NO-mediated regulation of NAD(P)H oxidase by laminar shear stress in human endothelial cells

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The flowing blood generates shear stress at the endothelial cell surface. In endothelial cells, NAD(P)H oxidase complexes have been identified as major sources of superoxide anion ($\cdot O_2^-$) formation. In this study, we analysed the effect of laminar shear stress on $\cdot O_2^-$ formation by cytochrome *c* reduction assay and on NAD(P)H oxidase subunit expression by standard calibrated competitive reverse transcription-polymerase chain reaction and Western blot in human endothelial cells. Primary cultures of human umbilical vein endothelial cells were exposed to laminar shear stress in a cone-and-plate viscometer for up to 24 h. Short-term application of shear stress transiently induced $\cdot O_2^-$ formation. This was inhibited by NAD(P)H oxidase inhibitor gp91ds-*tat*, but NAD(P)H oxidase subunit expression was unchanged. Long-term arterial laminar shear stress (30 dyne cm^{-2}, 24 h) down-regulated $\cdot O_2^-$ formation, and mRNA and protein expression of NAD(P)H oxidase subunits Nox2/gp91^{phox} and p47^{phox}. In parallel, endothelial NO formation and eNOS, but not Cu/Zn SOD, protein expression was increased. Down-regulation of $\cdot O_2^-$ formation, gp91^{phox} and p47^{phox} expression by long-term laminar shear stress was blocked by L-NAME. NO donor DETA-NO down-regulates $\cdot O_2^-$ formation, gp91^{phox} and p47^{phox} expression in static cultures. In conclusion, our data suggest a transient activation of $\cdot O_2^-$ formation by short-term shear stress, followed by a down-regulation of endothelial NAD(P)H oxidase in response to long-term laminar shear stress. NO-mediated down-regulation by shear stress preferentially affects the gp91^{phox}/p47^{phox}-containing NAD(P)H oxidase complex. This mechanism might contribute to the regulation of endothelial NO/$\cdot O_2^-$ balance and the vasoprotective potential of physiological levels of laminar shear stress.

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Oxidative stress and increased generation of reactive oxygen species like superoxide anions ($\cdot O_2^-$) has been associated with endothelial dysfunction and clinical risk factors of atherosclerosis (Griendling & FitzGerald, 2003). Furthermore, superoxide anions can reduce NO availability by peroxynitrite ($ONOO^-$) formation (Bachschmid *et al.* 2005). The major sources of endothelial superoxide anion generation are NAD(P)H oxidases (Gorlach *et al.* 2000). The endothelial NAD(P)H oxidase complex is composed of a membrane-bound flavocytochrome b_{558} consisting of gp91^{phox}/Nox2 and p22^{phox} and two cytosolic subunits (p47^{phox} and p67^{phox}) (Jones *et al.* 1996). The subunit gp91^{phox} seems to be the limiting subunit of the NAD(P)H oxidase complex in endothelial cells (Rueckschloss *et al.* 2001).

Endothelial cells *in vivo* are constantly exposed to shear stress by the flowing blood. The impact of shear stress on endothelial superoxide anion formation is not clear. Oscillatory shear stress induced the generation of $\cdot O_2^-$ in endothelial cells (De Keulenaer *et al.* 1998). This increased endothelial $\cdot O_2^-$ formation in response to oscillatory shear stress involved a p47^{phox}-containing NAD(P)H oxidase complex (Hwang *et al.* 2003*a*,*b*) and xanthine oxidase (McNally *et al.* 2003). Short-term application of pulsatile shear stress also increased $\cdot O_2^-$ formation in bovine and murine endothelial cells (Go *et al.* 1999; Hwang *et al.* 2003*b*). However, the impact of long-term application of laminar shear stress on $\cdot O_2^-$ formation in primary

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cultures of human endothelial cells has not been studied so far.

Increased endothelial NO synthase (eNOS) expression in response to long-term shear stress has been previously shown in bovine endothelial cells (Nishida *et al.* 1992). Short-term and long-term endothelial NO formation by shear stress can involve different mechanisms. Shear stress-induced NO production of an endothelium-intact arterial segment, as assessed by changes in the tone of a preconstricted endothelium-denuded detector ring, was biphasic and consisted of an initial transient Ca^{2+}-dependent phase followed by a Ca^{2+}-independent plateau phase (Ayajiki *et al.* 1996). While the first phase mainly represents a functional activation of eNOS, the second phase is accompanied by an up-regulation of eNOS expression. In addition, shear stress-dependent up-regulation of eNOS blocked activation of the caspase cascade in response to apoptosis-inducing exogenous oxygen radicals in endothelial cells (Dimmeler *et al.* 1999). Therefore, a major vasoprotective mechanism of shear stress could be the formation of NO (Fleming & Busse, 2003). The role of NO in the shear stress-dependent regulation of $\cdot O_2^-$ formation is not known. In addition, regulation of NAD(P)H oxidase in response to shear stress is not well understood.

Therefore, we determined the $\cdot O_2^-$ formation and expression of different NAD(P)H oxidase subunits in response to short-term and long-term laminar shear stress in human umbilical vein endothelial cells (HUVEC). Furthermore, the role of NO on NAD(P)H oxidase expression was analysed.

Methods

Cell culture and application of shear stress

Cell culture reagents and chemicals were purchased from Sigma-Aldrich, Munich, Germany, except when otherwise specified. Primary cultures of human umbilical vein endothelial cells (HUVEC) were isolated with collagenase II as previously described (Jaffe *et al.* 1973). The use of human umbilical vein endothelial cells for this study was approved (EK124082003) and followed the guidelines of the hospital's Ethical Committee at our University of Technology Dresden, Germany. In order to minimize variations of primary cultures, isolated endothelial cells were pooled and subsequently separated in medium M199 with 1.25 g l^{-1} sodium bicarbonate, 100 mg l^{-1} L-glutamine (Life Technologies), supplemented with 10% fetal calf serum, 100 000 U l^{-1} penicillin, 100 mg l^{-1} streptomycin, 250 mg l^{-1} fungizone (Life Technologies) and 16.7 μg l^{-1} endothelial cell growth supplement (C. C. Pro, Neustadt, Germany). HUVEC were subjected to laminar shear stress in a cone-and-plate viscometer as described (Morawietz *et al.* 2000; Schubert *et al.* 2000). Laminar shear stress of 1–50 dyne cm^{-2} (0.1–5 N m^{-2}) was applied in a humified environment with 5% CO_2 at 37°C. Each sample was accompanied by a control from the same cell preparation incubated for the same period of time without application of shear stress. In order to keep the cell culture medium volume constant and to avoid a spill-over of the medium even at high rotational speed, for application of arterial levels of shear stress (15, 30, or 50 dyne cm^{-2}) 5% dextran (M_r 71 400) was added to the cell culture medium to increase the viscosity of the medium 2.95-fold from 0.007 dyne cm^{-2} to 0.02065 dyne cm^{-2}. In these experiments, each cell culture dish was accompanied by two controls from the same HUVEC preparation incubated under static conditions with cell culture medium supplemented with or without 5% dextran for 24 h. Dextran had no effect on mRNA expression in this study (not shown). The achievement of equal degrees of shear stress at lower rotational speed by using additional dextran has been shown to give equal results (Malek & Izumo, 1992) and did not affect cell viability, detachment or increased release of lactate dehydrogenase (LDH) into the medium as an indicator of cell integrity (Schubert *et al.* 2000).

In further studies, cells were incubated with the NO synthase inhibitor N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME, 500 μmol l^{-1}) or the PI3 kinase inhibitor Ly294002 (10 μmol l^{-1}) during the application of shear stress (up to 24 h). In addition, static HUVEC were treated with the NO donor 2,2'-(hydroxynitrosohydrazino)*bis*-ethanamine (DETA–NO) (500 μmol l^{-1}) for up to 24 h.

Superoxide anion measurements

The amount of $\cdot O_2^-$ was determined by cytochrome *c* assay. In order to quantify the $\cdot O_2^-$-related part, this reactive oxygen species was selectively measured by addition of SOD and catalase. Confluent HUVEC were subjected to laminar shear stress (30 dyne cm^{-2}) for the indicated times (2, 8, 24 h) in medium M199 with 10% fetal calf serum without phenol red containing 5% dextran. Each experiment involved four dishes from the same primary HUVEC preparation (two dishes exposed to shear stress, two incubated in parallel for the same period of time without shear stress), and two dishes with the same amount of corresponding medium without cells (one treated like shear stress samples, one static control). Laminar shear stress was applied continuously for the same period of time to three of the six dishes (two with HUVEC, one control with medium). In two dishes with HUVEC (one with continuous exposure to laminar shear stress, and one as internal control without shear stress), superoxide dismutase (SOD: 40 μg ml^{-1}, Sigma) and catalase (CAT: 380 U ml^{-1}, ICN) were added 1.5 h before

the measurement to the medium. After an additional 30 min, cytochrome *c* (40 μmol l^{-1}) was added to all dishes. While HUVEC were continuously exposed to shear stress, every additional 30 min reduction of cytochrome *c* was determined spectrophotometrically at 550 nm in all dishes.

The NADPH oxidase-specific inhibitor gp91ds-*tat* prevents the intracellular activation of the NADPH oxidase complex by inhibiting p47^{phox} association with gp91^{phox} (Rey *et al.* 2001). The inhibitor gp91ds-*tat* (100 μmol l^{-1}) was added 30 min before application of short-term shear stress (2 h, 30 dyne cm^{-2}) and formation of reactive oxygen species was determined by cytochrome *c* assay. The samples were normalized to internal controls without or with shear stress containing a peptide with a scrambled gp91 sequence (scramb-*tat*) at the same concentration.

In the experiments using the NO donor DETA-NO, cells were treated with 500 μmol l^{-1} DETA-NO (Calbiochem) for 24 h and cytochrome *c* reduction assay performed as described. All experiments were normalized to the internal control with corresponding medium without cells in order to eliminate cell-independent changes of absorption. The generation of $\cdot O_2^-$ was normalized to protein content of HUVEC determined by BCA Protein Assay (Pierce Biotechnology, Inc., Rockford, IL, USA).

Nitric oxide measurements

Nitrite, the major metabolite of NO in aqueous solution, release was measured using the Griess reaction (Osanai *et al.* 2000; Wessells *et al.* 2006). One hundred microlitres of endothelial cell supernatant were removed, and 100 μl Griess reagent containing sulfanilamide (50 μl of a 1% solution) and *N*-(1-naphthyl)ethylenediamine (50 μl of a 0.1% solution) was added to each tube for diazotization of sulfanilic acid by NO. After 5–10 min incubation at room temperature for full colour development, the nitrite release was measured as the increase in absorbance at 540 nm (Biowave UV/Vis Diode Array Spectrophotometer, Biochrom, Cambridge, UK) and compared with known concentrations of nitrite using a standard curve (0–50 μmol l^{-1}). Absorbance was measured and nitrite release determined by use of linear regression analysis ($y = ax + b$, $R^2 > 0.99$).

RNA isolation and RT-PCR

Total RNA from HUVEC was isolated using TRI Reagent. In the reverse transcriptase (RT) reaction, equal amounts of total RNA (500 ng) from HUVEC were incubated for 3 min at 70°C, and subsequently reverse transcribed into cDNA using random hexamer primers and SuperScript^{TM} III reverse transcriptase (Invitrogen, Karlsruhe, Germany) for 1 h at 42°C. The mRNA expression of NAD(P)H oxidase subunits gp91^{phox}, p22^{phox}, p47^{phox} and p67^{phox} was quantified as previously described (Duerrschmidt *et al.* 2000; Rueckschloss *et al.* 2001). All PCR experiments were furthermore normalized to 18S rRNA RT-PCR reactions.

Protein isolation and Western blot analysis

Protein isolation and Western blot analysis was performed as described (Schubert *et al.* 2000) using antibodies specific for gp91^{phox} (kindly provided by Mark T. Quinn, Montana State University, Bozeman, MT, USA) (Rueckschloss *et al.* 2002), p67^{phox}, p47^{phox}, eNOS (BD Transduction Laboratories, San Diego, CA, USA) and Cu/Zn SOD (Novacastra Laboratories Ltd, Newcastle upon Tyne, UK). The protein expression was detected with Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, Boston, MA, USA) and quantified using AIDA Image Analyser software (Raytest, Berlin, Germany).

Statistics

Data are shown as means ± S.E.M. Statistical analysis was performed with the ANOVA procedure followed by Bonferroni's method (multiple comparison) or Student's *t* test (SigmaStat 3.11., Systat Software Inc., Richmond, CA, USA), as appropriate. A value of $P < 0.05$ was considered statistically significant.

Results

Regulation of superoxide anion and nitric oxide release by laminar shear stress

Primary cultures of human umbilical vein endothelial cells were exposed to laminar shear stress in a cone-and-plate viscometer for up to 24 h. The superoxide anion release during application of laminar shear stress was determined by cytochrome *c* reduction assay. As shown in Fig. 1*A*, short-term application of laminar shear stress (2 h, 30 dyne cm^{-2}) transiently augmented $\cdot O_2^-$ formation (to 7.5 ± 1.7 nmol (mg protein)$^{-1}$, Fig. 1*A*). After 8 h of shear stress, $\cdot O_2^-$ formation was comparable to control levels. However, long-term application of arterial levels of laminar shear stress decreased endothelial $\cdot O_2^-$ formation (control: 4.0 ± 0.3 nmol (mg protein)$^{-1}$; 24 h, 30 dyne cm^{-2}: 2.1 ± 0.4 nmol (mg protein)$^{-1}$, $n = 8$, $P < 0.01$, Fig. 1*A*). Superoxide anion formation was not significantly changed by application of long-term low laminar shear stress (1 dyne cm^{-2}, 24 h) in human endothelial cells (81 ± 8% of control).

Short-term application of high shear stress (2 h, 30 dyne cm^{-2}) was sufficient to increase the NO formation (from 0.11 ± 0.02 μmol l^{-1} NO in control to 0.68 ± 0.09 μmol l^{-1} NO in HUVEC after shear

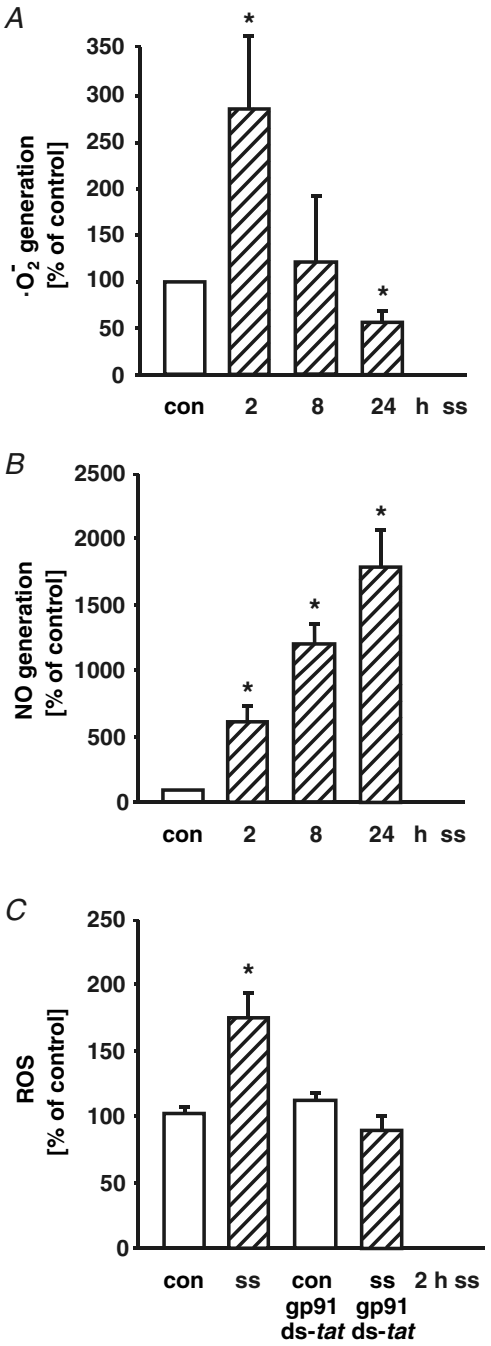

Figure 1. Time-dependent regulation of superoxide anion and nitric oxide generation by laminar shear stress in human endothelial cells

Human endothelial cells were exposed to laminar shear stress (ss, 30 dyne cm^{-2}) for 2, 8, or 24 h. Superoxide dismutase- and catalase-inhibited superoxide anion ($\cdot O_2^-$) formation was determined by cytochrome *c* reduction assay (*A*). Release of nitrite, the major metabolite of NO in aqueous solution, was measured using the Griess reaction (*B*). Impact of NADPH oxidase inhibitor gp91ds-*tat* (100 μmol l^{-1}) on formation of reactive oxygen species in response to short-term shear stress (ss, 2 h, 30 dyne cm^{-2}) was determined by cytochrome *c* assay. Samples were normalized to internal controls without or with shear stress containing a peptide with a scrambled gp91 sequence (scramb-*tat*) at the same concentration (*C*). Values are given as means ± S.E.M. as a percentage of control; $n \geq 3$ each; $^*P < 0.05$ *versus* control.

stress). Long-term low (1 dyne cm^{-2}, not shown) and high laminar shear stress (30 dyne cm^{-2}, Fig. 1*B*) further increased NO generation in human endothelial cells.

The NADPH oxidase-specific inhibitor gp91ds-*tat*, normalized to an internal control with scrambled gp91 sequence (scramb-*tat*) at the same concentration, inhibited the augmented reactive oxygen species formation in response to short-term laminar shear stress (2 h, 30 dyne cm^{-2}, Fig. 1*C*). These data suggest a crucial role of a $gp91^{phox}$-containing NADPH oxidase complex in superoxide anion formation in response to laminar shear stress in human endothelial cells.

Down-regulation of NAD(P)H oxidase subunit expression by long-term laminar shear stress

In order to find a molecular source of this $\cdot O_2^-$ formation in response to laminar shear stress, the expression of NAD(P)H oxidase subunits $gp91^{phox}$, $p22^{phox}$, $p47^{phox}$ and $p67^{phox}$ was determined.

Application of shear stress (30 dyne cm^{-2}) for 1 h did not increase the mRNA expression of the membrane-bound subunit $gp91^{phox}$ and the cytosolic subunit $p47^{phox}$ (Fig. 2). Furthermore, mRNA expression of $p22^{phox}$ (113 ± 12%) and $p67^{phox}$ (85 ± 13%) was not induced in response to 1 h of laminar shear stress as well.

In contrast to the short-term effects, long-term application of high laminar shear stress (30 dyne cm^{-2}) down-regulated $gp91^{phox}$ and $p47^{phox}$ mRNA expression in a time-dependent manner (Fig. 2*B* and *C*). This down-regulation of NAD(P)H oxidase subunits expression by long-term laminar shear stress was studied in more detail. Human endothelial cells were exposed to laminar shear stress of 1, 5, 10, 15, 30, or 50 dyne cm^{-2} for 24 h. The mRNA expression of NADPH oxidase subunits $gp91^{phox}$ and $p47^{phox}$ was down-regulated by long-term laminar shear stress in a dose-dependent manner (Fig. 2*D* and *E*). Because 30 dyne cm^{-2} is within the physiological range of arterial shear stress (Traub & Berk, 1998), all subsequent experiments studying high laminar shear stress were performed at this level.

Next, the protein expression of NAD(P)H oxidase subunits in response to shear stress was analysed. The specificity of the antibodies was confirmed by Western blot analysis including protein preparations from leucocytes (for $gp91^{phox}$ and $p47^{phox}$) as positive controls. After application of long-term high laminar shear stress (30 dyne cm^{-2}, 24 h), protein expression of NAD(P)H oxidase subunits $gp91^{phox}$ and $p47^{phox}$ was down-regulated (Fig. 3). In addition, NAD(P)H oxidase subunit $p67^{phox}$ was even slightly induced by long-term laminar shear stress on the mRNA level (140 ± 14% *versus* control; $n = 12$; $P < 0.05$), but not significantly changed on the protein level. The $p22^{phox}$ mRNA expression was not regulated by different levels of long-term (24 h) laminar shear stress.

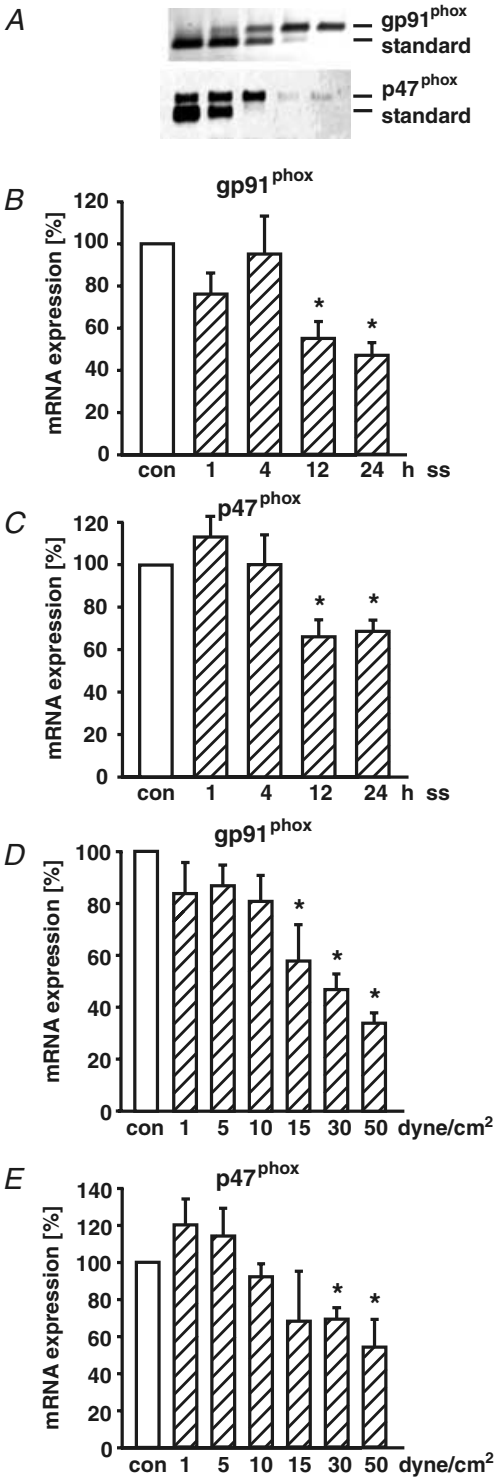

Figure 2. Time- and dose-dependent regulation of NAD(P)H oxidase subunit mRNA expression in response to laminar shear stress

The mRNA expression of NAD(P)H oxidase subunits gp91^{phox} and p47^{phox} was quantified by standard calibrated competitive reverse transcription-polymerase chain reaction (RT-PCR) (*A*) and normalized to 18S rRNA RT-PCR. The method compares amplification of an gp91^{phox} and p47^{phox} cDNA fragment from reverse transcribed total RNA of human endothelial cells (upper lane, longer fragment) *versus* different concentrations of an internally deleted and reverse transcribed cRNA standard (lower lane, shorter fragment) by PCR. Serial 1 : 3-dilution of appropriate cRNA standard was used. The PCR fragments were separated on agarose gels and stained with ethidium bromide. In time-course studies, human endothelial cells were exposed to laminar shear stress (ss) of 30 dyne cm^{-2} for 1, 4, 12 or 24 h and mRNA expression of NAD(P)H oxidase subunits gp91^{phox} (*B*) and p47phox (*C*) was quantified. The dose-dependent regulation of gp91^{phox} (*D*) and p47^{phox} (*E*) was studied in HUVEC exposed to long-term (24 h) laminar shear stress (ss, 1, 5, 10, 15, 30, 50 dyne cm^{-2}). Values are given as means ± S.E.M. as a percentage of control; $n \geq 5$ each; $^*P < 0.05$ *versus* control.

Up-regulation of eNOS protein expression by long-term arterial laminar shear stress

Human endothelial cells were exposed to long-term (24 h) arterial laminar shear stress (ss, 30 dyne cm^{-2}) and the protein expression of eNOS and Cu/Zn SOD was determined by Western blot and normalized to GAPDH protein (as a percentage of control without shear stress). After application of laminar shear stress, eNOS protein expression was up-regulated 3.5-fold (Fig. 4*A*). In contrast, Cu/Zn SOD protein expression was not changed in response to high laminar shear stress (Fig. 4*B*).

NO-dependent down-regulation of superoxide anion formation by long-term shear stress

Because shear stress induced endothelial NO synthase and NO formation, we analysed the effect of the NOS inhibitor L-NAME on shear stress-dependent $\cdot O_2^-$ formation in human endothelial cells. As shown in Fig. 5, L-NAME is not able to affect the shear stress-dependent induction of $\cdot O_2^-$ generation after 2 h. L-NAME had no effect on $\cdot O_2^-$ generation after 8 h as well. However, eNOS inhibition with L-NAME prevented the down-regulation of $\cdot O_2^-$ formation after 24 h of high laminar shear stress (Fig. 5). To get further insight into the NO-dependent regulation of endothelial $\cdot O_2^-$ formation, short- and long-term application of shear stress was repeated in the presence of the NO donor DETA-NO (500 μmol l^{-1}). In this context it has to be considered that the amount of NO formation by DETA-NO exceeds the shear stress-dependent NO formation (immediately after addition of DETA-NO: 407 ± 40 μmol l^{-1} NO, 2 h: 279 ± 31 μmol l^{-1} NO, 24 h: 245 ± 31 μmol l^{-1} NO). Additional NO release by the NO donor DETA-NO prevented the increased $\cdot O_2^-$ formation after 2 h of shear stress (Fig. 5). The $\cdot O_2^-$ formation was down-regulated by a combination of long-term laminar shear stress and DETA-NO as well.

Down-regulation of endothelial superoxide anion formation by NO donor

In order to further confirm the NO-dependent down-regulation of $\cdot O_2^-$ generation, static cultures of

human endothelial cells were exposed to DETA-NO (500 μmol l^{-1}) and $\cdot O_2^-$ formation measured. The NO donor reduced the endothelial superoxide anion formation to a similar extent to laminar shear stress. In additional experiments, human endothelial cells were incubated for 22.5 h with DETA-NO (500 μmol l^{-1}), the culture medium containing the NO donor was removed and subsequently $\cdot O_2^-$ generation was determined by SOD-specific cytochrome *c* assay (final measurement after 24 h). Even after removal of the compound, endothelial superoxide anion formation was down-regulated by the NO donor (control: 100 ± 25%, DETA-NO: 32 ± 12% of control, $n = 4$, $P < 0.05$). These data further support a NO-dependent down-regulation of superoxide anion formation.

Impact of NOS inhibition on shear stress-dependent down-regulation of NAD(P)H oxidase subunit expression

Next, we analysed the impact of NOS inhibition with L-NAME on the down-regulation of NAD(P)H oxidase subunit expression by high laminar shear stress. L-NAME itself had no effect on basal NAD(P)H oxidase expression. However, L-NAME prevented shear stress-dependent down-regulation of $gp91^{phox}$ and $p47^{phox}$ expression (Fig. 6). Inhibition of PI3 kinase by Ly294002 (10 μmol l^{-1}) had no effect on basal $gp91^{phox}$ expression (92 ± 13% of control) and did not prevent down-regulation of $gp91^{phox}$ by long-term high laminar shear stress (30 dyne cm^{-2}, 58 ± 8% of control, $n = 4$, $P < 0.05$).

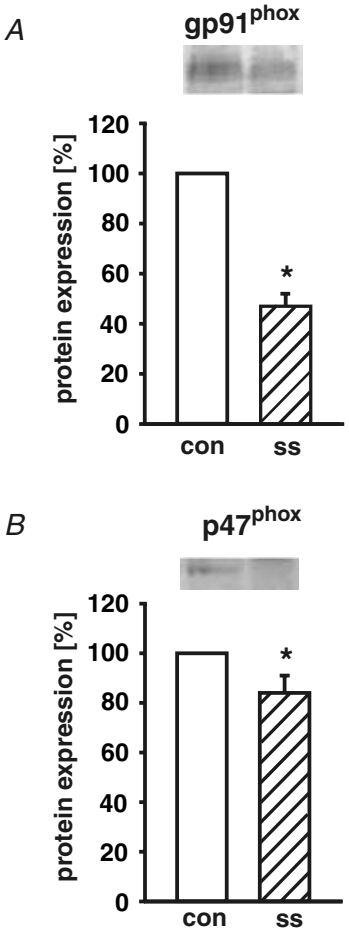

Figure 3. Down-regulation of NAD(P)H oxidase subunit protein expression by long-term arterial laminar shear stress
Human endothelial cells were exposed to long-term (24 h) laminar shear stress (ss, 30 dyne cm^{-2}). The protein expression of NAD(P)H oxidase subunits $gp91^{phox}$ (*A*) and $p47^{phox}$ (*B*) was determined by Western blot. Representative immunoblots for $gp91^{phox}$ and $p47^{phox}$ are shown above densitometric analyses. Values are given as means ± S.E.M. as a percentage of control without shear stress; $n \geq 5$ each; $^*P < 0.05$ *versus* control.

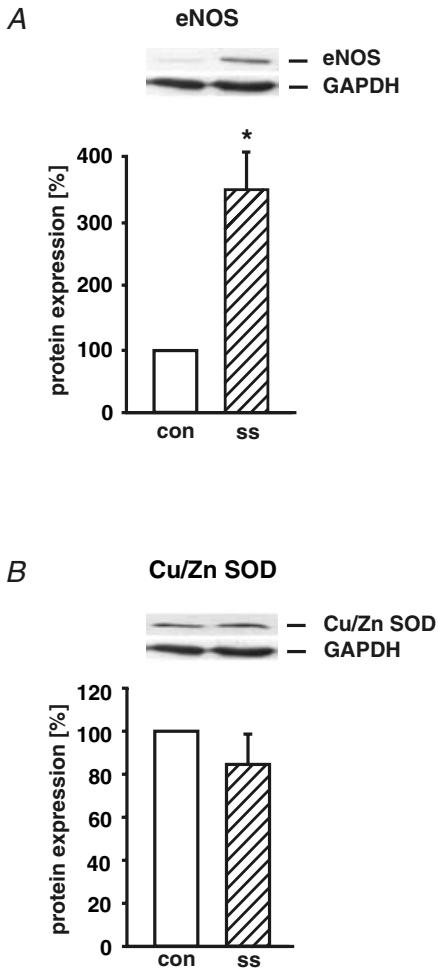

Figure 4. Up-regulation of eNOS protein expression by long-term arterial laminar shear stress
Human endothelial cells were exposed to long-term (24 h) laminar shear stress (ss, 30 dyne cm^{-2}). The protein expression of eNOS (*A*) and Cu/Zn SOD (*B*) was determined by Western blot and normalized to GAPDH protein. Values are given as means ± S.E.M. as a percentage of control without shear stress; $n = 4$; $^*P < 0.05$ *versus* control.

NO-dependent down-regulation of NAD(P)H oxidase subunit expression

To further confirm the impact of NO on different NAD(P)H oxidase subunits, we analysed the effect of DETA-NO on NAD(P)H oxidase expression in static endothelial cells. In agreement with our findings using L-NAME, we found a NO-dependent down-regulation of NAD(P)H oxidase subunit $gp91^{phox}$ and $p47^{phox}$ expression in static endothelial cells (Fig. 7).

Discussion

In this study, we show a short-term induction, but a NO-dependent down-regulation of superoxide anion formation during long-term exposure to laminar shear stress in primary cultures of human endothelial cells. The induction of superoxide anion formation by short-term shear stress in human endothelial cells in this study is in accordance with experiments using bovine aortic endothelial cells and a parallel-plate shear chamber (Go *et al.* 1999) or murine aortic endothelial cells (Hwang *et al.* 2003*b*). Oscillatory and low laminar shear stress (5 dyne cm^{-2}) has been shown to increase intracellular $\cdot O_2^-$ generation in a transient manner returning to baseline after 24 h in endothelial cells homogenized after application of shear stress (De Keulenaer *et al.* 1998). Oscillatory flow increased $\cdot O_2^-$ generation after 2–4 h in bovine endothelial cells as well (Hwang *et al.* 2003*a*). In parallel, laminar shear stress increased NO levels after 2–24 h and endothelial eNOS protein expression. This finding is in agreement with previous studies (Nishida *et al.* 1992; Ayajiki *et al.* 1996; Tsao *et al.* 1996; Dimmeler *et al.* 1999; Davis *et al.* 2001). Additional mechanisms might involve post-trancriptional modifications of eNOS. The eNOS can be activated by phosphorylation of serine 1177 by protein kinase Akt/PKB in a redox-sensitive manner (Du *et al.* 2001). Furthermore, increased endothelial eNOS Ser-1177 phosphorylation by shear stress could involve products of vascular oxidative stress such as hydrogen peroxide (Kojda & Hambrecht, 2005). The down-regulation of superoxide anion formation by long-term laminar shear stress observed in this study might support this increased flow-dependent NO availability in human endothelial cells.

Several putative sources of endothelial superoxide anion formation have been suggested (Griendling *et al.* 2000; Liu *et al.* 2003). Growing evidence supports NAD(P)H oxidase complexes as major sources of superoxide anions in

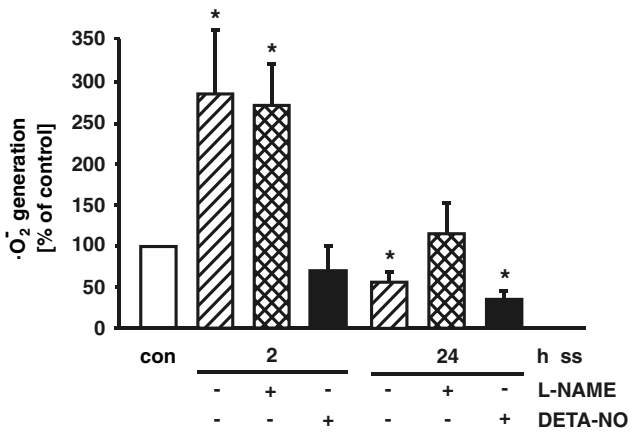

Figure 5. Impact of inhibition of endothelial NO synthase or additional NO formation on shear stress-dependent superoxide anion formation
Human endothelial cells were exposed to short-term (2 h) or long-term (24 h) laminar shear stress (ss, 30 dyne cm^{-2}) without or with the NO synthase inhibitor L-NAME (500 μmol l^{-1}), or the NO donor DETA-NO (500 μmol l^{-1}). Superoxide dismutase- and catalase-inhibited superoxide anion ($\cdot O_2^-$) formation was determined by cytochrome *c* reduction assay. Values are given as means ± S.E.M. as a percentage of control; $n \geq 3$ each; $^*P < 0.05$ *versus* control.

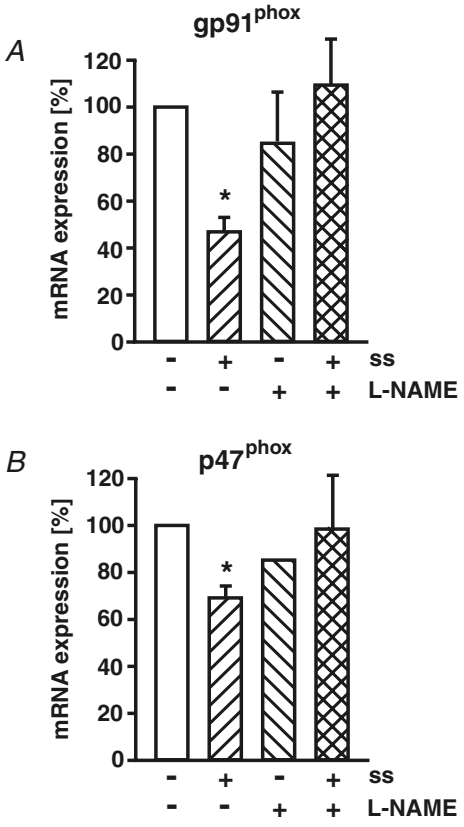

Figure 6. Endothelial NO synthase inhibitor prevents down-regulation of specific NAD(P)H oxidase subunits by shear stress
Human endothelial cells were exposed to long-term (24 h) laminar shear stress (ss, 30 dyne cm^{-2}) without or with NO synthase inhibitor L-NAME (500 μmol l^{-1}). The mRNA expression of NAD(P)H oxidase subunits $gp91^{phox}$ (*A*) and $p47^{phox}$ (*B*) was measured by standard calibrated competitive reverse transcription-polymerase chain reaction and normalized to 18S rRNA RT-PCR. Values are given as means ± S.E.M. as a percentage of control; $n \geq 5$ each; $^*P < 0.05$ *versus* control.

endothelial cells (Jones *et al.* 1996; Bayraktutan *et al.* 2000; Gorlach *et al.* 2000; Rueckschloss *et al.* 2001). Therefore, we focused in this study on the expression of the NAD(P)H oxidase subunits and the endothelial $\cdot O_2^-$ formation in response to laminar shear stress.

The short-term induction of endothelial $\cdot O_2^-$ formation by laminar shear stress might involve the gp91^{phox}/p47^{phox}-containing complex (Hwang *et al.* 2003*b*). Our data using the NADPH oxidase-specific inhibitor gp91ds-*tat* preventing the intracellular activation of the NADPH oxidase complex by inhibiting the association of p47^{phox} with gp91^{phox} (Rey *et al.* 2001) support this finding. Because we further did not observe an induction of NAD(P)H oxidase subunit expression by short-term shear stress, the increased $\cdot O_2^-$ generation under these conditions is most probably mediated by an activation of NADPH oxidase complexes using preformed subunits. It cannot be excluded that other sources of reactive oxygen species are involved in this short-term response as well. However, the major focus of this study was the impact of long-term laminar shear stress on endothelial $\cdot O_2^-$ formation.

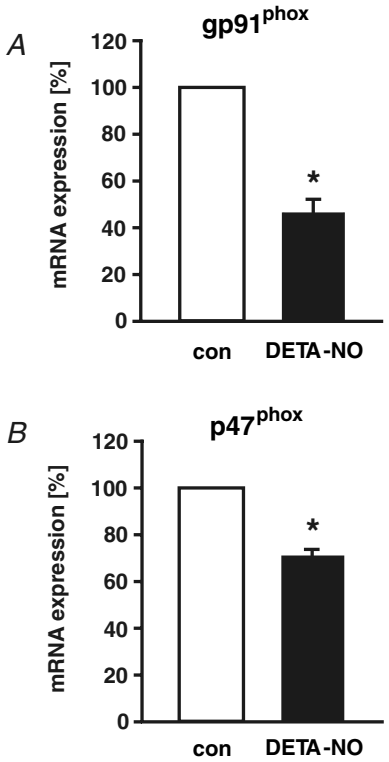

Figure 7. NO-dependent down-regulation of specific NAD(P)H oxidase subunit expression
Human endothelial cells were exposed in static culture to NO donor DETA-NO (500 μmol l^{-1}) for 24 h. The mRNA expression of NAD(P)H oxidase subunits gp91^{phox} (*A*) and p47^{phox} (*B*) was quantified by standard calibrated competitive reverse transcription-polymerase chain reaction and normalized to 18S rRNA RT-PCR. Values are given as means ± S.E.M. as a percentage of control; $n \geq 5$ each; $^*P < 0.05$ *versus* control.

The down-regulation of gp91^{phox} mRNA and protein expression in response to long-term laminar shear stress is in the same order of magnitude as the shear stress-dependent down-regulation of $\cdot O_2^-$ formation. This supports an essential role of gp91^{phox} as the rate-limiting subunit of the NAD(P)H oxidase complex in human endothelial cells (Rueckschloss *et al.* 2001; Rueckschloss *et al.* 2002). Gp91^{phox} shows a similar down-regulation on the mRNA and protein level to approximately 50% of control. The less pronounced down-regulation of p47^{phox} protein expression in comparison to mRNA expression might reflect additional regulation on the post-transcriptional level including translation and protein stability. However, even minor changes in protein expression could result in more pronounced effects on the functional superoxide anion formation. In addition, p47^{phox} is not a catalytic subunit and might therefore not directly be correlated with NAD(P)H oxidase activity.

NO synthase inhibitor L-NAME was not able to affect the shear stress-dependent induction of $\cdot O_2^-$ generation after 2 h. The suggested functional activation of preformed superoxide anion generating complexes therefore most probably does not involve NO. Nevertheless, additional NO release by the NO donor DETA-NO seemed to cause scavenging of the increased $\cdot O_2^-$ formation after 2 h of shear stress. In this context it has to be considered that the amount of NO formation by DETA-NO by far exceeds the shear stress-dependent NO formation. Thus, the down-regulation of $\cdot O_2^-$ generation after 24 h DETA-NO could, beside the NO-mediated down-regulation of NAD(P)H oxidase subunit expression, involve some scavenging effects of $\cdot O_2^-$ by NO. However, the NO-dependent down-regulation of endothelial superoxide anion formation even after previous removal of the NO donor supports an independent mechanism. Because L-NAME prevented the shear stress-dependent down-regulation of gp91^{phox} and p47^{phox}, this mechanism seems to involve an NO-dependent regulation of expression of subunits of the NAD(P)H oxidase complex. Interestingly, a slight increase of superoxide anion levels in samples with L-NAME compared to controls was observed which did not reach statistical significance. Under normal conditions, endothelial NO and $\cdot O_2^-$ formation might be in an equilibrium including some NO-dependent reduction of superoxide anion levels by peroxynitrite formation. If the NO production is inhibited by L-NAME, this could result in slightly increased $\cdot O_2^-$ levels (Munzel *et al.* 2000).

The down-regulation of endothelial $\cdot O_2^-$ levels might involve changes in SOD expression as well. Several studies have previously determined the regulation of different SODs in response to laminar shear stress in endothelial

cells (Inoue *et al.* 1996; De Keulenaer *et al.* 1998; Dimmeler *et al.* 1999; Chan *et al.* 2004). In our samples, laminar shear stress of 30 dyne cm^{-2} for 24 h increased protein expression of eNOS 3.5-fold, but the Cu/Zn SOD protein expression was not changed. Therefore, the decreased superoxide anion formation in response to laminar shear stress in our samples is not the result of an increased SOD expression.

In a recent study using a cranial window preparation in anaesthetized rats, increased intraluminal blood flow induced cerebral vasodilatation via activation of the NADPH oxidase by a PI3 kinase-dependent pathway (Paravicini *et al.* 2006). This is a very interesting finding suggesting even a transient vasodilatory effect of short-term released superoxide anions in response to rapid changes in blood flow in cerebral vessels. We did not observe an impact of PI3 kinase inhibition on down-regulation of $gp91^{phox}$ by long-term laminar shear stress. This further supports independent mechanisms of short-term and long-term effects of shear stress.

The *in vivo* relevance of the down-regulation of endothelial superoxide anion formation by long-term laminar shear stress observed in this study is supported by studies in porcine coronary arterioles (Sorop *et al.* 2003). Furthermore, cessation of flow in flow-adapted rat or mouse aorta increased generation of reactive oxygen species (Matsuzaki *et al.* 2005). In an interesting model of mouse arteriovenous fistula, creation of a shunt between right common carotid artery and jugular vein increased blood flow, shear stress, and subsequently a vascular NADPH oxidase complex comprising $p47^{phox}$ but not $gp91^{phox}$ (Castier *et al.* 2005). In this complex *in vivo* model, additional mechanisms in response to vessel dilatation-dependent increased mechanical stretch might be involved leading to activation of a Nox1/$p47^{phox}$-containing NADPH oxidase complex in the adjacent vascular smooth muscle cells. In contrast, increased blood flow in mice subjected to voluntary training reduced vascular superoxide release, Nox1 and $p47^{phox}$ expression (Laufs *et al.* 2005). Finally, chronic exercise training of patients with coronary artery disease before coronary artery bypass grafting surgery increased flow and decreased generation of reactive oxygen species and expression of $gp91^{phox}$ in internal mammary arteries (Adams *et al.* 2005).

In conclusion, our data suggest a transient activation of $\cdot O_2^-$ formation by short-term shear stress, but a down-regulation of endothelial $\cdot O_2^-$ formation in response to long-term laminar shear stress. The NO-mediated down-regulation of $\cdot O_2^-$ formation by long-term shear stress preferentially affects the $gp91^{phox}$/p47phox-containing NAD(P)H oxidase complex. This mechanism might contribute to the regulation of endothelial NO/$\cdot O_2^-$ balance and the antiatherosclerotic and vasoprotective potential of laminar shear stress.

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