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Antioxidants inhibit human endothelial cell functions through down-regulation of endothelial nitric oxide synthase activity

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Abstract

We have recently shown that superoxide and hydrogen peroxide are putative inducers of angiogenesis in vivo, possibly through up regulation of inducible nitric oxide synthase (NOS) and increased production of endogenous nitric oxide (NO). The aim of the present work was to elucidate the implication of reactive oxygen species in endothelial cell functions, using cultures of human umbilical vein endothelial cells (HUVEC). Superoxide dismutase (SOD), tempol (membrane permeable SOD mimetic) and the NADPH oxidase inhibitors, 4-(2-aminoethyl)-benzenesulfonyl fluoride and apocynin, but not allopurinol, inhibited HUVEC proliferation and migration, as well as activity of endothelial NOS (eNOS). Catalase and the intracellular hydrogen peroxide scavenger sodium pyruvate decreased, while hydrogen peroxide increased HUVEC proliferation, migration and activity of eNOS. Dexamethasone induced the proliferation and migration of HUVEC and activated eNOS. N^{ω}-nitro-L-arginine methyl ester (L-NAME), but not N^{ω}-nitro-D-arginine methyl ester, decreased endothelial cell functions and reversed the effects of dexamethasone and hydrogen peroxide. N_5-(1-iminoethyl)-L-ornithine dihydrochloride, but not the inducible NOS specific inhibitor *N*-[[3-(aminomethyl)phenyl]methyl]-ethanimidamide dihydrochloride also decreased endothelial cell functions, similarly to L-NAME. The guanylate cyclase inhibitor 1*H*-[1,2,4]Oxadiazole[4,3-a]quinoxalin-1-one inhibited HUVEC proliferation in a concentration-dependent manner and completely reversed hydrogen peroxide-induced proliferation, migration and cGMP accumulation. In conclusion, superoxide and hydrogen peroxide seem to play a significant role in promoting endothelial cell proliferation and migration, possibly through regulation of eNOS activity.

Keywords: Angiogenesis; Endothelial cell; Free radical; Nitric oxide; Endothelial nitric oxide synthase

1. Introduction

Reactive oxygen species are implicated in the pathophysiology of a variety of vascular diseases, including coronary artery disease, congestive heart failure, hypertension, atherosclerosis and diabetes (Maulik and Das, 2002). Although under oxidative stress reactive oxygen species have cytotoxic effects, evidence also exists concerning their implication in cell signalling (Dröge, 2001; Lander, 1997). It has recently been demonstrated that subunits of the NADPH oxidase complex are expressed in both vascular smooth muscle and endothelial cells (Babior, 2000; Bayraktutan et al., 2000; Gorlach et al., 2000). The amount of superoxide and hydrogen peroxide produced by endothelial cells is considerably less than that produced by leukocytes, suggesting that the low-output enzymes in the vessel wall produce reactive oxygen species for signalling rather than cytostasis (Babior, 2000; Bayraktutan et al., 2000). It is becoming increasingly recognized that reactive oxygen species trigger intracellular signalling that leads to enhanced angiogenesis in vivo (Maulik and Das, 2002; Polytarchou and Papadimitriou, 2004) and activation of endothelial cells in vitro (Lelkes et al., 1998; Maulik and Das, 2002; Stone and Collins, 2002; Yasuda et al., 1999). Moreover, reactive oxygen species, such as superoxide anion and hydrogen peroxide, play an important role in mediating angiogenic signals initiated by growth factors, such as vascular endothelial growth factor (Lin et al., 2003; Ushio-Fukai et al., 2002).

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Angiogenesis, the formation of new blood vessels from pre-existing ones, is a complex, multi-step process that characterizes a variety of physiological and malignant conditions (Carmeliet and Jain, 2000). Many molecules act as mediators of angiogenesis, among them nitric oxide (NO), which seems to be pro-angiogenic both in vivo and in vitro (Cooke, 2003; Morbidelli et al., 2003). Within mammalian cells, a family of NO synthase (NOS) enzymes generate NO, and all family members require a panel of substrates and co-factors to be fully functional. Three different isoforms of the NOS family have been identified; endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). The nNOS and eNOS isoforms are constitutively expressed in a variety of cell types and can be activated as a result of calmodulin binding following a rise in intracellular calcium. They may also be activated and/or inhibited by phosphorylation via various protein kinases. Unlike nNOS and eNOS, the activation of iNOS is not calcium-dependent and its expression can be transcriptionally regulated by factors, such as cytokines and oxidative stress (Alderton et al., 2001).

We have recently shown that superoxide and hydrogen peroxide are putative inducers of angiogenesis in vivo, possibly through up regulation of iNOS expression and activity (Polytarchou and Papadimitriou, 2004). In the present study, we investigated whether reactive oxygen species scavengers or inhibitors of reactive oxygen species production affect proliferation and migration of human umbilical vein endothelial cells (HUVEC) in vitro and if NO plays a role in these effects.

2. Materials and methods

The tested agents were:

- Superoxide dismutase (SOD) and its membrane permeable analogue 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl (tempol), which remove superoxide ions (Sigma, Greece).
- Apocynin and 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), both NADPH oxidase inhibitors (Sigma).
- Allopurinol, a xanthine oxidase inhibitor (Sigma).
- Hydrogen peroxide (Sigma).
- Catalase and sodium pyruvate, which detoxify hydrogen peroxide to H_2O extracellularly and intracellularly respectively (Sigma).
- Dexamethasone, a glucocorticoid receptor agonist which inhibits iNOS mRNA transcription (Sigma).
- N^{ω}-nitro-L-arginine methyl ester (L-NAME), a non-selective inhibitor of NOS activity and its inactive analogue N^{ω}-nitro-L-arginine methyl ester (D-NAME) (Sigma).
- N_5-(1-Iminoethyl)-L-ornithine dihydrochloride (L-NIO), a potent inhibitor of eNOS activity (Tocris Cookson Ltd., UK).
- *N*-[[3-(aminomethyl)phenyl]methyl]-ethanimidamide dihydrochloride (1400 W), a specific inhibitor of iNOS activity (Tocris Cookson Ltd.).
- 1*H*-[1,2,4]Oxadiazole[4,3-a]quinoxalin-1-one (ODQ), a potent, selective inhibitor of NO sensitive guanylyl cyclase (Sigma).

2.1. Cell culture

HUVEC were isolated and cultured as previously described (Hatziapostolou et al., 2003) and used only at passage 1. The cells were grown as monolayers in medium M199 supplemented with 15% fetal calf serum, 200 μg/ml endothelial cell growth supplement, 4 U/ml heparin sodium, 100 U/ml penicillin-streptomycin and 50 μg/ml gentamycin. Cultures were maintained at 37 °C, 5% CO_2 and 100% humidity.

2.2. Boyden chamber assay

Migration assays were performed as previously described (Hatziapostolou et al., 2003), in a 24-well microchemotaxis chamber (Costar), using untreated polycarbonate membranes with 8 μm pores. HUVEC were harvested and resuspended at a concentration of 10^5 cells/0.1 ml, in medium containing 0.25% bovine serum albumin. The bottom chamber was filled with 0.6 ml of medium containing 0.25% bovine serum albumin and the tested agents at the concentrations indicated. The upper chamber was loaded with 10^5 cells and incubated for 4 h at 37 °C. After completion of the incubation, the filters were fixed with saline-buffered formalin and stained using DiffQuick. The cells that migrated through the filter were quantified by counting the whole area of each filter, using a grid and an Optech microscope at a 20× magnification.

2.3. Cell proliferation assay

The effect of the tested agents on HUVEC growth was determined by directly measuring the number of cells, using a Neubauer hemocytometer and the Trypan blue exclusion assay. Briefly, cells were seeded at a density of 2×10^4 cells/well in 24-well tissue culture plates. The tested agents were added to the medium and the number of cells was measured after 48 h.

2.4. Western blot analysis of phosphorylated and total eNOs

HUVEC were incubated with the tested agents for 2 h and then directly lysed in lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.25% sodium dodecyl sulfate, 1 mM EDTA, 1 mM phenyl methyl sulfonyl fluoride, 1 μg/ml aprotinin and 1 mM sodium orthovanadate). The lysates were centrifuged at 20,000×*g* for 30 min at 4 °C and total protein concentration was determined in the supernatants, using the Bradford method (Bradford, 1976). Equal amounts

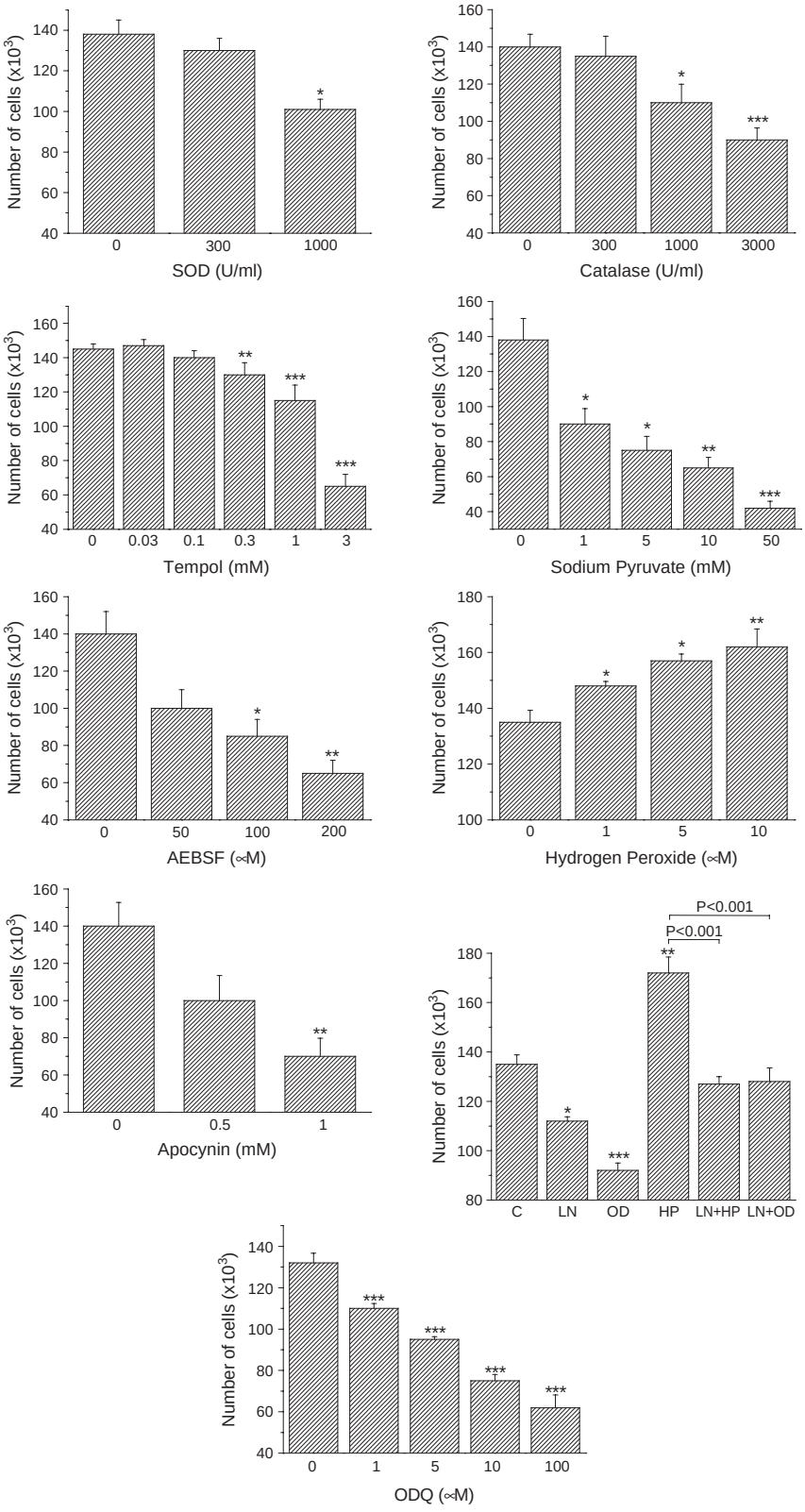

Fig. 1. Effect of antioxidants on HUVEC proliferation. Different concentrations of the tested agents were added in the culture medium of HUVEC and 48 h later, the number of the cells was estimated as described in Materials and methods. Results are expressed as mean±S.E.M. of the number of cells. Asterisks denote a statistically significant difference from untreated cells (*P<0.05, **P<0.01, ***P<0.001). C, control; HP, hydrogen peroxide 10 μM; LN, L-NAME 1 mM; OD, ODQ 10 μM.

of total protein were loaded on 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis mini gels, analyzed and transferred to Immobilon P membranes (Millipore). Blocking was performed by incubating the polyvinylidene fluoride membranes with 5% (w/v) non-fat dry milk in Tris buffered saline (TBS) pH 7.4, for 1 h at room temperature under continuous agitation. The membranes were then incubated with a polyclonal anti-phospho-eNOs (Serine 1177) antibody (Cell Signalling Technology, MA, USA) at a dilution of 1:1000 in 5% (w/v) bovine serum albumin in TBS containing 0.1% Tween-20 (TBS-T) overnight at 4 °C under continuous agitation and then with horseradish peroxidase conjugated goat anti-rabbit immunoglobulin G (IgG, Sigma) diluted 1:2500 in 3% (w/v) non-fat dry milk in TBS-T for 1 h at room temperature under continuous agitation. Detection of immunoreactive bands was performed by ChemiLuscent Detection System Kit (Chemicon, CA, USA), according to the manufacturer's instructions. The same membranes were stripped, blocked as described above and incubated with a polyclonal anti-eNOs antibody (Santa Cruz, CA, USA) at a dilution of 1:1000 in 3% (w/v) non-fat dry milk in TBS-T overnight at 4 °C under continuous agitation and then with horseradish peroxidase conjugated goat anti-rabbit IgG diluted 1:2500 in 3% (w/v) non-fat dry milk in TBS-T for 1 h at room temperature under continuous agitation. Detection of immunoreactive bands was performed by ChemiLuscent Detection System Kit, as described above. The pictures were digitized and the protein levels that corresponded to each immunoreactive band were quantified using the ImagePC image analysis software (Scion Corporation, Frederick, MD). The ratio phospho-eNOS/total eNOS represents the phosphorylation of eNOS after treatment with the tested agents.

2.5. cGMP assay

NO production from HUVEC in response to the tested agents was assessed by the intracellular accumulation of cGMP over a 4 h period. HUVEC were cultured in 60 mm dishes and used at confluence. The incubation with the tested agents was stopped by removal of the culture supernatant and the cells were then immediately covered with 0.1 N HCl and scraped off. The HCl extract was collected, centrifuged for 5 min at 5000×*g* and stored at −20 °C until analyzed. To normalize cGMP values, protein content in each dish was measured by the Bradford assay and the results were in the range of fmol cGMP/mg protein. Direct measurement of the intracellular cGMP levels was performed using the cGMP direct EIA kit (R&D Systems), according to the manufacturer's instructions.

2.6. Statistical analysis

The significance of variability between the results from each group and the corresponding control was determined by unpaired *t*-test or analysis of variance (ANOVA). Each experiment included triplicate measurements for each condition tested, unless otherwise indicated. All results are expressed as mean±S.E.M. from at least three independent experiments.

3. Results

3.1. Effect of antioxidants on HUVEC proliferation and migration

In order to elucidate whether and how antioxidants affect different angiogenic functions of endothelial cells, we studied their effect on HUVEC proliferation and migration.

As shown in Fig. 1, SOD, tempol, AEBSF and apocynin decreased the number of HUVEC in a concentration dependent manner. Allopurinol had no effect at any of the used concentrations (maximum concentration was 1 mM, data not shown). Catalase and sodium pyruvate also decreased HUVEC proliferation in a concentration dependent manner. Hydrogen peroxide at concentrations up to 10 μM increased HUVEC proliferation (Fig. 1), while at higher concentrations it decreased the number of HUVEC, possibly due to cytotoxicity (data not shown). The hydrogen peroxide-induced HUVEC proliferation was inhibited by L-NAME (Fig. 1), but not its inactive analogue D-NAME (data not shown). It was also inhibited by ODQ, which decreased HUVEC proliferation in a concentration dependent manner (Fig. 1).

All the tested agents, except allopurinol and hydrogen peroxide, significantly inhibited migration of HUVEC. Allopurinol had no effect, while hydrogen peroxide caused a significant increase in the migration of HUVEC, which was inhibited by ODQ and L-NAME (Fig. 2) but not D-NAME (data not shown).

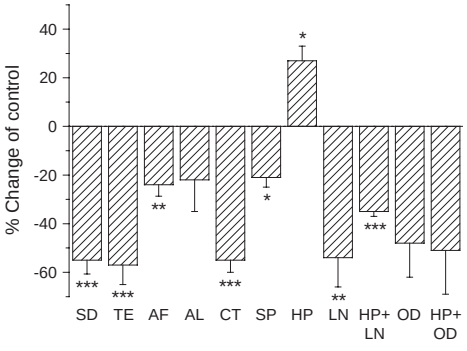

Fig. 2. Effect of antioxidants on HUVEC migration. Migration was measured using the Boyden chamber assay, as described in Materials and methods. Results are expressed as mean±S.E.M. of the % change of the cells that migrated in treated compared to untreated wells. The number of untreated cells that migrated through the membranes were 260±36 cells. Asterisks denote a statistically significant difference from untreated cells (*P<0.05, **P<0.01, ***P<0.001). SD, SOD 600 U/ml; TE, tempol 1 mM; AF, AEBSF 200 μM; AL, allopurinol 1 mM; CT, catalase 200 U/ml; SP, sodium pyruvate 1 mM; HP, hydrogen peroxide 10 μM; LN, L-NAME 1 mM; OD, ODQ 10 μM.

3.2. Effect of antioxidants on eNOS phosphorylation and cGMP production by HUVEC

We tested the effect of antioxidants on eNOS phosphorylation, a key event during the activation of the enzyme (Alderton et al., 2001). As shown in Fig. 3A and B, all the tested agents, except hydrogen peroxide and allopurinol, significantly decreased the phosphorylation of HUVEC eNOS. Allopurinol had no effect, while hydrogen peroxide increased eNOS phosphorylation.

cGMP production was measured in order to access the effect of the tested agents on NO production by HUVEC. All the tested agents, except hydrogen peroxide and allopurinol, significantly decreased the formation of cGMP by HUVEC. Allopurinol had no effect, while hydrogen peroxide increased the formation of cGMP by HUVEC, an effect which was inhibited by ODQ (Fig. 3C).

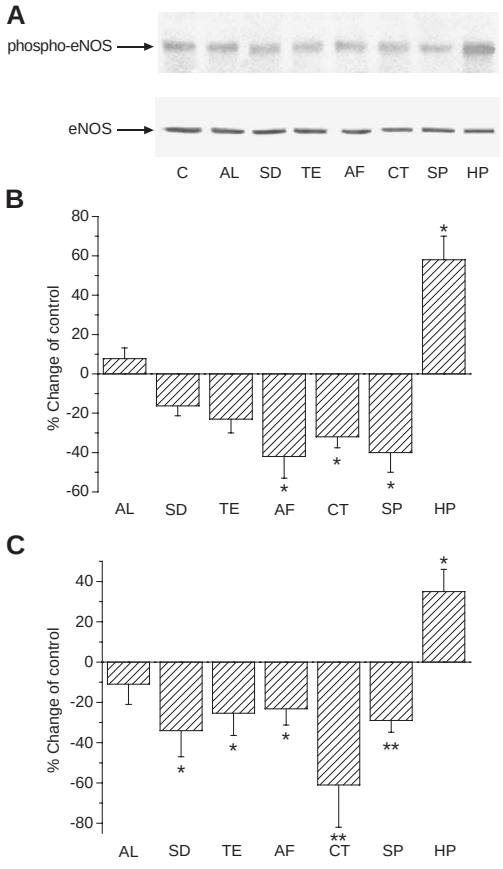

Fig. 3. (A) Effect of antioxidants on the phosphorylation of eNOS (Serine 1177) in HUVEC. Western blot analysis for phospho- and total eNOS 2 h after addition of the tested agents into the HUVEC culture medium. The figure shows a representative picture of five independent experiments. (B) The protein amounts were quantified by densitometric analysis of the corresponding bands and the ratio phospho-eNOS/total eNOS was calculated in each lane. Results are expressed as mean±S.E.M. of the % change compared to untreated cells. (C) Effect of antioxidants on cGMP formation by HUVEC. Results are expressed as mean±S.E.M. of the % change of cGMP amounts in treated compared to untreated cells. The amounts of cGMP produced by unstimulated HUVEC were 799.8±45.7 fmol/mg protein. Asterisks in all cases denote a statistically significant difference from untreated cells (*P<0.05, **P<0.01). C, control; AL, allopurinol 1 mM; SD, SOD 600 U/ml; TE, tempol 1 mM; AF, AEBSF 200 μM; CT, catalase 200 U/ml; SP, sodium pyruvate 1 mM; HP, hydrogen peroxide 10 μM.

3.3. Effect of NOS inhibitors on HUVEC proliferation and migration

In order to see if the effect of the antioxidants on eNOS phosphorylation and cGMP formation could be related to their effect on angiogenic endothelial cell functions, we tested the effect of several NOS inhibitors on HUVEC proliferation and migration. As shown in Fig. 4, L-NAME, but not its inactive analogue D-NAME, decreased HUVEC proliferation in a concentration dependent manner, reaching a maximum inhibition at a concentration of 5 mM. L-NIO, but not 1400 W, also decreased HUVEC proliferation in a concentration dependent manner, reaching a maximum inhibition at a concentration of 1 mM. Dexamethasone caused a dose-dependent increase in the number of HUVEC, reaching a maximum at a concentration of 40 nM. The effect of dexamethasone was completely reversed by L-NAME but not D-NAME.

All the tested agents, except 1400 W, D-NAME and dexamethasone, significantly inhibited migration of HUVEC. 1400 W and D-NAME had no effect, while dexamethasone caused a significant increase, which was inhibited by L-NAME but not D-NAME (Fig. 5).

As shown in Fig. 6A, all the tested agents, except 1400 W, D-NAME and dexamethasone significantly decreased the formation of cGMP by HUVEC. 1400 W and D-NAME had no effect, while dexamethasone caused a significant increase, which was inhibited by L-NAME but not D-NAME. The dexamethasone-induced increase in cGMP formation was in line with an increase in eNOS phosphorylation observed with the same concentration of dexamethasone (Fig. 6B).

4. Discussion

We have recently shown that superoxide anion and hydrogen peroxide affect physiological angiogenesis in vivo through regulation of iNOS expression and activity (Polytarchou and Papadimitriou, 2004). In the present study, we found that the same reactive oxygen species play a significant role in regulating human endothelial cell proliferation and migration in vitro, through regulation of eNOS activity.

All antioxidants that affected HUVEC proliferation and migration inhibited NO production by HUVEC and both L-NAME and L-NIO inhibited HUVEC migration and proliferation. Moreover, L-NAME totally inhibited the hydrogen peroxide induced increase in HUVEC proliferation and migration. These data support a pro-angiogenic role of NO, in agreement with several previous studies

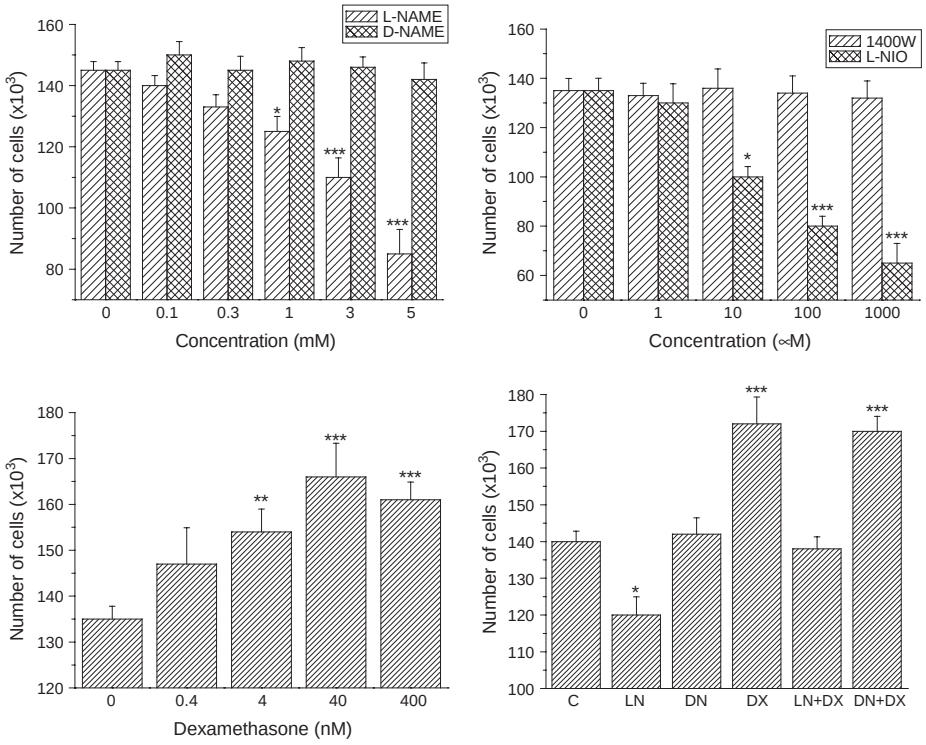

Fig. 4. Effect of NOS inhibitors and dexamethasone on HUVEC proliferation. Different concentrations of the tested agents were added in the culture medium of HUVEC and 48 h later, the number of cells was estimated as described in Materials and methods. Results are expressed as mean±S.E.M. of the number of cells. Asterisks denote a statistically significant difference from untreated cells (*P<0.05, **P<0.01, ***P<0.001). C, control; LN, L-NAME 1 mM; DN, D-NAME 1 mM; DX, dexamethasone 400 nM.

(reviewed in Morbidelli et al., 2003). They also suggest that reactive oxygen species affect endothelial cell functions, at least partly, through regulation of NO amounts. Activation of eNOS through increased phosphorylation of serine 1177 by hydrogen peroxide has recently been suggested by several studies (Cai et al., 2003; Drummond et al., 2000; Thomas et al., 2002). The present study, however, shows a direct link of hydrogen peroxide-induced eNOS activation

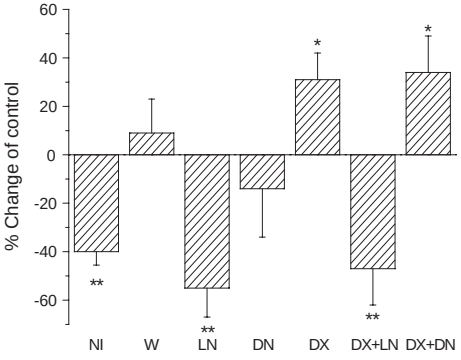

Fig. 5. Effect of NOS inhibitors and dexamethasone on HUVEC migration. Migration was measured using the Boyden chamber assay, as described in Materials and methods. Results are expressed as mean± S.E.M. of the % change of the cells that migrated in treated compared to untreated wells. The number of untreated cells that migrated through the membranes were 260±36 cells. Asterisks denote a statistically significant difference from untreated cells (*P<0.05, **P<0.01). NI, L-NIO 100 μM; W, 1400 W 100 μM; LN, L-NAME 1 mM; DN, D-NAME 1 mM; DX, dexamethasone 400 nM.

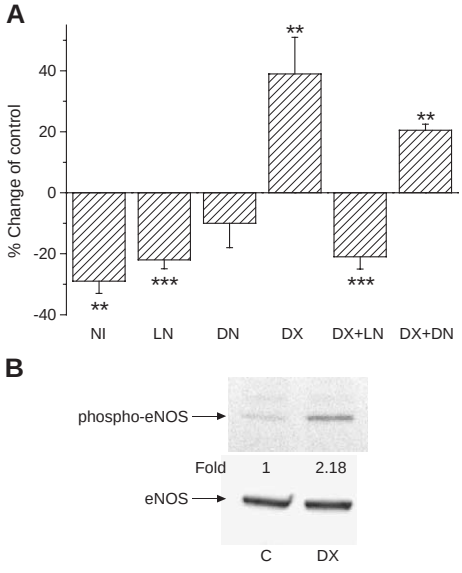

Fig. 6. (A) Effect of NOS activity modulators on cGMP formation by HUVEC. Results are expressed as mean±S.E.M. of the % change of cGMP amounts in treated compared to untreated cells. The amounts of cGMP produced by unstimulated HUVEC were 799.8±45.7 fmol/mg protein. Asterisks denote a statistically significant difference from untreated cells (**P<0.01, ***P<0.001). NI, L-NIO 100 μM; LN, L-NAME 1 mM; DN, D-NAME 1 mM; DX, dexamethasone 400 nM. (B) Effect of dexamethasone on the phosphorylation of eNOS (Serine 1177) in HUVEC. Western blot analysis for phospho- and total eNOS 2 h after addition of dexamethasone into the HUVEC culture medium. C, control; DX, dexamethasone 400 nM. The figure shows a representative picture of four independent experiments.

to stimulation of angiogenic endothelial cell functions. Moreover, it suggests that eNOS is also activated by superoxide anions, through phosphorylation on serine 1177, and this activation leads to increased endothelial cell proliferation and migration.

A major source of endothelial superoxide anion generation is the NADPH oxidase (Babior, 2000). The inhibitors of NADPH oxidase used in the present study inhibited HUVEC proliferation and migration, in agreement with previous studies (Abid et al., 2000). Our data furthermore suggest that this effect is mediated by down-regulation of eNOS activity. Allopurinol had no effect on either HUVEC proliferation and migration or eNOS activation, which suggests that xanthine oxidase activity is not involved in physiological endothelial cell functions, in line with a lack of effect on angiogenesis in vivo (Polytarchou and Papadimitriou, 2004) and with the fact that xanthine oxidase accounts for only a minor proportion of total reactive oxygen species production under normal conditions (Dröge, 2001).

Surprisingly, dexamethasone caused a significant increase on HUVEC proliferation and migration, which were abolished by L-NAME but not its inactive analogue D-NAME. Dexamethasone is considered to inhibit angiogenesis (Badruddoja et al., 2003; Luo et al., 2004) through down-regulation of transcription factors and the subsequent decrease in the expression of genes, among which is iNOS (Matsumura et al., 2001; Radomski et al., 1990). The iNOS protein and mRNA were not detectable in HUVEC of passage 1, something that is also supported by a lack of effect of the specific iNOS inhibitor 1400 W in the present study and is in line with previous studies (De Assis et al., 2002). Our results suggest that the stimulatory effect of dexamethasone on HUVEC is mediated by a rapid activation of eNOS, without affecting the amounts of the protein (Fig. 6B). These data support and extend those of a recent report that suggests that corticosteroids exert cardiovascular protection through a novel mechanism involving the rapid, non-transcriptional activation of eNOS by the non-nuclear actions of glucocorticoid receptor (Hafezi-Moghadam et al., 2002).

It has been suggested that antioxidants may enhance the activity of NOS by preserving the bioactivity of the NOS cofactor tetrahydrobiopterin (Huang et al., 2000), or by increasing NO bioactivity (Tomasian et al., 2000). However, our data suggest that antioxidants inhibit iNOS (Papadimitriou and Polytarchou et al., 2004) and eNOS (this study) and thus decrease NO amounts, in line with their well-documented anti-angiogenic effects (this study and Ashino et al., 2003; Lin et al., 2003; Nishimura et al., 1999; Polytarchou and Papadimitriou, 2004).

In conclusion, this study suggests that superoxide anion and hydrogen peroxide are putative inducers of endothelial cell functions in vitro, possibly through up regulation of eNOS activity that leads to increased production of endogenous NO.

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