Shear stress increases the amount of S-nitrosylated molecules in endothelial cells: important role for signal transduction

Jörg Hoffmann, Stefanie Dimmeler, Judith Haendeler*

Molecular Cardiology, Department of Internal Medicine IV, University of Frankfurt, Theodor-Stern-Kai 7, Frankfurt, Germany

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Abstract Laminar flow (shear stress) is an important stimulus for nitric oxide (NO) synthesis in endothelial cells. NO can react with free SH-groups of different proteins leading to S-nitrosylation. Since S-nitrosylation of proteins is an important regulator of protein functions, we investigated the effect of endogenously synthesized NO. Exposure to shear stress significantly increased the overall S-nitrosylation of proteins in endothelial cells. Interestingly, shear stress increased S-nitrosylation of specific target proteins, i.e. the catalytic p17 subunit of caspase-3, the GTPase p21ras and the oxidoreductase thioredoxin. S-nitrosylation resulted in an inhibition of caspase-3 and in an augmented activity of p21ras and thioredoxin. These data suggest that long term exposure to shear stress exerts its different atheroprotective effects at least in part via increased S-nitrosylation of specific signaling proteins.

Key words: Caspase; Endothelial cell; GTPase p21ras; S-nitrosylation; Shear stress; Thioredoxin

1. Introduction

The mechanical forces in the vessel wall such as shear stress are crucial factors for the integrity of the endothelium and the functional role of endothelial cells. Shear stress appears to be a particularly important hemodynamic force because it stimulates the release of vasoactive substances and changes gene expression, cell metabolism, and cell morphology [1]. Several studies delineated that shear stress exerts atheroprotective, anti-thrombotic and anti-apoptotic functions. The atheroprotective and anti-apoptotic effect is in part due to the upregulation of superoxide dismutase and the endothelial nitric oxide synthase (eNOS) thereby increasing the amount of bioavailable release of nitric oxide (NO) [2,3]. The mechanisms underlying the mechanosensing by which endothelial cells convert shear stress stimulation into biochemical signals have been studied intensively. Recent findings deciphering the role of integrins in this process have been reviewed by Shyy and Chien [4]. By dynamically interacting with the extracellular matrix integrins lead to the activation of signaling cascades, for example via the caveolin-1/Fyn pathway or the focal adhesion kinase FAK/c-Src pathway both combining activation of mitogen-activated protein (MAP) kinases and extracellular signal-regulated kinase (ERK) through ras or rap1 [4].

NO is an important factor for the function of endothelial cells. Beside its well established role in regulation of the blood flow and acting anti-inflammatory, it also functions as a signaling molecule in a cyclic guanosine monophosphate (cGMP)-dependent and a cGMP-independent manner [5]. One important cGMP-independent mechanism is the modification of protein function by S-nitrosylation. S-nitrosylation is the reversible covalent binding to the SH-group of a reactive cysteine of a target protein. In the past few years more and more target proteins for S-nitrosylation have been identified as summarized by a recent review [6]. For example the activity of the pro-apoptotic caspase-3 is inhibited by S-nitrosylation of a critical cysteine in the catalytic center of the enzyme [7–9]. Beside caspase-3, we recently identified the oxidoreductase thioredoxin as a target protein for S-nitrosylation [10]. Thioredoxin plays a role in regulating the redox status of the cell and in apoptosis inhibition. S-nitrosylation of thioredoxin on cysteine 69 enhanced the redox regulatory capacity and anti-apoptotic function in endothelial cells [10]. Additionally, a variety of proteins involved in mediating the effects of shear stress are S-nitrosylated.

S-nitrosylation was demonstrated after induction of the inducible NOS (iNOS) or by exogenous NO donors. Recent studies also showed that S-nitrosylation occurred in cells containing the constitutive neuronal NOS (nNOS) and the eNOS [11,12]. Thus, endogenous synthesis of NO via the eNOS, which leads to approximately 0.025 ± 0.005 nmol/10^5 cells NO [13], appears sufficient to S-nitrosylate proteins. Since shear stress is an important activator of the eNOS, we wanted to investigate the role of physiological concentrations of endogenously produced NO by induction through shear stress on S-nitrosylation. Given the diverse biological effects provided by shear stress, we investigated whether S-nitrosylation of different target proteins may contribute to functional changes in signal transduction of endothelial cells. Here, we report that the content of S-nitrosylation was significantly increased after application of shear stress for 24 h in venous and arterial human endothelial cells. Further, the S-nitrosylation of the catalytic subunit of caspase-3, p17, the small GTPase p21ras and thioredoxin was enhanced after application of shear stress. The enhanced S-nitrosylation of the investigated target proteins resulted in a modulation of the functional activity of p21ras and thioredoxin. Thus, one pos-

*Corresponding author. Fax: (49)-69-6301 83462.
E-mail address: j.haendeler@em.uni-frankfurt.de (J. Haendeler).

Abbreviations: HUVEC, human umbilical vein endothelial cells; HUAEC, human arterial endothelial cells; NOS, nitric oxide synthase; NO, nitric oxide; DAN, 2,3-diamino-naphthalene; L-NMMA, N^G-monomethyl-L-arginine

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sible mechanism of the signal transduction of shear stress might be the enhanced S-nitrosylation of various target proteins.

2. Materials and methods

2.1. Cell culture

Human umbilical vein endothelial cells (HUVEC) and human arterial endothelial cells (HUAEC) 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. Shear stress exposure was performed with a cone-and-plate apparatus as described previously [7,14]. Cells were incubated with 1 mM N^G-monomethyl-L-arginine (L-NMMA) for the indicated time points.

2.2. Transfection

The plasmid encoding eNOS was a kind gift from Dr. Nakane and was subcloned as previously described [3]. The eNOS S1177D construct was generated using site-directed mutagenesis (Stratagene, Heidelberg, Germany). The plasmid encoding caspase-3 p17 wt subunit was cloned as previously described [7]. HUVEC were transfected with 3 μg plasmid and 25 μl Superfect as described previously with a transfection efficiency of 40% [15].

2.3. Immunostaining of S-nitrosylated proteins

Immunostaining of S-nitrosylated proteins was performed as described [10]. In brief, cells were fixed with 4% paraformaldehyde for 15 min at 25°C. After permeabilization and blocking (3% bovine serum albumin fraction V, 0.1% Triton X-100, 5% horse serum) for 15 min at 25°C, cells were incubated with an antiserum against S-nitrosocysteine (1:50, Alexis) at 4°C overnight. After incubation with a biotinylated antibody against rabbit IgG (1:500, Vector), cells were labeled with streptavidin-fluorescein (1:200, Vector) and visualized by fluorescence microscopy (magnification 1:40). As a negative control fixed and permeabilized cells were preincubated with 0.8% $HgCl_2$ for 1 h at 37°C.

2.4. Detection of S-nitrosylated proteins with the 2,3-diaminonaphthalene (DAN) assay

Detection of S-nitrosylated proteins was performed as described [10]. In brief, cells were lysed in 50 mM Tris (pH 8.0), 150 mM NaCl, 1% Nonidet-P40, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate (SDS) and subjected to immunoprecipitation using the appropriate antibody. Immunoprecipitates were washed twice with lysis buffer and two times with phosphate buffered saline (PBS). The pellet was resuspended in 500 μl PBS. After addition of 100 μM $HgCl_2$ and 100 μM DAN, samples were incubated in the dark at room temperature for 30 min and 1 M NaOH was added. The generated fluorescent triazole was measured using an excitation wavelength of 375 nm and an emission wavelength of 450 nm.

2.5. Detection of S-nitrosylated proteins by immunoblotting

Detection of S-nitrosylated proteins was performed as described [10,16]. In brief, cells were lysed in HENS buffer (25 mM HEPES pH 7.7, 0.1 mM ethylenediamine tetraacetic acid (EDTA), 0.01 mM neocuproine and 1% SDS) for 20 min and centrifuged at 20 000 × *g* for 15 min. 800 μg of total protein was incubated with 20 mM methylmethanthiosulfate (MMTS) for 20 min at 50°C and vortexed every 2 min for 5 s. MMTS was removed by protein precipitation with acetone. After resuspending the pellet in HENS buffer, 400 μM *N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide (biotin-HPDP) and 1 mM sodium ascorbate was added and incubated for 1 h at room temperature in the dark. Gel electrophoresis and Western blotting were performed in the dark. Biotinylated proteins were detected by using a horseradish peroxidase linked streptavidin according to the manufacturer's protocol (Amersham).

2.6. Immunoblot

After stimulation for the indicated times, HUVEC 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, 0.1% SDS). After removing cell debris (15 min, 4°C, 20 000 × *g*), 30 μg protein/slot were resolved on SDS–polyacrylamide gels and were blotted on polyvinylidene difluoride (PVDF) membranes. For detection of protein expression membranes were incubated with antibodies against tubulin (1:1000, overnight, Neo Markers), myc (1:250, overnight, Santa Cruz), thioredoxin (1:500, overnight, Pharmingen), ras (1:1000, overnight, BD Transduction Laboratories). After incubation for 2 h with the corresponding secondary antibody tagged with horseradish peroxidase, signals were detected by the enhanced chemiluminescence system (Amersham).

2.7. S-nitrosylated molecules

S-nitrosylated molecules were measured using the Saville–Griess assay as described previously [10]. In brief, HUVEC were lysed in Griess lysis buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 5 mM KCl, 1% Nonidet-P40, 1 mM phenylmethylsulfonyl fluoride, 1 mM bathocuproinedisulfonic acid, 1 mM diethylenetriamine pentaacetic acid, 10 mM *N*-ethylmaleimide) and 100 μg of cell lysate was incubated with 1% sulphanilamide and 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride in the presence or absence of 3.75 mM *p*-chloromercuribenzosulfonic acid or 10 mM $CuCl_2$ for 20 min and the content of S-nitrosylated molecules was measured photometrically at 540 nm. The amount was calculated using defined GSNO concentrations as a standard.

2.8. Preparation of recombinant proteins

Recombinant proteins were prepared as described previously [17]. In brief, the Raf1-RBD construct was transformed into *Escherichia coli* $DH5_\alpha$. Protein production was initiated by addition of 1 mM isopropyl β-D-thiogalactopyranoside to the culture. Fusion proteins were affinity purified on glutathione Sepharose 4B to obtain glutathione *S*-transferase (GST)-Raf1-Ras binding domain (RBD) to isolate Ras^{GTP}.

2.9. Ras activation assays

Ras activation assay was performed as described previously [17]. In brief, cells were lysed in lysis buffer containing 10 mM $MgCl_2$. 20 μg GST-Raf1-RBD was added to the supernatant and incubated overnight at 4°C with slight agitation. After washing the beads three times with lysis buffer, Laemmli sample buffer was added and samples were resolved on a 12% SDS–polyacrylamide gel electrophoresis (PAGE).

2.10. Thioredoxin activity

Cells were lysed in Griess lysis buffer as described above. For thioredoxin activity, 70 μl of cell lysate was incubated with 140 mU of thioredoxin reductase and 450 nM reduced nicotinamide adenine dinucleotide phosphate (NADPH). We measured NADPH consumption using a fluorescence photometer at 340 nm over 2 min, with points taken every 30 s. Thioredoxin activity was calculated as $(A_2-A_1)/(T_2-T_1)$ per mg of protein. A_1 is the absorbance measured at T_1. A_2 is the absorbance measured at T_2. T_1 and T_2 are the first and second time points, respectively.

2.11. Statistics

Statistical analysis was performed with Student's *t*-test.

3. Results

3.1. Shear stress increased the amount of S-nitrosylated molecules in endothelial cells

To investigate the effect of shear stress on the overall content of S-nitrosylation, HUVEC were exposed to 15 dynes/cm^2 shear stress for 24 h in a cone and plate apparatus as previously described [18]. In a first approach S-nitrosylated molecules were visualized by immunofluorescence analysis with an anti-S-nitrosocysteine antiserum. Fig. 1A showed that after application of shear stress the fluorescence signal was much brighter than in static controls indicating that more S-nitrosylated molecules were present. The specificity of the anti-S-nitrosocysteine antiserum was demonstrated by preincubating fixed and permeabilized cells with $HgCl_2$ which disrupts the S-nitrosylation bound (Fig. 1A).

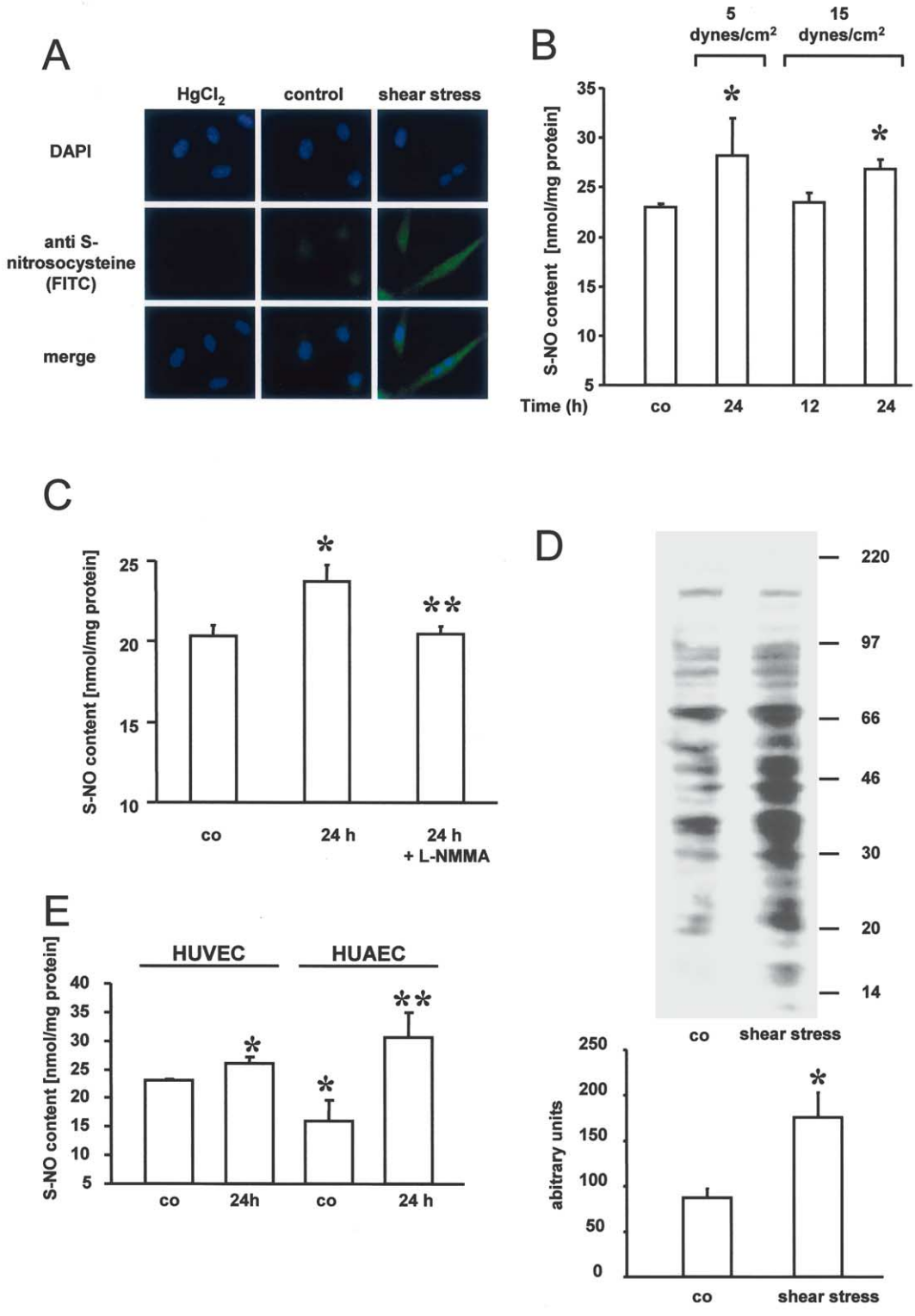

Fig. 1. Shear stress increased the amount of S-nitrosylated molecules. A: HUVEC were exposed to shear stress (15 dynes/cm^2) for 24 h. S-nitrosylated molecules were detected using an anti-S-nitrosocysteine antiserum. A representative figure is shown ($n=3$). The specificity of the staining was approved by incubation with $HgCl_2$. B: HUVEC were exposed to shear stress (5 and 15 dynes/cm^2 for the indicated time points). The content of S-nitrosylated molecules was quantified with the modified Saville–Griess assay ($n=5$). Data are means ± S.E.M. ($^*P<0.05$ versus static control). C: HUVEC were exposed to shear stress (15 dynes/cm^2) with or without 1 mM L-NMMA. The content of S-nitrosylated molecules was quantified with the modified Saville–Griess assay ($n=5$). Data are means ± S.E.M. ($^*P<0.05$ versus static control; $^{**}P<0.05$ versus 24 h of shear stress). D: HUVEC were exposed to shear stress (15 dynes/cm^2) for 24 h. S-nitrosylated proteins were detected by immunoblotting using the biotin switch method. A representative blot is shown ($n=3$). The densitometric analysis using Scion Image program is shown below ($^*P<0.05$ versus static control). E: HUVEC and HUAEC were exposed to shear stress (15 dynes/cm^2 for 24 h). The content of S-nitrosylated molecules was quantified with the modified Saville–Griess assay ($n=5$). Data are means ± S.E.M. ($^*P<0.05$ versus static control HUVEC; $^{**}P<0.05$ versus static control HUAEC).

To quantify the increase of S-nitrosylated molecules, we used a modified Saville–Griess assay. We could previously demonstrate that the majority of S-nitrosylated molecules in HUVEC are proteins with a mass greater than 5000 Da [16]. To investigate physiological relevant levels of shear stress, HUVEC were exposed to 5 dynes/cm^2 and 15 dynes/cm^2 shear stress for 24 h. Both levels of shear stress increased the amount of S-nitrosylated molecules in HUVEC to a similar extent (Fig. 1B). Moreover, application of shear stress time dependently increased the amount of S-nitrosylated molecules in HUVEC, with a maximum after 24 h of shear stress (Fig. 1B). Incubation of HUVEC with 1 mM of the NOS inhibitor L-NMMA completely abolished this increase, indicating that this increase was dependent on NOS activity (Fig. 1C). To further analyze the amount of S-nitrosylation for different target proteins the biotin switch method was used [11], which allows the analysis of S-nitrosylated proteins by immunoblotting. As shown in Fig. 1D shear stress enhanced the S-nitrosylation of different target proteins as displayed by an augmented chemiluminescence signal.

Next, we compared the amount of S-nitrosylated molecules in HUVEC and HUAEC. HUAEC showed significantly lower basal levels of S-nitrosylated molecules when compared to HUVEC (Fig. 1E). However, S-nitrosylation was enhanced in HUAEC after application of 15 dynes/cm^2 shear stress for 24 h to levels comparable to HUVEC (Fig. 1E).

3.2. Shear stress increased S-nitrosylation of caspase-3 subunit p17

To get further insights, we determined the S-nitrosylation of known target proteins. Among them is the catalytic subunit of caspase-3 p17. We could previously demonstrate that the activity of the pro-apoptotic caspase-3 is reduced by S-nitrosylation of the catalytic subunit p17 [7] and that shear stress inhibits apoptosis in HUVEC [18]. Therefore we investigated whether shear stress modulates the S-nitrosylation of caspase-3 p17. In order to demonstrate the S-nitrosylation of a specific target we immunoprecipitated the protein and used the DAN assay. Indeed, after application of shear stress the S-nitrosylation of caspase-3 p17 was significantly increased in comparison to the static control (Fig. 2). The shear stress-induced increase in S-nitrosylation was blocked by coincubation with L-NMMA, indicating the dependence on NOS activity (Fig. 2). Furthermore, cotransfection of a phosphomimetic eNOS (S1177D) construct, which has an increased NOS activity [3], further enhanced S-nitrosylation of caspase-3 p17 (Fig. 2).

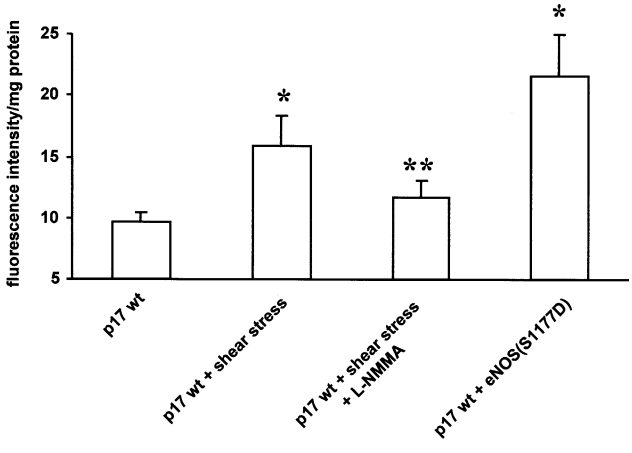

Fig. 2. Shear stress augmented S-nitrosylation of caspase-3 p17. HUVEC were cotransfected with caspase-3 p17 wt and empty vector or with caspase-3 p17 wt and eNOS (S1177D) and were then exposed to shear stress (15 dynes/cm^2) for 24 h with or without 1 mM L-NMMA. After immunoprecipitation with *myc* antibody the S-nitrosylation of caspase-3 p17 wt was detected using the DAN assay ($n = 3$–4). Data are means ± S.E.M. (*$P < 0.05$ versus static control; **$P < 0.05$ versus p17 wt+shear stress).

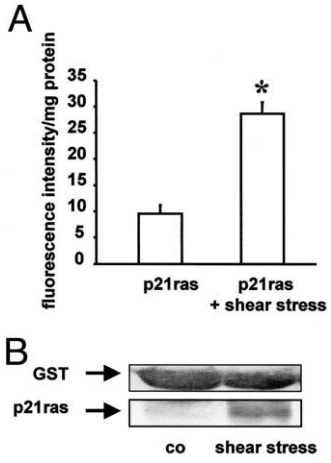

Fig. 3. Shear stress enhanced S-nitrosylation and activity of p21ras. A: Endothelial cells were exposed to shear stress (15 dynes/cm^2) for 24 h. After immunoprecipitation with an antibody against p21ras, S-nitrosylation of endogenous p21ras was detected using the DAN assay ($n = 4$). Data are means ± S.E.M. (*$P < 0.01$ versus static control). B: Ras activation was determined using Raf1-RBD-GST (20 μg). Immunoblots were performed with an antibody against Ras (lower panel). Equal amount of the Raf1-RBD-GST was demonstrated using an antibody against GST (upper panel). A representative figure is shown ($n = 3$).

3.3. Shear stress augmented S-nitrosylation and activity of p21ras

There is rising evidence that the small GTPase p21ras is involved in the mechanotransduction of shear stress via activation of integrins and a shc/Grb2/Sos-dependent pathway [4]. Furthermore, p21ras can be S-nitrosylated on cysteine 118, which results in guanine nucleotide exchange and subsequent downstream signaling [19]. Therefore, we wanted to investigate whether shear stress also modulates S-nitrosylation and activity of p21ras. After application of shear stress the S-nitrosylation of p21ras was significantly increased in comparison to the static controls (Fig. 3A). Simultaneously the activation of p21ras was assessed with a GST-Raf1-RBD fusion protein [17]. As shown in Fig. 3B, immunoprecipitation of the active form of p21ras was drastically augmented after application of shear stress. Thus, application of shear stress for 24 h resulted in an enhanced S-nitrosylation and activation of p21ras.

3.4. S-nitrosylation and reductase activity is enhanced by shear stress

We recently demonstrated that the oxidoreductase thioredoxin is S-nitrosylated on cysteine 69 [10]. This S-nitrosylation on cysteine 69 is important for redox regulatory and anti-apoptotic function of thioredoxin in endothelial cells. Therefore, we hypothesized that shear stress modulates the S-nitrosylation and activity of thioredoxin. Indeed, after application

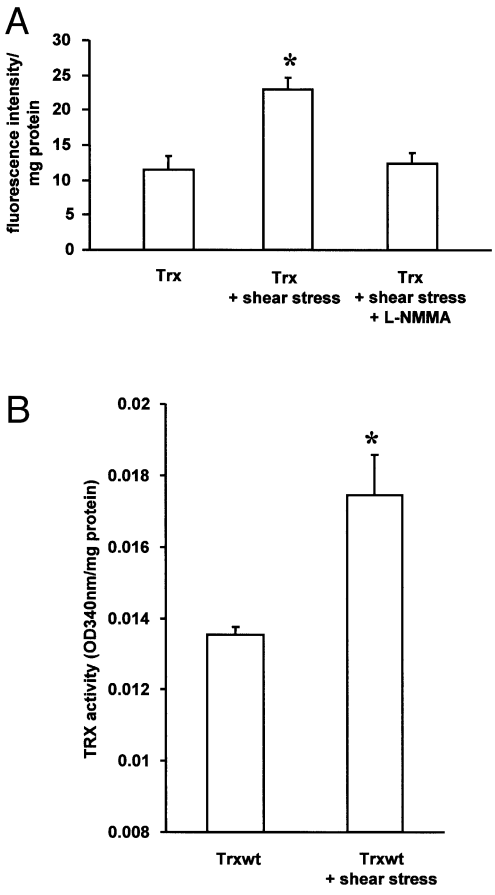

Fig. 4. Shear stress increased S-nitrosylation and activity of thioredoxin. A: Endothelial cells were exposed to shear stress (15 dynes/cm^2) for 24 h with or without 1 mM L-NMMA. After immunoprecipitation with an antibody against thioredoxin, S-nitrosylation of endogenous thioredoxin was detected using the DAN assay. Data are means ± S.E.M. (*$P < 0.05$ versus static control). B: Endothelial cells were exposed to shear stress (15 dynes/cm^2) for 24 h. Reductase activity of thioredoxin was monitored by a NADPH oxidation assay ($n = 5$). Data are means ± S.E.M. (*$P < 0.05$ versus static control).

of shear stress the S-nitrosylation of thioredoxin was significantly increased compared to the static control (Fig. 4A). This enhanced S-nitrosylation of thioredoxin was blocked by coincubation with the NOS inhibitor L-NMMA (Fig. 4A). Furthermore, the reductase activity of thioredoxin was enhanced after application of shear stress (Fig. 4B).

4. Discussion

Mechanical forces like shear stress are important stimuli for the function of endothelial cells in the vascular system. The present study gives several lines of evidence that one possible mechanism how these mechanical signals are converted into biochemical signals is S-nitrosylation of various target proteins, which are involved in mediating the effects of shear stress. The overall content of S-nitrosylated molecules was increased after exposure of endothelial cells to shear stress for 24 h, indicating a possible role in the long term signal transduction of shear stress. These data are in line with a previous study by Gow et al., who showed an increase in overall S-nitrosylation by acetylcholine-induced eNOS activation [12]. The present study now extends these observations by demonstrating an increase in S-nitrosylation of specific targets, such as the catalytic subunit p17 of caspase-3, p21ras and thioredoxin, by application of shear stress, which was accompanied by an augmented activity of p21ras and thioredoxin.

The pro-apoptotic caspase-3 consists of two subunits, p12 and p17, which form a heterodimer to build the active protease. The subunit p17 contains a reactive cysteine at position 163 in the catalytic center, which is essential for the enzyme activity. NO has been shown to inhibit caspase-3 p17 via S-nitrosylation of this critical cysteine [7,20]. Thus, the increased S-nitrosylation of caspase-3 p17 after application of shear stress might in part contribute to its anti-apoptotic effect in endothelial cells. These data go in line with the finding that shear stress leads to activation of eNOS and, thus, enhances the release of NO.

The conversion of shear stress as a mechanical force into biochemical signals has been addressed by several studies. One major player in this process are the integrins, a family of heterodimeric transmembrane receptors (for review see [4]). Although integrins predominantly activate the focal adhesion kinase, the integrins, $\alpha_1\beta_1$, $\alpha_v\beta_1$ and $\alpha_5\beta_1$, also activate Shc [21]. After association with Fyn, a member of the src kinase family, and subsequent activation of Shc and recruitment of the Grb2/Sos, the small GTPase p21ras is activated in short term transient manner via the integrins [4]. An additional mechanism to modulate p21ras activity is S-nitrosylation. S-nitrosylation of p21ras on cysteine 118 results in a guanine nucleotide exchange and subsequent downstream signaling [19]. Activation of p21ras by shear stress has only been shown as a transient activation with a peak at 1 min, which returns to baseline after 10 min [22]. Therefore, we hypothesized that S-nitrosylation of p21ras might be the trigger for a second prolonged activation of p21ras as a long term mechanism after application of shear stress for 24 h. And indeed, we demonstrate that long term application of shear stress for 24 h leads to an increased S-nitrosylation of p21ras, which is accompanied by an enhanced activation of p21ras. Thus, this might contribute to activation of MAP kinase signaling under long term conditions.

Finally, we showed that S-nitrosylation of thioredoxin is increased by shear stress.

The oxidoreductase thioredoxin is part of one essential component of the anti-oxidative defense system in cells. Via its reductase domain thioredoxin is able to reduce reactive oxygen species [23]. In addition to its oxidoreductase activity, thioredoxin interacts with proteins like transcription factors or the apoptosis signaling kinase (ASK)-1 [24–28]. Furthermore, thioredoxin possesses anti-apoptotic functions [10]. We have recently demonstrated that the anti-apoptotic and redox regulatory capacity of thioredoxin in endothelial cells is dependent on S-nitrosylation of cysteine 69 [10,29]. Thus, the activity of thioredoxin in endothelial cells is in part regulated by S-nitrosylation. In the present study we provide evidence that application of shear stress for 24 h leads to a significant increase of S-nitrosylation of the endogenous protein. This increase is not due to a transcriptional upregulation of the protein (data not shown) but rather due to a better bioavailability of NO. Strikingly, the reductase activity of thioredoxin was also increased after application of shear stress. Thus, activation of thioredoxin might additionally contribute to

the observed adaptive response of endothelial cells towards laminar flow for longer periods [30]. Furthermore, this activation might also be the reason why the development of atherosclerosis is prone in regions of turbulent or no flow, whereas regions with laminar flow are protected, since oxidative stress is discussed to be one reason for the development of atherosclerosis (for review see [31,32]). Another independent risk factor for atherosclerosis is aging. We previously demonstrated that upon aging of endothelial cells, the expression of eNOS and the amount of S-nitrosylated molecules is reduced [33]. In contrast to our findings here, shear stress was unable to increase the expression of eNOS and the amount of S-nitrosylated molecules in aged HUVEC [33]. Therefore, it is tempting to speculate that signaling transduction pathways were disrupted in aged HUVEC in comparison to young HUVEC, which interfere with shear stress-induced S-nitrosylation. Moreover, aged HUVEC are more sensitive to stimulus-induced apoptosis [33]. Since we show here that shear stress-induced S-nitrosylation regulates a variety of major signaling molecules in young HUVEC, this may in part account for the anti-apoptotic protective effect of shear stress in young HUVEC. Moreover, comparing the content of S-nitrosylated molecules in young HUVEC to that of old HUVEC, we may speculate that the endogenous NO synthesis by the eNOS in young endothelial cells in culture is sufficient to S-nitrosylate proteins in HUVEC.

Taken together the data of the present study provide several lines of evidence that the increased S-nitrosylation of various target proteins due to exposure of endothelial cells to shear stress might modulate their protein function. Thus, these results give new insights in how long term effects of shear stress might be mediated in endothelial cells.

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