, are poorly understood. Previous studies suggested that ROS can inhibit caspases [43], activate the serine/threonine kinase Akt [44] and the mitogen-activated protein kinase [45] to enhance cell survival. Here, we demonstrate that low doses of H_2O_2 upregulate the redox-regulator Trx-1. It is known that Trx-1 directly reacts with H_2O_2 to detoxify cells [46,47]. Thus, one may speculate that a feedback regulation occurs, in which H_2O_2 regulates Trx-1 expression. A similar mechanism was shown for catalase, another detoxifier of H_2O_2, which expression is regulated by intracellular and extracellular H_2O_2 [48]. How can increased expression of Trx-1 protect endothelial cells from apoptosis? Several studies demonstrated that overexpression of Trx-1 or recombinant Trx-1 inhibits cell death in different cell types [29–31,49,50]. In contrast, oxidative stress has been shown to inhibit Trx-1 function, expression and activity in vitro and in vivo [26,27], which seems to contribute to the onset of vascular disease and hypertension. Thus, it is tempting to speculate that enhanced Trx-1 expression could be necessary to prevent endothelial cell apoptosis. Moreover, Trx-1 also increases the activation of transcription factors like NFκB and AP1 by binding Ref1 [25,51]. This study now provides evidence that low doses of H_2O_2 protect endothelial cells from apoptosis dependent on Trx-1 expression. It has to be noted that the protective effect occurred as early as 6 h after treatment with low doses of H_2O_2. Therefore, a reasonable explanation for our findings would be the upregulation of

immediate early genes like the transcription factor AP-1. The induction of Trx-1 expression should most likely involve the antioxidant responsive element present in the Trx-1 promoter [52] as well as the oxidative stress responsive element [15]. Since both elements have been shown to enhance Trx-1 expression in T cells and neutrophils upon oxidative stress in the form of radiation or menadione [15,53], the induction of Trx-1 mRNA by low doses of H_2O_2 could be due to the two responsive elements in the Trx-1 promoter. The induction of nuclear transcription factors by Trx-1 is also underscored by translocation of Trx-1 from the cytosol into the nucleus upon induction of oxidative stress by ferric nitrilotriacetate [54]. After translocation into the nucleus, Trx-1 enhances the DNA-binding of AP-1 and/or NFκB, which finally results in transcriptional activation of several downstream targets [51,55]. Trx-1 directly binds to NFκB and, thereby, reduces disulfide bonds, which leads to enhanced DNA-binding of NFκB [24]. This process of induction of enhanced NFκB-DNA binding capacity is reversible. Therefore, our study may shed some light on the discussion of NFκB with regard to apoptosis inhibition or induction in response to oxidative stress. Low doses of H_2O_2 can reversibly induce NFκB activation in endothelial cells due to the increase of Trx-1 expression, which results in a short term response. In contrast, high concentrations of ROS may directly destroy the disulfide bond involving cysteine 62 in NFκB and, therefore, leads to an irreversible activation of NFκB, which finally results in endothelial cell death.

Taken together, upregulation of Trx-1 expression appears to importantly modulate the effects of low doses of H_2O_2 to exert anti-apoptotic functions.

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