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ORIGINAL ARTICLE

Exogenous nitric oxide regulates activity and synthesis of vascular endothelial nitric oxide synthase

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ABSTRACT

Background Nitric oxide (NO) – a major signalling molecule of the vascular system – is constitutively produced in endothelial cells (EC) by the endothelial NO synthase (eNOS). Since a reduced NO synthesis is an early sign of endothelial dysfunction and NO delivering drugs are used to substitute the impaired endothelial NO production, we addressed the effect of exogenous NO on eNOS in human umbilical venous endothelial cell cultures.

Materials and methods The synthetic NO donor DETA/NO (trade name, but in the following we refer to detNO), that releases NO in a strictly first order reaction with a half life of 20 h, was used in our experiments.

Results Short-term (20–30 min) detNO treatment of EC increases the Ser^{1177} phosphorylation of the constitutively expressed endothelial NOS and the production of endogenous NO generated by eNOS from [^{3}H]arginine. The phosphorylation of eNOS is Akt-dependent and completely reverted by the phosphatidylinositol-3 kinase (PI-3K) inhibitor LY294002. A prolonged continuous exposure of EC to detNO 150 μmol L^{-1} over a period of 24–48 h causes a reversible cell cycle arrest at G_1-phase associated with a larger cell volume and increased cell protein content (hypertrophic phenotype of EC). The eNOS protein and mRNA of the hypertrophic cells and the generation of endogenous NO are reduced but eNOS phosphorylation could still be elevated by stimulation with vascular endothelial growth factor.

Conclusions Our data explain clinical studies describing a short-term but not a long-term benefit of NO treatment for patients with cardiovascular risk factors. The results could be a rational approach to develop a generation of NO donors accomplishing a retarded release from NO donors that mimic the low continuous pulsatile stress-induced release of endogenous NO.

Keywords cell culture, endothelial dysfunction, endothelial nitric oxide synthase, synthetic NO donors.

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Introduction

Nitric oxide (NO) is an important messenger that regulates numerous physiological functions and also participates in the pathogenesis of various diseases (for review see [1]). In vascular endothelium, NO is constitutively generated by the isoform 3 of the NO synthases (eNOS). NO is a pleiotropic agent that acts via cGMP-dependent or cyclic guanosine monophosphate (cGMP)-independent pathway, in part by S-nitrosylation of intracellular or extracellular proteins [2–4] or by inhibiting intraendothelially generated superoxide anions [5].

Risk factors for arteriosclerosis, such as dyslipoproteinaemia, hypertension, diabetes mellitus and smoking are assumed to be associated with endothelial dysfunction, a condition/setting with reduced endothelial generation or bioavailability of NO [6]. Endothelial dysfunction is probably due to eNOS uncoupling (decreased NO synthesis) and/or oxidative inactivation of NO by superoxide (decreased NO bioavailability) [7]. Possible diagnostic techniques are impaired endothelium-dependent dilatation of coronary artery and reduced flow-mediated dilatation measured by ultrasonography [8,9].

NO-delivering drugs (NO donors) are used for their potential therapeutic benefit in coronary heart disease risk patients [10] by increasing coronary blood flow and dilating coronary arteries. The superoxide anion derived from the NADPH oxidases and/or xanthine oxidase may combine with NO generated by a still functional eNOS. This would lead to increased formation of peroxynitrite that has been shown to oxidize the essential eNOS

co-factor tetrahydrobiopterin to biologically inactive products thereby leading to uncoupling of eNOS and production of high amounts of reactive oxygen species instead of NO [11,12]. Especially organic nitrates have been described to cause vascular reactive oxygen species formation which was associated with endothelial dysfunction (cross-tolerance) and nitrate tolerance [13]. Several previous studies have described the action of NO donors on vascular smooth muscle cells [14–16]. The pathway leading to NO formation differs among individual NO donor classes: indirect NO donors such as organic nitrates (nitroglycerol, isosorbide mononitrate, isosorbide dinitrate) require enzymatic catalysis, other NO donors require interaction with thiols to release NO, some have to undergo oxidation or reduction. In contrast, direct NO donors generate NO non-enzymatically. Examples are nicorandil, SIN-1 (the active metabolite of molsidomine) and the group of 1-substituted diazen-1-ium-1,2-diolates that releases NO spontaneously with a half life from minutes to hours [17].

For our experiments we have selected the nitric oxide donor DETA/NO (trade name), chemical name see 'Materials and methods'. This compound, in the following detNO, belongs to the class of direct NO donors, is stable in 0·01 mol L^{-1} NaOH, and can be stored at 0 °C for 24 h. In culture medium at pH 7·4, detNO spontaneously releases NO with a half life of about 20 h at 37 °C in a strictly first order reaction [17–19], thereby disintegrating to two NO and diethylentriamine. Diethylentriamine, the by-product of detNO disintegration, is known to be non-effective. [16,17]. DetNO has been successfully used by previous investigators [14,20,21].

We addressed the question of which effects are exerted by short-term and prolonged exposure of vascular endothelial cells to exogenously NO delivered by detNO and found a stimulating as well as an inhibiting effect on eNOS. The use of other nitric oxide donors such as SNAP (S-nitroso-N-acetylpenicillamine) or the molsidomine derivative SIN-1 (3-morpholino-sydonimine) is complicated by the need for metabolic activation and their short half life (< 12 h). Therefore they were not used in this study.

Materials and methods

Reagents

DETA/NO (trade name), chemical name: (Z)-1-[2-(2-Aminoethyl)–N–(2-aminoethyl) amino] (diazen-1-ium-1,2-diolate) is a product of Alexis Biochemicals, Q-BIOgene-Alexis GmbH, Grünberg, Germany. In the following the abbreviation detNO is used throughout. Rec. hum. vascular endothelial growth factor (VEGF) was purchased from R & D Systems (# 293-VE), Wiesbaden, Germany. [Methyl-^{3}H]thymidine (spec. activity 1·3 TBq $mmol^{-1}$) was obtained from ICN Biomedicals GmbH, Eschwege, Germany. Primer pairs were designed for DNA sequences of eNOS (NCBI: NM_000603, Fw: GCGGCTGCATGACATTGAG, Re:TCGCGGTAGAGATGGTCAAGTT), and p21 (NCBI: NM_00389, Fw: GACACCACTGGAGGGTGACT, Re: CAGGTCCACATGGTCTTCCT).

Cell culture

Human umbilical venous endothelial cells (HUVEC) were harvested, characterized and cultured in RPMI 1640 medium with supplements (standard medium) as described [22]. The detNO effect was determined 24–96 h after seeding and incubation was continued in the presence or absence of 50–200 μmol L^{-1} detNO for the specified time. In parallel long-term experiments treatment of the cells with 150 μmol L^{-1} detNO for 48 h was followed by 1 h stimulation by 50 ng mL^{-1} VEGF of control and detNO treated cells. Then, medium and cells were processed for analysis according to the described protocol. Diethylentriamine (DETA), the reaction product of detNO, known to be non-effective [16,17] was confirmed by our experiments and used in our experiments as a negative control. Cell growth and cell growth inhibition was estimated on the basis of [^{3}H]thymidine incorporation and cell counting by standard methods as previously described [23].

cDNA electrophoresis and real-time RT-PCR

Total RNA was isolated by Qiagen RNeasy kit and further purified by DNase digestion. 2 μg of total RNA was then reversely transcribed into cDNA using forward and reverse oligonucleotide primers for PCR as previously described [24]. PCR products were visualized by agarose gel electrophoresis or by real-time RT-PCR on the ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems, Darmstadt, Germany) and the QuantiTect SYBR®Green PCR kit (Qiagen, Hilden, Germany) as previously described [25].

Immunoblotting

The total protein content was determined using the BCA Assay (Pierce, Rockford, IL, USA). For immunoblot analysis the cell layer was washed, lysed and subjected to SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis). Proteins were transferred to PVDF (polyvinyldifluoride) membranes. Bands of eNOS and phosphorylated eNOS, Akt and phospho-Akt were detected with the cognate primary antibodies (Akt polyclonal AB, Cell Signalling #9272 (New England BioLabs, Frankfurt/Main, Germany); p-Akt (Ser^{473}) rabbit mAB, Cell Signalling #4058; eNOS, Sigma N-3893 (Sigma-Aldrich, Taufkirchen, Germany); p-eNOS (Ser^{1177}), Cell Signalling #9571; p-eNOS (Thr^{495}), BD Transduction Laboratory #612706 (BD Transduction Laboratory, Heidelberg, Germany); iNOS mAB, R & D Systems # MAB9502 (R & D Systems, Wiesbaden, Germany); Actin rabbit mAB, Cell Signalling #4970). The membrane was washed and incubated with peroxidase labelled antirabbit or mouse secondary antibody.

Antigens were visualized by enhanced chemiluminescence (ECL, Amersham/GE Healthcare, Freiburg, Germany).

Metabolic labelling

For metabolic labelling of eNOS Tran^{35}S-Label Met/Cys (specific activity 43·5 TBq/mmol) from ICN Biomedicals GmbH (Eschwege, Germany) was used as a precursor. Cell protein was labelled for 48 h in standard medium containing 10 μCi mL^{-1} [4,5^{-3}H]leucine. Labelled cells were lysed in 200 μL 1% SDS. The protein content and the ^{3}H-radioactivity were determined by the BCA-assay and scintillation counting.

NOS activity assay

Total NOS activity in HUVEC was determined by measuring the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline using the NOSdetect®Assay Kit (Stratagene, Heidelberg, Germany) according to the instructions of the producer. Briefly, HUVEC were washed after incubation with detNO at the specified time, centrifuged to pellet the cells and homogenized to disrupt the cells. After high speed centrifugation at 4 °C for 5 min 10 μL of the supernatant (normalized for protein content) was incubated in 40 μL reaction mixture containing 10 mmol L^{-1} NADPH (Sigma), L-[2,3,4,5–^{3}H]-arginine (1 μCi μL^{-1}) (Amersham/GE-Healthcare, Code TRK 698, UK), and 6 mM $CaCl_2$. Incubation of the citrulline assay reaction was carried out for 30 min at 37 °C. Blank values included 1 mM N-nitro-L-arginine methyl ester HCl (L-NAME) (a competitive NOS inhibitor). To measure the ratio of [^{3}H]arginine to [^{3}H]citrulline, 400 μL elution buffer was added to each spin column and centrifuged in a microcentrifuge at full speed for 1 min. Each eluate was quantified in a liquid scintillation counter.

Cell volume measurement

Casy® technology (I Model TT) (Innovatis A.G., Reutlingen, Germany) was used for cell volume measurement according to the description of the manufacturer. Cells suspended in a conductive isotonic buffer solution are drawn through a measuring pore defined in size, separated by two platinum electrodes producing a low voltage field cycled with 1 MHz. Each cell passing the measuring pore generates an individual electric pulse that is proportional to the individual cell volume. The electric pulses sampled with 1 MHz are analysed by amplitude, pulse width, time course and resulting pulse area. The analysed pulse areas of each cell are accumulated in a multichannel analyser. The resolution of the pulse area analysis ensures ultra precise cell volume measurement. Results are expressed in fL/cell (femtoliter/cell).

Other methods

Apoptosis and necrosis of EC were monitored by the cellular DNA fragmentation ELISA (enzyme-linked immunosorbent assay) and cell death detection ELISA^{Plus} (Roche Diagnostics GmbH Mannheim, Germany). Densitometric measurements of electrophoretically separated protein and cDNA bands were carried out by the NIH (National Institute of Health) image and analysis program (http://rsb.info.nih.gov/ij/; accessed 12 June 2005). Antiproliferation effects were assayed by a standard method as previously described [23].

Statistics

Results are expressed as means± standard deviation (SD) of the specified number of experiments carried out on different cultures in duplicates or triplicates. Statistical significance was assessed using the non-parametric Wilcoxon-test for paired samples or Student's paired *t*-test. $P < 0{\cdot}05$ (*) was accepted as significant, $P < 0{\cdot}001$ (**) as highly significant.

Results

eNOS alterations by short-term exposure to detNO

EC express eNOS constituently and continuously and regulate its activity by phosphorylation of serine^{1177} residue. We found that in cultured cells the phosphorylation can be achieved by exposure of EC to 150 μmol L^{-1} detNO in short-term experiments. Western blot analysis (Fig. 1a) shows a clear increase of eNOS phosphorylation at Ser^{1177} after a short lag phase, detectable 20 min after detNO addition. Densitometric measurements indicate an increase of phosphorylation by 100–150% ($n = 4$, $P < 0{\cdot}001$). No significant changes of the eNOS protein content (Fig. 1a) were expected during this time. The phosphorylation is mediated by the protein kinase Akt that is converted into p-Akt within 10 min after addition of detNO in a concentration-dependent manner and was found highly significant by densitometry (Fig. 1b). The phosphorylated Akt increases in turn Ser^{1177} phosphorylation of eNOS. This phosphorylation cascade Akt → Ser^{473} p-Akt → Ser^{1177} p-eNOS could be reverted by pre-incubation of the cells with the PI-3 kinase inhibitor LY294002 (5 μmol L^{-1}) that prevents phosphorylation of both Akt and eNOS (Fig. 1c). Non-phosphorylated Akt and eNOS served as controls.

Thr^{495} is constitutively phosphorylated in all endothelial cells [26] and is a negative regulatory site, i.e. phosphorylation leads to a decrease of eNOS activity. Stimulation of endothelial cells with agonists does not result in a change in the phosphorylation of Thr^{495} [26]. In our short-term studies detNO causes no unambiguous changes in Thr^{495} phosphorylation in response to detNO (Fig. 1a).

Since the measurement of Ser^{1177} and Thr^{495} phosphorylation give only indirect information about changes of eNOS activity we determined the net effect of the release of endogenous NO in response to exogenous detNO using L-[2,3,4,5–^{3}H]arginine as indicator. The eNOS-mediated conversion of [^{3}H]arginine to NO and [^{3}H]citrulline was measured and the results are given in [^{3}H]citrulline equivalents (Fig. 1d). This result confirms the data of Fig. 1a in so far as a statistically significant increase of endogenous

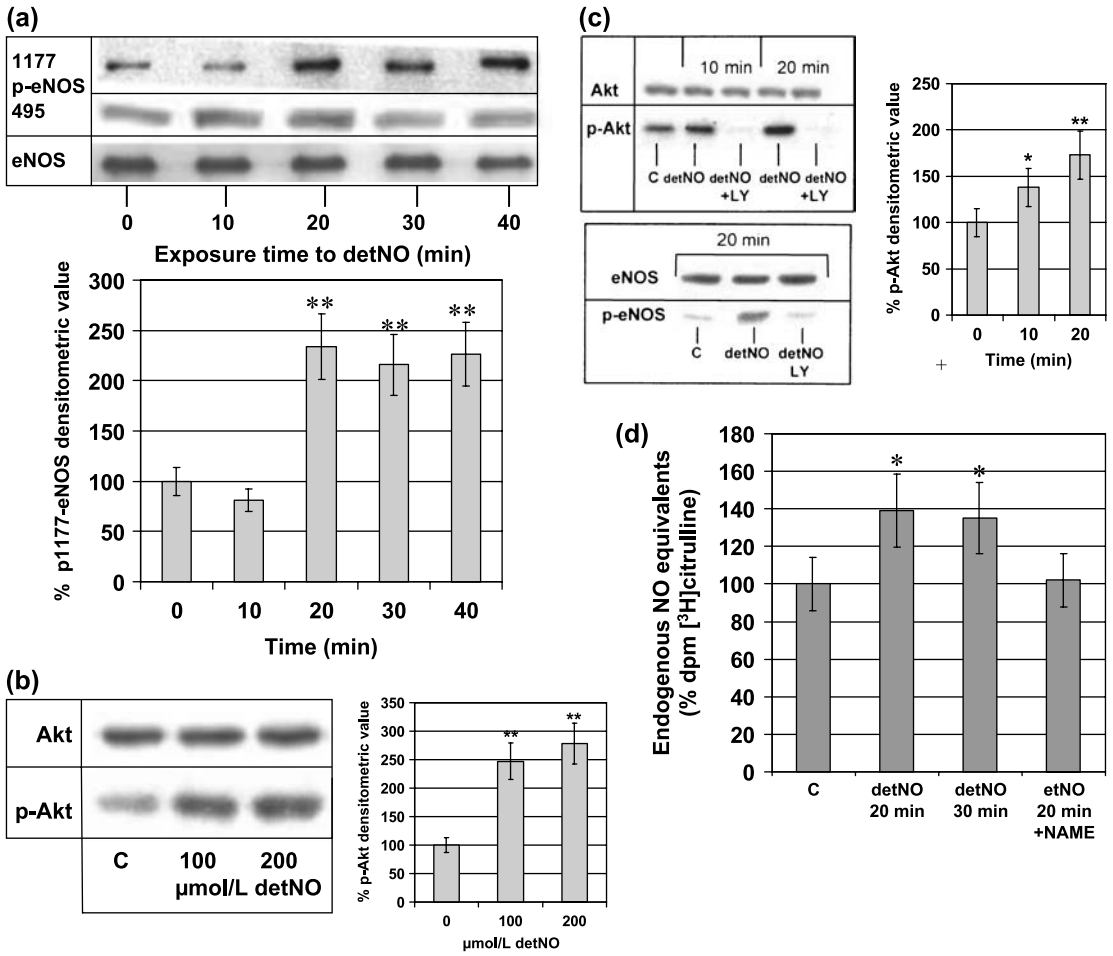


Figure 1 (a) Nitric oxide (detNO) effect on endothelial NO synthase (eNOS) phosphorylation (p-eNOS) at Ser^{1177} and Thr^{495}. Confluent cells (5–6 × 10^5 cells/dish) were exposed to 150 μmol L^{-1} detNO for the specified time. Cells were lysed and cell proteins were separated on 8% SDS-PAGE. Western blot analysis was performed with rabbit monoclonal antiphospho-eNOS antibodies against Ser^{1177} and Thr495 and analysed by densitometry. $n = 4$; P (10 min vs. 20/30 min) < 0·001. (b) Effect of detNO on Akt phosphorylation (p-Akt) at Ser473. Confluent cells were incubated in serum free medium SFM™ (Gibco) with 100 and 200 μmol L^{-1} detNO for 10 min each. For Western blot analysis antiphospho-Akt antibodies were used. $n = 3$; P (control vs. 100/200 μmol L^{-1} detNO) < 0·05. (c) NO-induced phosphorylation of Akt and eNOS is reversible. Cells were preincubated for 15 min with 5 μmol L^{-1} LY294002 and then treated with detNO for 10 and 20 min. Primary antibodies against p-Akt and p-eNOS were used. $n = 4$; P (control vs. p-Akt10 min) < 0·05; P (control vs. p-Akt20 min) < 0·05; P (control vs. p-eNOS 20 min) < 0·001. In A, B and C the phosphorylation effects of detNO on eNOS or Akt were normalized by comparison with non-phosphorylated eNOS or Akt. (d) Enzymatic activity of eNOS after short-term exposure to detNO. Cells were incubated in the presence or absence of detNO for the specified time together with [2,3,4,5–^{3}H]arginine that is converted to NO and [^{3}H]citrulline. Cultures were processed according to the instructions of the NOSdetect®Assay Kit (see Methods). The amount of released NO is expressed in [^{3}H]citrulline equivalents. N-nitro-L-arginine methyester HCl was used as arginase inhibitor. $n = 4$; P (control vs. 20 min and 30 min) < 0·05.

NO production after 20 and 30 min exposure to detNO is shown. N-nitro-L-arginine methyl ester HCl (NAME), a competitive NOS inhibitor, verifies the reaction conditions of the assay.

Upon exposure to cytokines or lipopolysaccharides iNOS can be expressed in most cardiovascular tissues including endothelial cells. However, in many *in vivo* settings a major proportion of iNOS expression and activity may be in inflammatory cells [27] and numerous studies have shown that induction of iNOS in vessels is mainly a host defence response that may lead also to vasoconstriction and myocardial

dysfunction [28]. In our short-term experiments an effect of iNOS can be excluded and in our long-term experiments (see below) iNOS was not involved in the decreased eNOS expression as indicated by immunoblot analysis that failed to detect iNOS protein.

detNO-induced cell cycle arrest and hypertrophy

Cultured quiescent EC released from the G_o-phase by seeding at a low density re-enter the cell cycle and proliferate up to confluency. In this phase, detNO causes a dose-dependent suppression of proliferation of EC indicated by a decreased incorporation of [^{3}H]thymidine (Fig. 2a) in the presence of 100 μmol L^{-1} or 200 μmol L^{-1} and a cell cycle arrest (Fig. 2a, inset). The antiproliferative effect of detNO was associated with a remarkable increase of cell protein content that continued up to a 2–3-fold amount of control cells within 3 days (Fig. 2b) while the cell number indicates an inhibition of cell proliferation and shows neither increase nor decrease. The elevated total cell protein was the result of *de novo* synthesis indicated by measurements of [^{3}H]leucine incorporation into total cellular protein. After 48 h incubation of subconfluent cultures in the presence of [4,5–^{3}H]leucine the incorporated radioactivity was $24{\cdot}8 \times 10^3$ dpm/10^5 control cells and $34{\cdot}6 \times 10^3$ dpm/10^5 cells exposed to 150 μmol L^{-1} detNO ($n = 4$; $P = 0{\cdot}0041$).

The concomitant up-regulation of p21 refers to a block at the G_1-phase of the cell cycle. The inset of Fig. 2(b) shows an increased transcription of the p21 gene after 48 h administration of detNO that exceeds that of control by 156 ± 14% when evaluated by densitometry ($n = 3$, $P = 0{\cdot}0053$). TGF-β_1, used as a positive control, increases the p21 mRNA synthesis as compared with controls ($P = 0{\cdot}0033$). The detNO-induced metabolic alterations convert the cells into a hypertrophic phenotype (Fig. 3). Measurements of cell volume ($n = 37$, $P < 0{\cdot}001$) show an increase from 2·49 ± 0·18 (controls) to 2·89 ± 0·29 (150 μmol L^{-1} detNO) and to 3·38 ± 0·36 (200 μmol L^{-1} detNO) fL/cell, all *P*-values were highly significant.

The inhibition of proliferation is cytostatic but not cytotoxic as evaluated by cell death determination and is reversible. A quantitative determination of mono- and oligonucleosomes by cell death detection ELISA^{plus} system (Roche Diagnostics GmbH, 11 774425 001) in the cytoplasmic fraction of cell lysates revealed no significant apoptotic cell death in control or detNO pre-treated cells under the experimental conditions used (100–200 μmol L^{-1} detNO). The average ratio absorbance (A_{405nm}-A_{480nm}) of detNO pre-treated cells/control cells (no stimulation, no pre-treatment) was 1·21 (± 0·14) 2 h (short-term), 24 and 48 h (long-term) after detNO stimulation. When the medium of growth-arrested cells is replaced by a standard medium, cell proliferation recovers within the following 48 h (Fig. 2c) with a continuous increase of cell number.

detNO effect after prolonged continuous exposure of EC

Exposure of EC to 150 μmol L^{-1} detNO for 24 and 48 h reduces the eNOS protein content as compared with controls. Densitometry revealed a reduced eNOS protein content by 38 ± 4·6% (24 h) and 32 ± 4·1% (48 h) ($n = 3$, $P < 0{\cdot}05$) as compared with control cultures according to densitometry (Fig. 4a, first and second row of blot and attached diagram). The phosphorylation profile of p-eNOS (Fig. 4a) shows an increased Thr^{495} phosphorylation from 24 to 48 h and an inversed degree of Ser^{1177} phosphorylation. Real-time RT-PCR confirms the reduced transcription of eNOS-specific mRNA (Fig. 4b). In spite of the reduced eNOS, the hypertrophic EC retain their phosphorylated protein. Moreover, phosphorylation can be accelerated by stimulation with VEGF (vascular endothelial growth factor) (Fig. 4c). VEGF is known to induce a rapid activation of eNOS in EC [29]. For direct determination of eNOS enzyme activity after long-term exposure to detNO, [2,3,4,5–^{3}H]arginine was added to the culture medium (see methods). The radioactivity of [^{3}H]citrulline formed by the NADPH-dependent NOS oxidoreductase is directly proportional to the NO produced and released by the endothelial cells. Figure 4(d) shows a significant reduction of NO production expressed as [^{3}H]citrulline equivalents in accordance to the reduced Ser^{1177} phosphorylation of eNOS. In order to detect NO-induced changes of the rate of eNOS protein synthesis, we performed metabolic labelling of eNOS using [^{35}S]Met/Cys as precursors. After 48 h incubation of the cells in the presence or absence of 150 μmol L^{-1} detNO followed by a 6 h labelling with [^{35}S]Met/Cys we analysed the eNOS protein band obtained by Western blot for ^{35}S-radioactivity and for protein content by densitometry. According to the reduced eNOS protein content of NO-treated cultures, the ^{35}S-radioactivity incorporated into eNOS protein was reduced to 68% (control = 100%, $n = 4$, $P < 0{\cdot}05$). However, when the ^{35}S-radioactivity of eNOS expressed as counts/integrated densitometric units, values of 224 ± 28 for control and 258 ± 32 for NO-treated cultures were recorded. Thus, the specific radioactivity of eNOS protein remained unchanged under detNO treatment.

Discussion

NO is a major signalling molecule in the vascular system enhancing smooth muscle cell relaxation and vasodilation. In arteriosclerosis an impaired generation of NO leads to endothelial dysfunction – an early event in the development of arteriosclerotic lesions.

In our experiments we demonstrate that exogenously applied NO released from the NO donor detNO has under *in vitro* conditions a dual function in the regulation of eNOS expression. The applied dose of detNO (150 μM) used in our study is

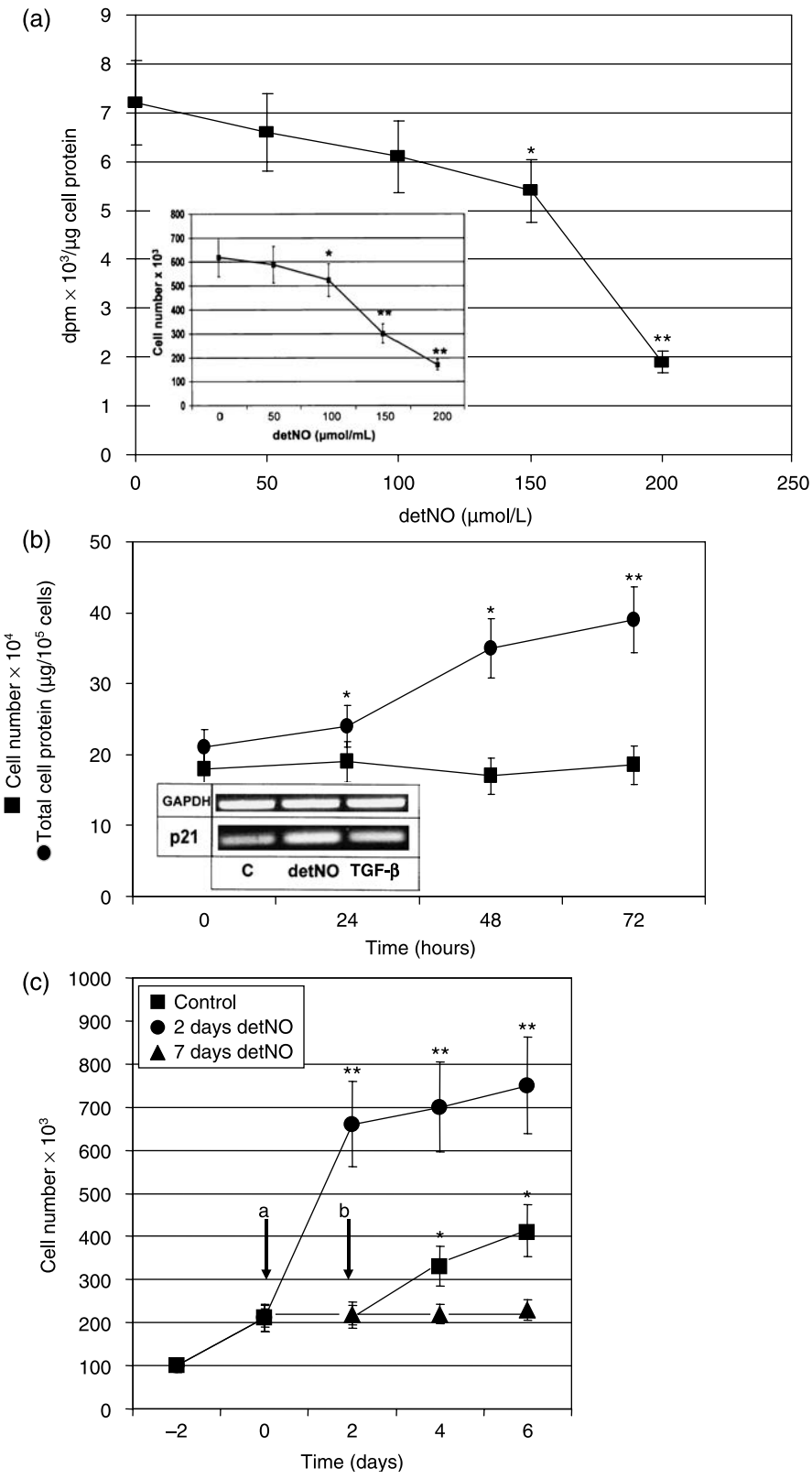
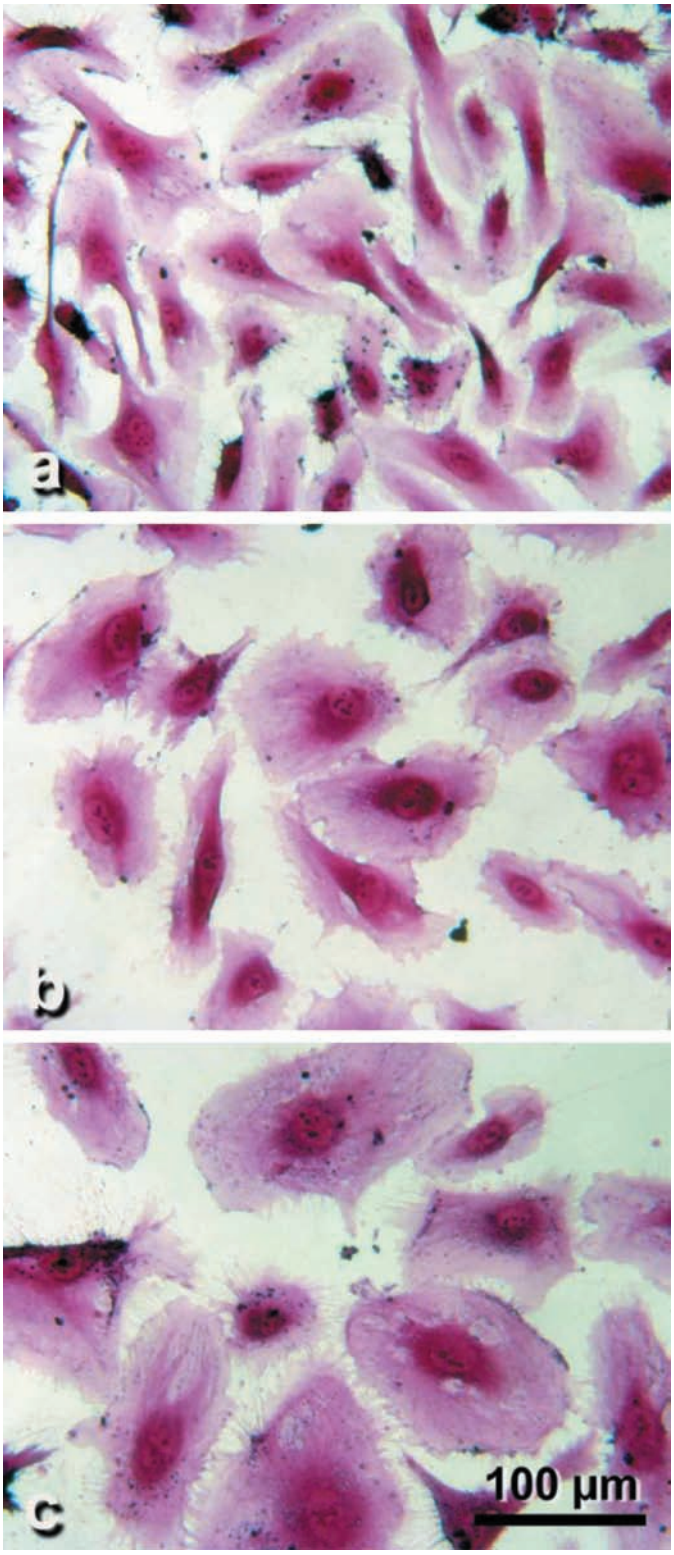


Figure 2 (a) Concentration–response curves of the effect of detNO on [^{3}H]thymidine incorporation and cell number (inset). Cells were seeded at a density of 100·000 cells/dish (35 mm ∅) and after two days incubated in the presence of the specified detNO concentrations for further 48 h[^{3}H]thymidine was added 8 h prior to the end of experiment. Values are means of 4 ([^{3}H]thymidine) and 5 (cell number, inset) experiments performed in duplicate. (b) Time-dependent variation of cell number and total cell protein of human umbilical venous endothelial cells (EC) in the presence of 150 µmol L^{-1} detNO. Cells were seeded at a density of 100·000 cells/dish and cell number and cell protein were determined at the specified time points. Values are means of 4–8 experiments each performed in duplicates. Inset: Cells were incubated in the presence of 150 µmol L^{-1} detNO or 10 ng mL^{-1} TGF-β_1 for 48 h. Total RNA was isolated and quantified using GENE Quant (Pharmacia), transcribed to cDNA and submitted to PCR with p21 primers. 12 ng were applied to agarose gel electrophoreses and amplicons were visualised by UV radiation. Values are normalized by GAPDH as a housekeeping gene. (c) Proliferation kinetics of EC in the absence (●) and presence (■, ▲) of 150 µmol L^{-1} detNO. Each experiment comprised three cultures in duplicate. At time zero (a) detNO was added to two (▲, ■) of three culture sets. After two days (b) the medium was replaced by a detNO-free medium (■) and detNO containing medium again (▲). The overall control shows a normal proliferation kinetic of endothelial cells (●). Change of medium in all cultures was every other 48 h.



2–3-fold higher than physiological NO plasma levels in healthy adults (36–42 µM) [30]. However, the detNO is applied in a latent/retard form from which the active NO is released in a first order reaction with a half life of 20 h. Taking into account the ultra short half life of NO (< 1 min) the actual concentration of NO is in the order of ≅ 2 ng × min^{-1} × mL^{-1} medium over the experimental time window. This value, however, is in accordance with the findings of Santoni *et al*. [31] who determined the concentration of isosorbide dinitrate (ISDN) and its active metabolites (2-ISMN and 5-ISMN) after a 5 mg infusion of ISDN to six patients with moderate heart failure over 8 h and found peak levels after 75 min of 6–24 ng and a continuous decrease to 2–16 ng mL^{-1} plasma after 8 h. During short–term exposure of endothelial cells, exogenous detNO enhances the phosphorylation of the protein kinase Akt (Fig. 1b) that in turn activates eNOS of endothelial cells by increasing its phosphorylation (Fig. 1a) leading to a higher release of endogenous NO as judged by a conversion of [^{3}H]arginine to [^{3}H]citrulline and by an increase of eNOS phosphorylation at Ser^{1177}. Taking this reaction sequence into account, the effect of the NO donor could be considered partially a trigger for the acceleration of endogenous NO production that finally effects vasodilation via the physiological pathway. This leads to the hypothesis of a potential switch from an exogenously applied to an endogenously generated NO stimulation. This hypothesis could be presumed because endothelial cells are the first destination of exogenous NO that undergoes complex interactions with various cellular components. Depending on the chemical structure of the NO donor, other reactive species apart from NO itself can be generated such as S-nitroso derivatives of proteins, nitroso thiols like S-nitrosocystein or other low molecular weight thiols [32].

Prolonged administration of exogenous detNO to EC reduces the expression of eNOS at protein and mRNA level (Fig. 4a,b). The reduced share of eNOS (38–47%) in total cell protein is associated with a similar reduction of eNOS protein synthesis (33%) as indicated by ^{35}S incorporation into eNOS protein and a reduced release of endogenous NO. This shows that in spite of a general increase of cell protein, the synthesis of eNOS protein does not participate in this overexpression under prolonged detNO treatment. However, the eNOS remains a target for phosphorylation by VEGF (Fig. 4c).

Figure 3 Light micrographs of normal (a) and detNO treated EC (b, c). Cells were fixed, stained by Fuchsin and examined for light microscopy. a, control; b, 100 µmol L^{-1}; c, 150 µmol L^{-1} detNO. The light micrographs are representative for 5 experiments. The morphological alterations of EC incubated with detNO were also analysed for cell volume measurements (see text).

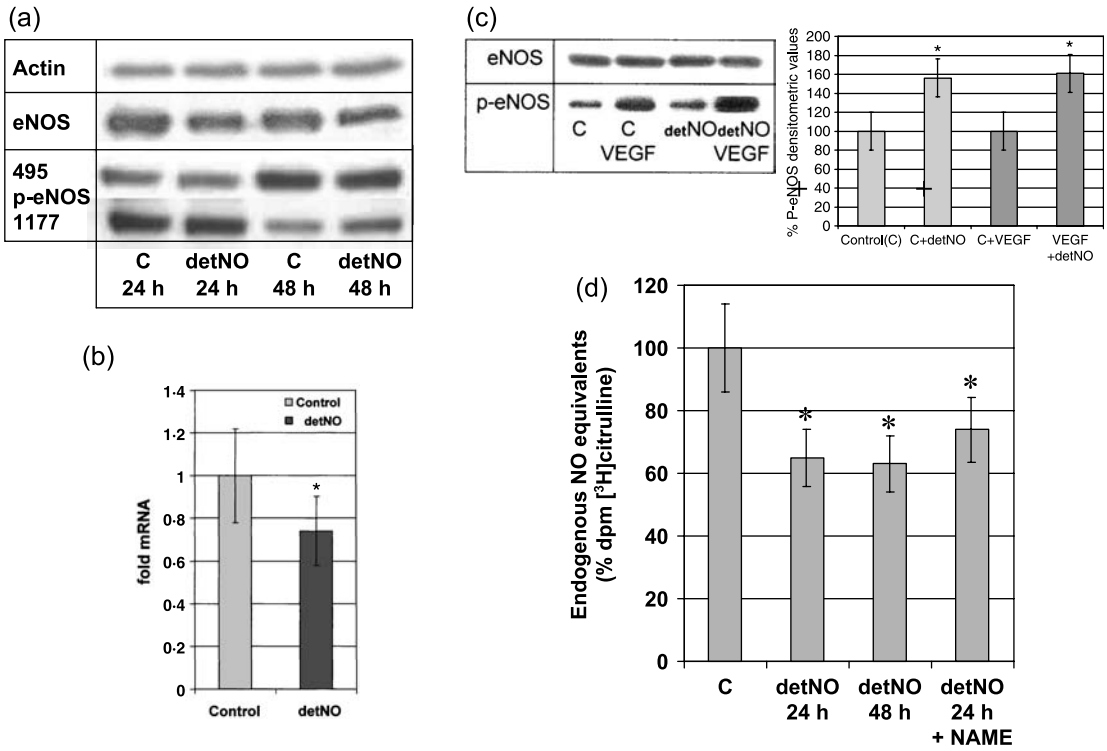


Figure 4 (a) Effect of prolonged detNO treatment on eNOS protein expression. EC were submitted to a continuous exposure of detNO for 24 and 48 h followed by Western blot analysis for eNOS with corresponding antibodies. $n = 3$; P (control vs. 24 h/48 h) < 0·05. Actin was used as a housekeeping protein. The phosphorylation status of eNOS was determined by Western blot analysis by means of antibodies against Thr^{495} and Ser^{1177} phosphorylation sites. (b) detNO-induced changes of eNOS-specific cDNA after 48 h administration to EC. Confluent cells were maintained in the presence or absence of 150 μmol L^{-1} detNO. Total RNA was isolated and used for real-time RT-PCR. (c) VEGF effect of detNO on eNOS phosphorylation of EC after prolonged NO treatment. EC were treated with 150 μmol L^{-1} detNO for 48 h followed by 1 h stimulation by 50 ng mL^{-1} VEGF of control and NO treated cells. $n = 3$; P (control vs. VEGF and detNO vs. detNO + VEGF) < 0·05. For Western blot antiphospho-eNOS and anti-eNOS antibodies were used. (d) Enzymatic activity of eNOS after long-term exposure to detNO. Cells were incubated in the presence or absence of 150 μmol L^{-1} detNO for the specified time. Then, [2,3,4,5–^{3}H]arginine or [2,3,4,5–^{3}H]arginine plus N-nitro-L-arginine methyl ester was added and the release of NO as [^{3}H]citrulline was measured over 30 min $n = 3$; P (control vs. 24 h/48 h) < 0·05.

Conclusions

Taken together, our findings emphasize the limitation of NO donors as long-term therapeutics owing to the inhibition of eNOS synthesis. Our studies were carried out on cultured human umbilical venous endothelial cells under *in vitro* conditions but a large body of our knowledge about endothelial cells originates from HUVEC and the most principal findings made on HUVEC also count for other types of endothelial cells [33].

However, whether NO donors are operative and effective in a similar way in humans is still uncertain. In numerous clinical studies the outcome of repeated administration of indirect or direct NO donors to patients with coronary artery disease (CAD) was investigated. The results were ambiguous. The potential benefit of long-acting nitrates has remained controversial (for review see [6]). Pathways leading to NO formation differ significantly among individual NO donor classes. In the Fourth International Study of Infarct Survival (ISIS-4), there was no significant reduction in 5 week mortality and no survival advantage [34]. Chronic administration of long acting nitrates in patients with healed myocardial infarction resulted in an increased number of patients with cardiac events [35] and an increased risk of cardiac deaths occurred in CAD patients with long acting nitrates [36]. Furthermore, a study on 19 healthy volunteers documented that isosorbide mononitrate given over 7 days impaired endothelial function due to formation of free radicals [37]. In a 700 patient study (ACCORD) the direct NO donor molsidomine, or its

active metabolite SIN-1, given for six months reduced the rate of restenosis from 47 to 38% [38].

The endothelium plays a crucial role in the pathogenesis of vascular diseases. An imbalance of the intracellular NO from endogenous or exogenous sources and the formation of deleterious oxygen species transform the endothelium towards a proatherogenic and vasoconstrictor organ. A new generation of NO donors and drugs that block $O_2^{\bullet-}$ production might be a rational approach to improve this balance.

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