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Antioxidants Inhibit Angiogenesis *In Vivo* through Down-regulation of Nitric Oxide Synthase Expression and Activity

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Although reactive oxygen species (ROS) participate in many cellular mechanisms, only few data exist concerning their involvement in physiological angiogenesis. The aim of the present work was to elucidate possible mechanisms through which ROS affect angiogenesis *in vivo*, using the model of the chicken embryo chorioallantoic membrane (CAM). Superoxide dismutase (SOD) and its membrane permeable mimetic tempol, dose dependently decreased angiogenesis and down-regulated inducible nitric oxide synthase (iNOS) expression and nitric oxide (NO) production. The NADPH oxidase inhibitors, 4-(2-amino-ethyl)-benzenesulfonyl fluoride (AEBSF) and apocynin, but not allopurinol, also had a dose dependent inhibitory effect on angiogenesis and NO production *in vivo*. Catalase and the intracellular hydrogen peroxide (H_2O_2) scavenger sodium pyruvate decreased, while H_2O_2 increased in a dose-dependent manner the number of CAM blood vessels, as well as the expression and activity of iNOS. Dexamethasone, which down-regulated NO production by iNOS and L-NAME, but not D-NAME, dose dependently decreased angiogenesis *in vivo.* These data suggest that antioxidants affect physiological angiogenesis *in vivo*, through regulation of NOS expression and activity.

Keywords: Angiogenesis; Hydrogen peroxide; NADPH oxidase; Nitric oxide; Reactive oxygen species; Superoxide

INTRODUCTION

Reactive oxygen species (ROS) are produced by several intracellular systems, such as cyclooxygenases, lipoxygenases, cytochrome P450, mitochondrial respiration, NADPH oxidase and xanthine oxidase and are generated under various physiological and pathological conditions, such as inflammation, ischemia, reperfusion, sepsis and ionizing irradiation.[1] Major ROS are superoxide anion ($O_2^{-\cdot}$), hydroxyl radicals ($OH^{\cdot}$) and hydrogen peroxide (H_2O_2). Although under oxidative stress, ROS have cytotoxic effects, evidence also exists on their implication in cell signalling.[1–4] For example, ROS increase protein phosphorylation[5,6] and activate the transcription factor NF-κB.[3,7,8] In the same line, H_2O_2 produced by macrophages, activates NF-κB and leads to enhanced inducible nitric oxide synthase (iNOS) expression and nitric oxide (NO) production.[9] Finally, there is increasing evidence that ROS are involved in the mitogenic signal transduction cascades initiated by several growth factors.[10,11]

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.[12] Many molecules act as mediators of angiogenesis, among them NO, which has been shown to possess both pro- and antiangiogenic properties.[13–15] This discrepancy has been attributed to the different amounts of NO produced in each assay and/or the type of NO involved in these effects.

ROS have recently been implicated in the regulation of tube formation by endothelial cells *in vitro*.[16] ROS stimulate vascular endothelial growth factor (VEGF) release[17,18] and mediate activation of mitogen activated protein kinase,

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a signalling pathway for VEGF.[3,10] Moreover, VEGF-induced migration, signalling and angiogenesis were suppressed by NADPH oxidase inhibitors.[19,20]

In order to clarify the role of ROS in physiological angiogenesis *in vivo*, we investigated whether ROS scavengers or inhibitors of ROS production affect angiogenesis in the *in vivo* chicken embryo CAM model of angiogenesis. Moreover, we investigated the effect of the same antioxidants on the iNOS expression and activity.

MATERIALS AND METHODS

The tested agents (Sigma, Athens, Greece) were:

- Superoxide dismutase (SOD) and its membrane permeable analogue 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl (tempol), which remove $O_2^{-\cdot}$.
- Apocynin and 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), which are NADPH oxidase inhibitors.
- Allopurinol, a xanthine oxidase inhibitor.
- Hydrogen peroxide.
- Catalase and sodium pyruvate, which detoxify H_2O_2 to H_2O extracellularly and intracellularly, respectively.
- Dexamethasone, an inhibitor of iNOS mRNA transcription.
- N^{ω}-nitro-L-arginine methyl ester (L-NAME), a non-specific inhibitor of NOS activity and its inactive analog D-NAME.

CAM Assay

The *in vivo* chicken embryo CAM angiogenesis model was used, as previously described.[21] Leghorn fertilized eggs (Pindos, Greece) were incubated for 4 days at 37°C, when a window was opened on the egg shell, exposing the CAM. The window was covered with tape and the eggs were returned to the incubator. The tested agents were diluted in 20 μl H_2O and applied on an area of 1 cm^2 (restricted by a plastic ring) of the CAM at day 9 of embryo development. Application of tested agents in liquid form avoids the misinterpretation of focal inflammation or toxic effects that are frequently observed when agents are applied on plastic discs or filters.[22,23] In order to evaluate the effect of each substance on angiogenesis, 48 h after treatment and subsequent incubation at 37°C, CAMs were fixed *in situ*, excised from the eggs, placed on slides and left to air-dry. Pictures were taken through a stereoscope equipped with a digital camera and the total length of the vessels was measured using image analysis software, as previously described.[21] Assays for each test sample were carried out three times and each experiment included 8–10 eggs per data point. Results are expressed as percentage of the control, untreated CAMs, which are a different group of eggs. The percentage is calculated by dividing the value of each treated egg by the mean value of the untreated eggs.

For the biochemical studies, the tested agents were applied on the CAM and after different time periods of incubation at 37°C, the CAMs were excised from the eggs and processed as described below.

Western Blot Analysis of iNOS

In CAM paraffin sections, iNOS was detected only on blood cells.[24] Therefore, in order to increase the sensitivity of the assay on the determination of iNOS protein levels, we performed Western blot analysis of iNOS on CAM blood cell lysates. Blood cells were lysed in lysis buffer (50 mM Tris–HCl, pH 7.4, 0.25% SDS, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/ml aprotinin, 1 mM Na_3VO_4) with agitation for 4 h at 4°C. The lysates were then centrifuged at 20,000 *g* for 30 min at 4°C. Total protein concentration was determined in the supernatants, using the Bradford method.[25] Equal amounts of total protein were loaded on 7.5% SDS-PAGE mini gels, analyzed and transferred to Immobilon P membranes (Millipore). Blocking was performed by incubating the PVDF 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-iNOS antibody at a dilution of 1:1000 (Upstate Biotechnologies, NY, USA) in 3% (w/v) non-fat dry milk in TBS containing 0.05% Tween-20 (TBS-T) overnight at 4°C under continuous agitation and then with horseradish peroxidase conjugated goat antirabbit IgG (Sigma, Greece) diluted 1:2500 in 3% (w/v) non-fat dry milk in TBS-T for 1.5 h at room temperature under continuous agitation. Detection of immunoreactive bands was performed by Super Signal West Pico chemiluminescence substrate (Pierce, USA), according to the manufacturer's instructions. The protein levels that corresponded to the immunoreactive bands were quantified using the image PC analysis software (Scion Corporation, Frederick, MD).

Measurement of Nitrite Levels

CAMs of at least 5 eggs were dissected, cut into small pieces and washed 5 times with sterilized PBS pH 7.4. They were then placed in 24-well plates (each well contained approximately 1 mg protein) in Ham's F10 medium that did not contain phenol red. The tested agents were added at the indicated concentrations and the CAMs were incubated for 24 h at 37°C and 5% CO_2. After completion of the incubation,

the samples were collected and centrifuged at 7000*g* for 2 min in a microcentrifuge. Nitrites were measured in the supernatant with the use of the Griess reagent, as previously described.[26] Total protein amounts were measured in the tissue pellet using the Bradford assay and results are expressed as nmoles NO_2^-/mg protein.

Statistical Analysis

The significance of variability between the results from each group and the corresponding control was determined by unpaired *t*-test or ANOVA. Each experiment included triplicate measurements for each condition tested. All results are expressed as mean ± SEM from at least three independent experiments.

RESULTS

Effect of Antioxidants on CAM Angiogenesis

Initially, we investigated whether antioxidants affect physiological angiogenesis in the *in vivo* chicken embryo CAM model of angiogenesis. At the 9th day of development, the tested agents were applied on the CAM and the vascular density was measured 48 h later, as described in Methods section. As shown in Fig. 1, SOD, tempol, AEBSF and apocynin decreased the number of CAM vessels in a dose dependent manner. Allopurinol did not affect angiogenesis at any of the doses tested (0.2 and 1 μmol/cm^2, data not shown). Higher doses could not be applied because of solubility restrictions.

Catalase and sodium pyrouvate also decreased angiogenesis in a dose dependent manner, while H_2O_2 dose-dependently increased angiogenesis in the chicken embryo CAM (Fig. 2).

The decrease in the number of CAM vessels in all the above cases was not due to toxicity, as verified on CAM paraffin sections stained with eosin-hematoxylin or treated with a kit for *in situ* detection of apoptosis (data not shown).

Effect of Antioxidants on iNOS Expression and Activity

We tested the effect of the antioxidants on iNOS expression and NO production in the CAM. iNOS is the only NOS isoform detected in the CAM[26]

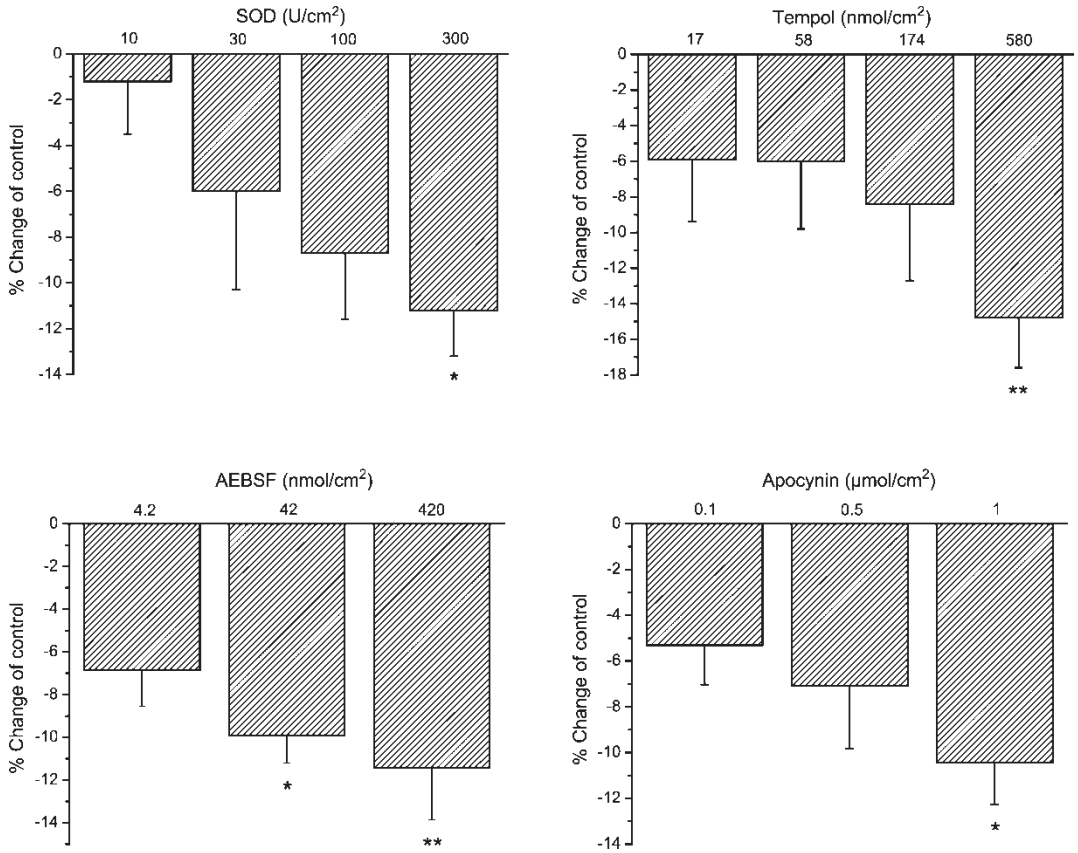

FIGURE 1 Effect of superoxide scavengers and NADPH inhibitors on physiological *in vivo* angiogenesis in the chicken embryo CAM. Various amounts of the tested agents in the same final volume of 20 μl were applied on an area of 1 cm^2 restricted by a plastic ring, at CAMs of day 9, as described in "Materials and Methods" section. After 48 h of incubation at 37°C, the CAMs were fixed, excised from the eggs, photographed and the total length of the vessel network was measured using image analysis software. Results are expressed as mean ± SEM of the percentage change of the number of vessels in treated compared to untreated tissue (control). Asterisks denote a statistically significant difference (unpaired *t*-test) from the control (*$P < 0.05$, **$P < 0.01$).

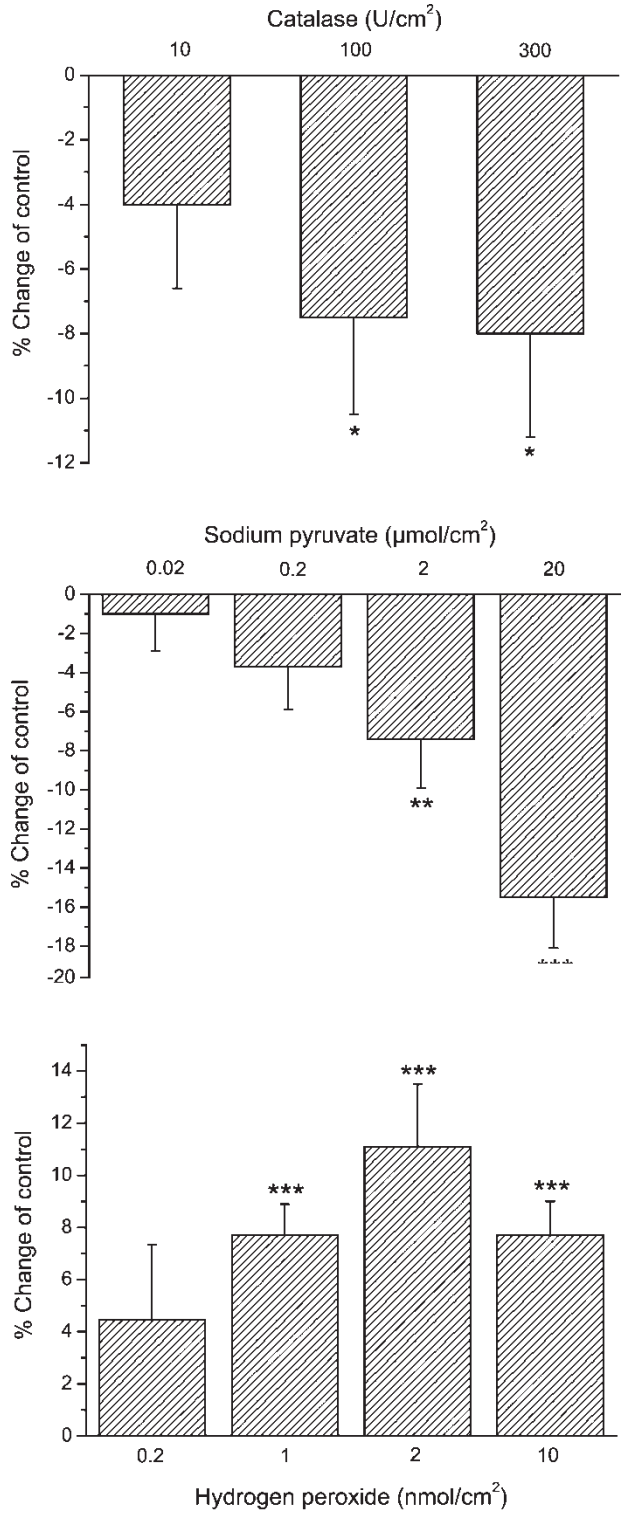

FIGURE 2 Effect of agents that affect hydrogen peroxide amounts on physiological *in vivo* angiogenesis in the chicken embryo CAM. Various amounts of the tested agents in the same final volume of 20 μl were applied on an area of 1 cm^2 restricted by a plastic ring, at CAMs of day 9, as described in "Materials and Methods" section. After 48 h of incubation at 37°C, the CAMs were fixed, excised from the eggs, photographed and the total length of the vessel network was measured using image analysis software. Results are expressed as mean ± SEM of the percentage change of the number of vessels in treated compared to untreated tissue (control). Asterisks denote a statistically significant difference (unpaired *t*-test) from the control (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

and is immunolocalised on CAM blood cells.[24] As shown in Fig. 3, all the tested antioxidants, except allopurinol, significantly decreased the protein amounts of iNOS, 24 h after their application. In line with this, nitrite production was significantly decreased 24 h after application of all the antioxidants, except allopurinol (Fig. 4). H_2O_2 significantly increased both expression (Fig. 3) and activity (Fig. 4) of iNOS.

Effect of NOS Inhibitors on CAM Angiogenesis

In order to see if the effect of the antioxidants on iNOS expression and NO production could be related to their effect on angiogenesis, we tested the effect of two NOS inhibitors on angiogenesis in the chicken embryo CAM. As shown in Fig. 5, dexamethasone, an inhibitor of iNOS mRNA transcription, decreased the number of vessels in a dose dependent manner. L-NAME, a non specific NOS antagonist, also inhibited angiogenesis in a dose dependent manner. D-NAME, the inactive analogue of L-NAME, had no effect on angiogenesis in the chicken embryo CAM (data not shown). The effect of both NOS inhibitors was not due to toxicity, as verified on CAM paraffin sections stained with eosin-hematoxylin or treated with a kit for *in situ* detection of apoptosis (data not shown).

As shown in Fig. 6, dexamethasone significantly down-regulated, while L-NAME or D-NAME had no effect on iNOS protein levels. Nitrite concentration was significantly decreased after treatment of the CAM with dexamethasone or L-NAME but not D-NAME (Fig. 7).

DISCUSSION

Although ROS are usually linked to cytotoxic effects, evidence also exists on their implication in cell signaling.[1,2,7,8] In the present study, we found that ROS play a significant role in regulating physiological angiogenesis *in vivo*, through regulation of iNOS expression.

In several *in vitro* studies, it has been shown that ROS or H_2O_2 induce tubular morphogenesis of endothelial cells.[16,28–30] It has also been suggested that ROS may trigger intracellular signalling that might accelerate angiogenesis *in vivo*.[29,30] This last notion is supported by the present study, which suggests that ROS affect physiological angiogenesis *in vivo*. In the chicken embryo CAM, there are several growth factors, such as bFGF[31] and/or VEGF,[27] which are involved in angiogenesis of the tissue. It has been shown that ROS stimulate the release of VEGF *in vitro*[18] or mediate VEGF-induced signaling. [11,19,20] Whether ROS affect the expression of these or other growth factors or the signaling

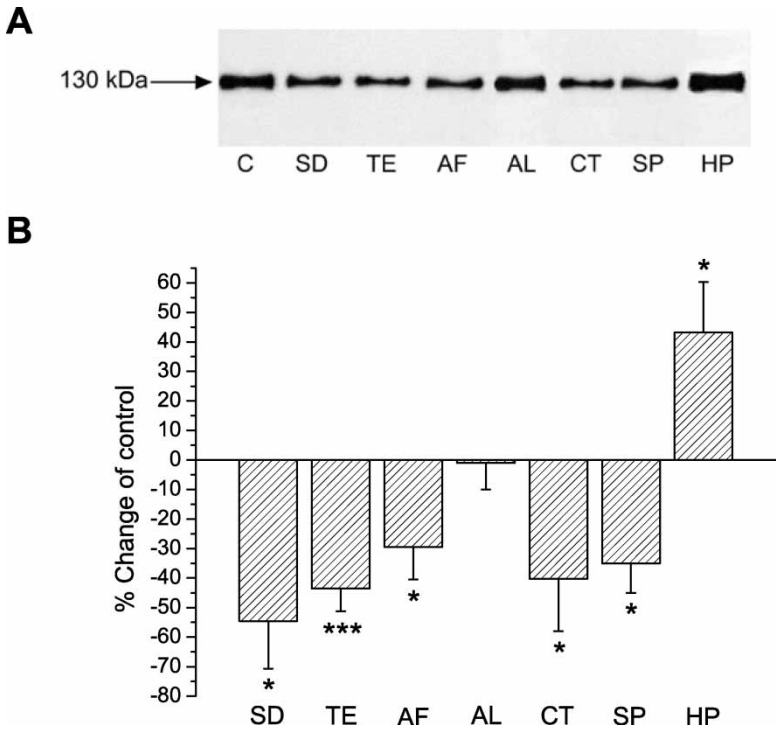

FIGURE 3 Effect of antioxidants on iNOS protein levels. **A** Western blot analysis for iNOS 24 h after application of the tested agents on the chicken embryo CAM. C, control; SD, SOD 300 U/cm^2; TE, Tempol 580 nmol/cm^2; AF, AEBSF 420 nmol/cm^2; AL, Allopurinol 1 μmol/cm^2; CT, Catalase 100 U/cm^2; SP, Sodium pyruvate 2 μmol/cm^2; HP, Hydrogen peroxide 2 nmol/cm^2. Representative picture of six independent experiments. **B** The protein amounts that corresponded to iNOS immunoreactive band were quantified using image analysis software. Results are expressed as mean ± SEM of the percentage change of iNOS protein amounts in treated compared to untreated tissue (control). Asterisks denote a statistically significant difference (unpaired *t*-test) from the control (*$P < 0.05$, ***$P < 0.001$).

pathways that the latter mediate in the CAM, is not known and is under further investigation.

In the present study, we showed that scavengers for superoxide and H_2O_2 down regulated expression and activity of iNOS *in vivo*. This is in line with previous *in vitro* studies showing that ROS enhance,[9] while the endogenous antioxidant ascorbate inhibits[32] iNOS expression and activity. Down-regulation of iNOS seems to be at least one of the mechanisms through which antioxidants regulate angiogenesis *in vivo*. This is supported by the fact that inhibitors of iNOS also decreased angiogenesis in the chicken embryo CAM in a dose dependent manner and to comparable levels with antioxidants. Our data also suggest that iNOS may play a significant role in the formation and/or the stability of blood vessels under physiological angiogenesis *in vivo*, in line with its role in tumor angiogenesis.[33,34]

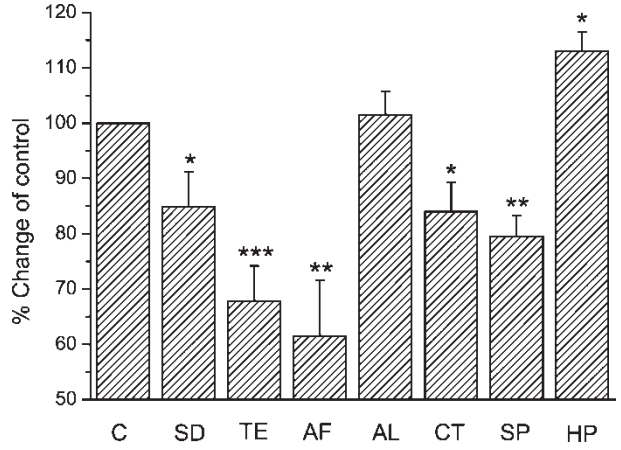

FIGURE 4 Effect of antioxidants on NO_2^- production. Nitrites were measured 24 h after application of the tested agents on the chicken embryo CAM, as described in "Materials and Methods" section. C, control; SD, SOD 500 U/ml; TE, Tempol 966 nmol/ml; AF, AEBSF 700 nmol/ml; AL, Allopurinol 1.67 μmol/ml; CT, Catalase 167 U/ml; Sodium pyruvate 3.33 μmol/ml; HP, Hydrogen peroxide 3.33 nmol/ml. Results are expressed as mean ± SEM of the percentage change of nitrite amounts treated compared to untreated tissue (control, 100%), of six independent experiments performed in quadruplicates. Asterisks denote a statistically significant difference (unpaired *t*-test) from the untreated tissue (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

A major source of endothelial superoxide generation is NADPH oxidase,[35] which is required for endothelial cell proliferation and migration, induced or not by VEGF.[19,20] The inhibitors of NADPH oxidase inhibited HUVEC proliferation and migration[19] and moreover, inhibited physiological angiogenesis *in vivo* (this study). The results from our study support the notion that NADPH oxidase plays a significant role in superoxide generation *in vivo*, but cannot exclude the involvement of other intracellular systems that produce superoxide in the CAM. Allopurinol had no effect, which suggests that xanthine oxidase activity is not involved in physiological angiogenesis, in line with the fact that

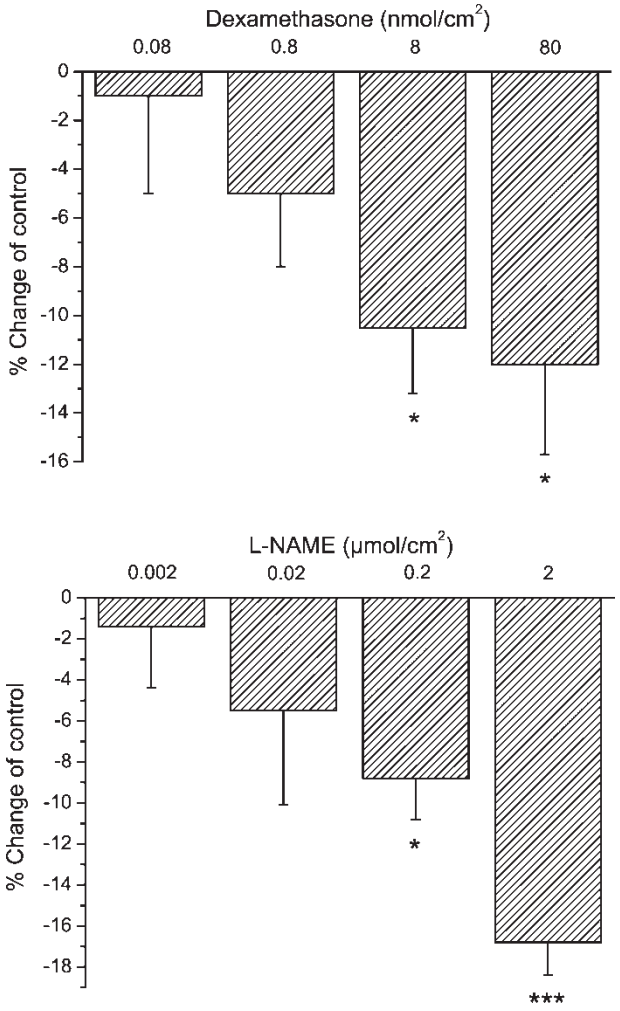

FIGURE 5 Dexamethasone and L-NAME decreased the number of vessels in the chicken embryo CAM. Various amounts of dexamethasone or L-NAME in the same final volume of 20 μl were applied on an area of 1 cm^2 restricted by a plastic ring, at CAMs of day 9, as described in "Materials and Methods" section. After 48 h of incubation at 37°C, the CAMs were fixed, excised from the eggs, photographed and the total length of the vessel network was measured using image analysis software. Results are expressed as mean ± SEM of percentage change of the number of vessels in treated compared to untreated tissue (control). Asterisks denote a statistically significant difference (unpaired *t*-test) from the control (*$P < 0.05$, ***$P < 0.001$).

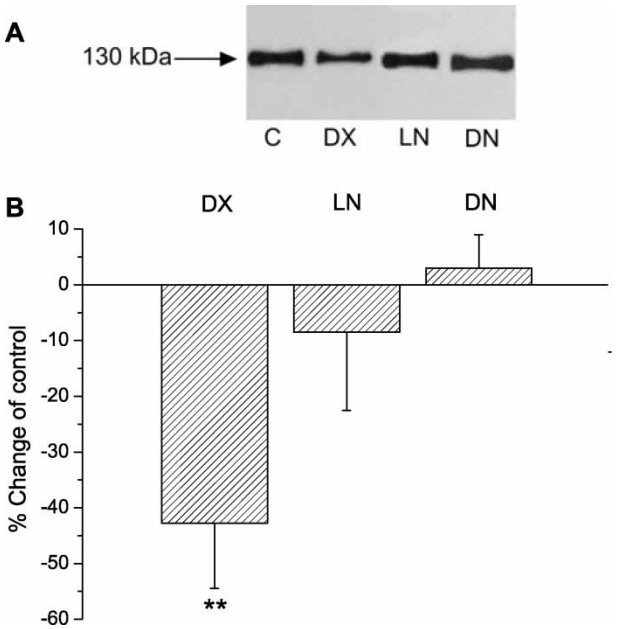

FIGURE 6 Effect of NOS inhibitors on iNOS protein levels. A. Western blot analysis for iNOS 24 h after application of the tested agents on the chicken embryo CAM. C, control; DX, Dexamethasone 80 nmol/cm^2; LN, L-NAME 2 μmol/cm^2; DN, D-NAME 2 μmol/cm^2. Representative picture of five independent experiments. B. The protein amounts that corresponded to iNOS immunoreactive band were quantified using image analysis software. Results are expressed as mean ± SEM of the percentage change of iNOS protein amounts in treated compared to untreated tissue (control). Asterisks denote a statistically significant difference (unpaired *t*-test) from the control (**$P < 0.01$).

xanthine oxidase accounts for only a minor proportion of total ROS production under normal conditions.[1]

Dexamethasone *in vivo* inhibited angiogenesis and NO production, to a similar degree with L-NAME. The only NOS isoform detected in the CAM so far has been iNOS,[24,26] which is inhibited by dexamethasone.[36,37] Although these data are in line with an absence of eNOS in the chicken embryo CAM and despite the fact that we haven't been able to detect it so far, the possibility of its existence in the CAM cannot be excluded. It is possible that eNOS is present in very small amounts compared to iNOS and/or its activity is also much lower, so that it cannot be detected by the methods used up to date.

All data in this study support a proangiogenic role of NO *in vivo* under physiological conditions. This correlates with our previous *in vivo* data showing

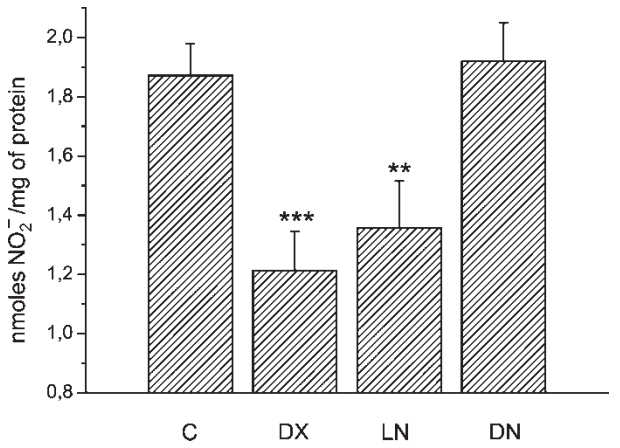

FIGURE 7 Effect of NOS inhibitors on NO_2^- production. Nitrites were measured 24 h after application of the tested agents on the chicken embryo CAM, as described in "Materials and Methods" section. C, control; DX, Dexamethasone 13.3 nmol/ml; LN, L-NAME 1 μmol/ml; DN, D-NAME 1 μmol/ml. Results are expressed as nmoles NO_2^-/mg of total protein ± SEM of six independent experiments performed in quadruplicates. Asterisks denote a statistically significant difference (unpaired *t*-test) from the untreated tissue (**$P < 0.01$, ***$P < 0.001$).

that amifostine, a radioprotectant and ROS scavenger, inhibited iNOS expression and angiogenesis in the chicken embryo CAM.[27] It is also in agreement with the increasing evidence in the literature that NO correlates with increased angiogenesis and tumor growth and aggressiveness.[13,34] The influences generated by NO can be divided into cGMP- and non-cGMP mediated effects, e.g. nitrotyrosine production.[38] The proangiogenic effects of NO on endothelial cells seem to be mediated by cGMP.[13] Whether its proangiogenic effect on the chicken embryo CAM is cGMP dependent or independent is not known and is currently being investigated.

In conclusion, this study suggests that superoxide and H_2O_2 are putative inducers of angiogenesis *in vivo*, possibly through up regulation of iNOS and increased production of endogenous NO.

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