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Vitamin C inhibits NO-induced stabilization of HIF-1α in HUVECs

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Abstract
HIF-1α represents the oxygen-regulated sub-unit of the transcription factor HIF-1, which regulates the transcription of numerous genes involved in cellular response to hypoxia and oxidative stress. It is shown here that nitric oxide (NO) induces HIF-1α stabilization in human endothelial cells from umbilical cords (HUVECs) under normoxic conditions. HIF-1α protein was increased ~ 36-fold after incubation with 500 µM DETA-NO, which releases a steady state NO concentration of roughly one thousandth of the initial concentration of the donor. Loading of the cells with vitamin C counteracted NO-induced HIF-1α accumulation. Based on the observations that oxidative and nitrosative stress can influence the activity of the proteasomal system, which is responsible for the non-lysosomal degradation of proteins, among them HIF-1α, it was investigated whether NO-induced stabilization of HIF-1α might be due to reduced 20S proteasomal activity. This hypothesis could not be proved, because NO concentrations to inhibit 20S proteasomal activity were about one order of magnitude higher than that to inhibit HIF-1α degradation.

Keywords: *Nitric oxide, ascorbate, DHA, HIF-1α, 20S proteasomal activity.*

Abbreviations: *HIF-1, hypoxia-inducible factor; DHA, dehydroascorbic acid; HUVECs, human umbilical vein endothelial cells; DETA-NO, diethylenetriamine/nitric oxide adduct.*

Introduction

Hypoxia-inducible factor-1 (HIF-1) is a transcription factor that induces oxygen-regulated genes in response to reduced oxygen conditions (hypoxia) [1]. The two sub-units of HIF-1, HIF-1α and HIF-1β, are constitutively expressed, but HIF-1α is rapidly degraded by the proteasome system under normoxic conditions and is stabilized during hypoxia. The oxygen content of the cells is sensed by specific prolyl hydroxylases, which hydroxylate HIF-1α and thus make it a substrate for a ubiquitin ligase [2–6]. The prolyl hydroxylases utilise vitamin C (ascorbate) to keep non-heme iron in the active Fe(II) form. Thus, vitamin C has been identified as modulator of the hypoxic response [7,8]. It is one of the major antioxidants in the human organism and numerous publications report its putative anti-ageing, healing and anti-carcinogenic properties. Recently, a novel function of ascorbate has gained broad interest in the field of angiology and cardiology and in research on functions of the arterial endothelium. The vitamin protects the blood vessels from the symptoms of endothelial dysfunction, which are a hallmark of arterial diseases, such as arteriosclerosis, coronary artery disease, chronic heart failure, diabetes mellitus and others [9–14]. The mechanisms involved are not resolved so far.

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However, patients suffering from one of the diseases associated with endothelial dysfunction often have lowered concentrations of ascorbate in plasma, which normally is ~ 50 μM. This may be a consequence of enhanced consumption resulting from compensation of increased oxidative stress. Other aspects apart from direct antioxidative effects of ascorbate should be considered in this context: (i) ascorbate was described as a co-factor of the endothelial form of NO-synthase (eNOS), more specifically as a stabilizer of its coenzyme tetrahydrobiopterin [15,16]; (ii) ascorbate can reduce nitrosothiols and nitrite which recently have been suggested to play an important role in regulating the arterial blood flow [17–21]; (iii) ascorbate deficiency may be analogous to hypoxia with respect to the prolyl hydroxylase activity and may thus alter the effect of NO. Although the exact relations are by no means clear, it is established that NO interferes with HIF-1α expression and that this response depends on the degree of oxygen supply [22–27].

In the present study, we show that NO-induced HIF-1α stabilization under normoxic conditions is reversed by loading human endothelial cells from umbilical cords (HUVECs) with vitamin C.

It has been assumed that high concentrations of NO can cause nitro-oxidative stress and reactive nitrogen species are known for post-translational protein modification [28]. The proteasome system is responsible for the non-lysosomal degradation of proteins. However, modified, cross-linked and aggregated proteins can act as irreversible inhibitors of the 20S proteasome [29]. Furthermore, it has been reported that NO directly interferes with 20S proteasomal activity and subsequently can block proteasome-mediated protein degradation [30].

Based on these findings, we investigated whether NO-induced HIF-1α stabilization might be due to inhibition of the 20S proteasome.

Materials and methods

Standard laboratory chemicals were obtained from Sigma (Deisenhofen, Germany), Merck (Darmstadt, Germany) or Fluka (Buchs, Switzerland) if not stated otherwise.

N-Succinyl-Leu-Leu-Val-Tyr7-Amido-4-Methylcoumarin (S-LLVY-AMC) was dissolved in dimethylsulphoxide (2 mM), kept at –20°C and was freshly diluted in buffer prior to use.

Cell culture

Human umbilical vein endothelial cells (HUVECs) were obtained by collagenase digestion of umbilical veins and grown in endothelial cell basal medium (EBM, PromoCell, Heidelberg, Germany) supplemented with 20% (v/v) foetal calf serum (Gibco, NY), 2 mM glutamine, 50 U/ml streptomycin and 0.1 mg/ml penicillin (PAA-Laboratories, Linz, Austria) and 50 ng/ml endothelial cell growth supplement (BD Biosciences, Schwechat, Austria). Cell viability was determined with trypan blue exclusion or the electronic cell counter CASY Model TTC (Schärfe System, Reutlingen, Germany).

Uptake of vitamin C

HUVECs were grown in fibronectin-pre-coated 6-well plates and washed twice with pre-warmed incubation buffer (TBSG; 140 mM NaCl, 20 mM Tris, 5 mM glucose, pH 7.4). All additions used for the specific experiments were included in a volume of 2 ml and the experiment was started by the addition of ascorbate. Dehydroascorbic acid (DHA) was generated by the conversion of ascorbate to DHA with ascorbate oxidase. Cells were incubated at 37°C and the uptake experiments were stopped at various time points by two washing steps with 1 ml of ice-cold PBS. Then, cells were detached by scraping in PBS, centrifuged at 1000 g for 60 s and re-suspended in 300 μl PBS. For the determination of intracellular vitamin C content, an aliquot of 200 μl was mixed with an equal volume of 10% (w/v) meta-phosphoric acid. The residual suspension was used for determination of protein concentration by Bradford assay. The samples were either analysed immediately by HPLC or frozen at −80°C.

Measurement of ascorbate and DHA by HPLC

Ascorbic acid was assayed by HPLC and electrochemical detection as described previously [31,32]. Conversion of DHA to ascorbate was achieved by addition of 10 mM dithiothreitol in 0.5 M Tris. The reduction was stopped after 10 min with 0.2 M sulphuric acid and DHA concentrations were calculated by subtracting concentrations of ascorbate from those of total ascorbate determined in paired aliquots of the same sample.

Measurement of NO release

The release of NO from DETA-NO was monitored with a nitric oxide sensitive electrode (ISO-NOP, WPI, Berlin, Germany) at 37°C and concentrations were calculated with a calibration curve based upon the chemical reduction of $NaNO_2$ by KJ and H_2SO_4 according to the manufacturer's protocol.

Induction of HIF-1α under normoxic conditions and immunoblotting

HUVECs were grown in 6-well plates, loaded with either ascorbate for 24 h or DHA for 30 min, washed twice and increasing concentrations of DETA-NO

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were added for 4 h. Cells were washed twice with PBS and lysed in 60 µl lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 100 µM NaF, 10 µM Na_3VO_4, 10% (v/v) glycerol, 1% (v/v) NP40, 1% (v/v) protease inhibitor cocktail) for 30 min on ice and sonicated. After centrifugation at 28 000 g for 10 min at 4°C, the protein content in the supernatant was determined by the Bradford assay, proteins were resolved on 8% SDS-polyacrylamide gels and blotted onto nitrocellulose membranes (0.45 µm; Bio-Rad, Vienna, Austria). Non-specific binding was blocked with 5% milk solution in TBS washing buffer (20 mM Tris-HCl pH 7.4 with 145 mM NaCl, 0.1% (v/v) Tween-20) for 1 h and membranes were incubated with HIF-1α (BD Biosciences, Schwechat, Austria) and β-actin antibodies in TBS washing buffer containing 3% (w/v) bovine serum albumin (1:250 and 1:2000, respectively) overnight at 4°C. Then, the blots were washed three times for 10 min with washing buffer and incubated with a 1:4000 dilution of goat anti-mouse IgG-horseradish peroxidase-linked antibody (Bio-Rad) diluted in blocking buffer (washing buffer with 5% (w/v) fat-free dry milk) for 1 h. After another washing with TBS, the proteins were revealed using Super Signal Pico (Pierce, Rockford, IL) and a chemiluminescence imager system (ChemiImager 4400 with AlphaEase FC Sofware, Biozym, Vienna, Austria).

Activity of the 20S proteasome

HUVECs were grown in culture flasks (25 cm^2), washed twice with ice-cold PBS, detached by scraping in 100 µl lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 10 % (v/v) glycerol, 1 % (v/v) NP40), transferred into reaction tubes and lysed for 1 h on ice. After centrifugation at 28 000 g at 4°C, the pellets were discarded and supernatants with a protein content of 60 µg were transferred into reaction tubes with a final volume of 1 ml PBS containing up to 10 mM DETA-NO. Aliquots were transferred into black 96 well plates (Greiner bio-one, Kremsmünster, Austria) and cleavage of AMC from 20 µM S-LLVY-AMC was monitored by the increase in fluorescence emission at 450 nm with an excitation wavelength set at 355 nm (Wallac, Turku, Finland) over a time period of 100 min at 37°C. Protein concentrations were determined by the Bradford assay.

Measurement of cytotoxicity by neutral red assay

Cells were incubated with the respective compounds, washed twice with PBS and freshly diluted neutral red solution (50 mg/l RPMI 1640-medium) was added [33]. After incubations with the dye for 3 h, cells were washed twice with PBS, lysed in 50% (v/v) ethanol/1% (v/v) acetic acid for 30 min and neutral red retention was measured in a plate reader at 540 nm (Wallac, Turku, Finland).

Data analysis

All experiments were performed at least three times and the results are presented as means ± SD. Statistical differences were analysed with GraphPad Prism 3.0 software and comparisons were made by one-way analysis of variance (ANOVA) with *post-hoc* testing using Newman-Keuls' test. A value of $p < 0.05$ was considered significant.

Results

Transport of vitamin C in HUVECs

Vitamin C is accumulated in primary endothelial cells (HUVECs) by two distinct mechanisms: first, there exists a transporter for ascorbate. At a starting concentration of 100 µM, uptake rates of 26.7±1.4 pmol/min per mg cell protein were measured (Figure 1A). This transporter showed high affinity (K_M 28.3 µM) and specificity for ascorbate with v_{max} values of 99.8±12.6 pmol/min per mg cell protein (Figure 1B) and was sodium-dependent (Figure 1C). Only 12.5% of intracellular ascorbate was observed with choline instead of sodium in the uptake buffer and a starting concentration of 100 µM ascorbate. Secondly, when ascorbate was oxidized to dehydroascorbate (DHA), a sodium-independent transporter system mediates uptake. Within the cells, only ascorbate and no DHA was found, indicating that DHA is immediately reduced back to ascorbate. The uptake rates for 100 µM DHA were 264±65 pmol/min per mg cell protein (Figure 1D) and are ~ 10-fold higher than the rates observed with ascorbate. The K_M-value was 3.5 mM and v_{max} was 10.7±1.7 nmol/min per mg cell protein (Figure 1E). To test whether DHA transport in these cells was mediated by the facilitative glucose transporters, competition studies were performed using O-methylglucose (3.13–100 mM OMG), a competitive inhibitor of glucose transporters. Uptake of 100 µM DHA was inhibited half maximal with ~ 10 mM OMG (Figure 1F). In the presence of 10 µM cytochalasin B, an uncompetitive inhibitor of glucose transporters, ~ 12% of the original amount of DHA uptake was observed (data not shown). These findings underline that DHA enters HUVEC via glucose transporters.

After verifying that HUVECs transport vitamin C in the oxidized as well as in the reduced form, cells were loaded with either 200 µM ascorbate for 24 h or with 200 µM DHA for 30 min. Due to the distinct transport rates both incubations led to an intracellular ascorbate concentration of ~ 2.5 mM, reflecting concentrations found in neutrophils, lymphocytes and monocytes [34]. The actual concentration of ascorbate in endothelial cell *in vivo* is still unknown.

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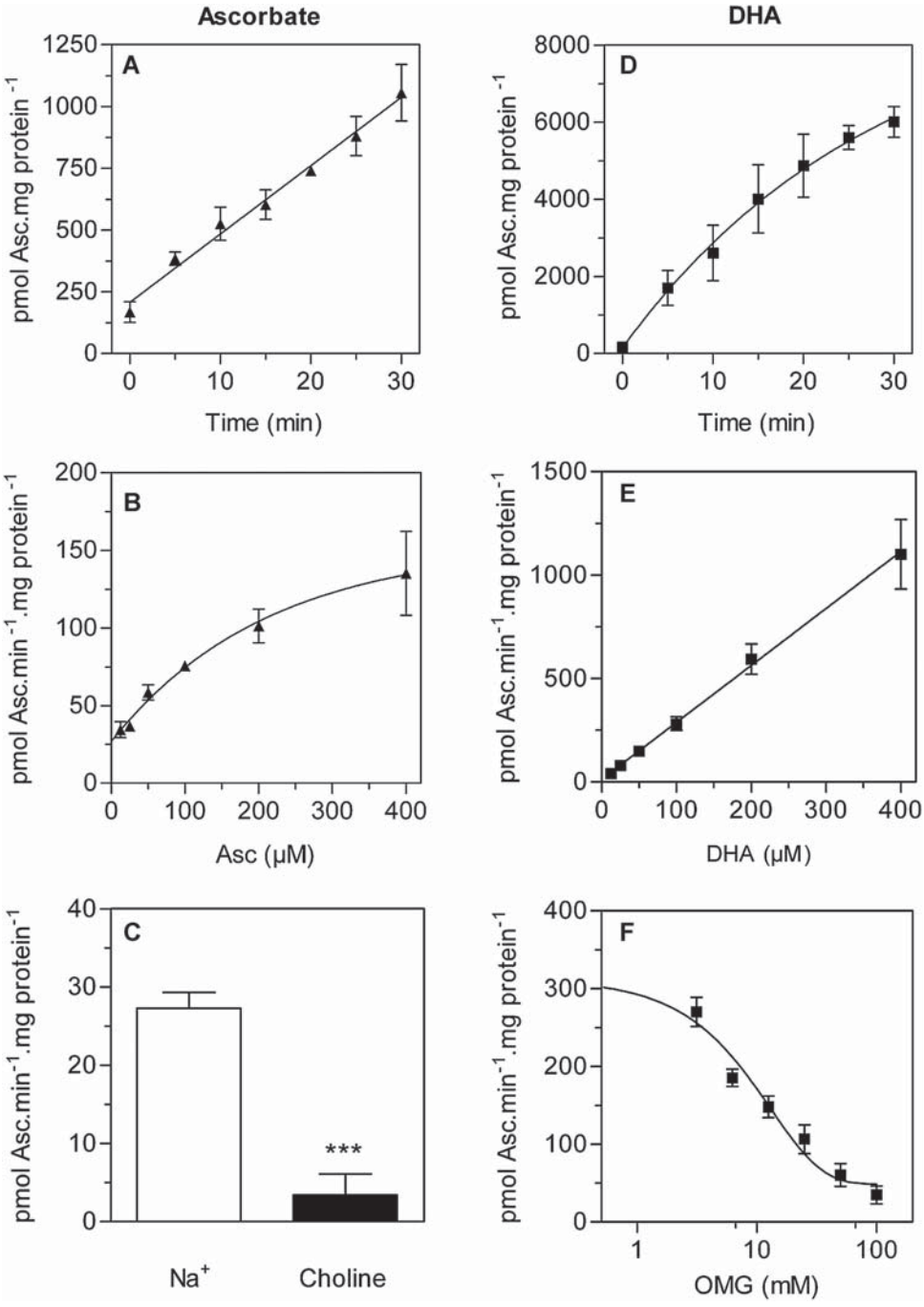

Figure 1. Uptake of ascorbate (A–C) and DHA (D–F) in HUVECs. Time dependence: HUVECs were incubated with 100 μM ascorbate (A) or 100 μM ascorbate together with 3 U/ml ascorbate oxidase to obtain DHA (D) for the indicated times before measurement of the cell content of ascorbate (Asc) by HPLC. Concentration dependence: cells were incubated with up to 400 μM ascorbate (B) or 400 μM ascorbate with 3 U/ml ascorbate oxidase for 20 min (E). Transport specificity: HUVECs were incubated in Tris-buffer with sodium or Tris-buffer with choline and uptake of 100 μM ascorbate was followed for 20 min (C). Transport of 100 μM DHA was followed in the presence of 3.1–100 mM O-methylglucose (F). Results are means ± SD for three experiments, $^{***}p < 0.001$.

Effect of vitamin C on NO-induced HIF-1α protein stabilization

The importance of NO as a signalling molecule with vasorelaxant character in the vascular endothelium is well established. To determine the effect of NO on HIF-1α protein degradation, we used the donor DETA-NO, which releases NO slowly with a constant rate and a half-life of ~ 20 h [35]. By using a sensitive NO-electrode we quantified steady state NO concentrations of roughly one thousandth of the initial concentrations of DETA-NO.

HIF-1α protein was present in HUVECs at very low amount due to fast degradation by the proteasome complex during normoxic conditions (20% O_2, 5% CO_2) but was accumulated ~ 5.5-fold by 250 μM, ~ 36-fold by 500 μM and ~ 40-fold by 1 mM DETA-NO, respectively (Figures 2A–C).

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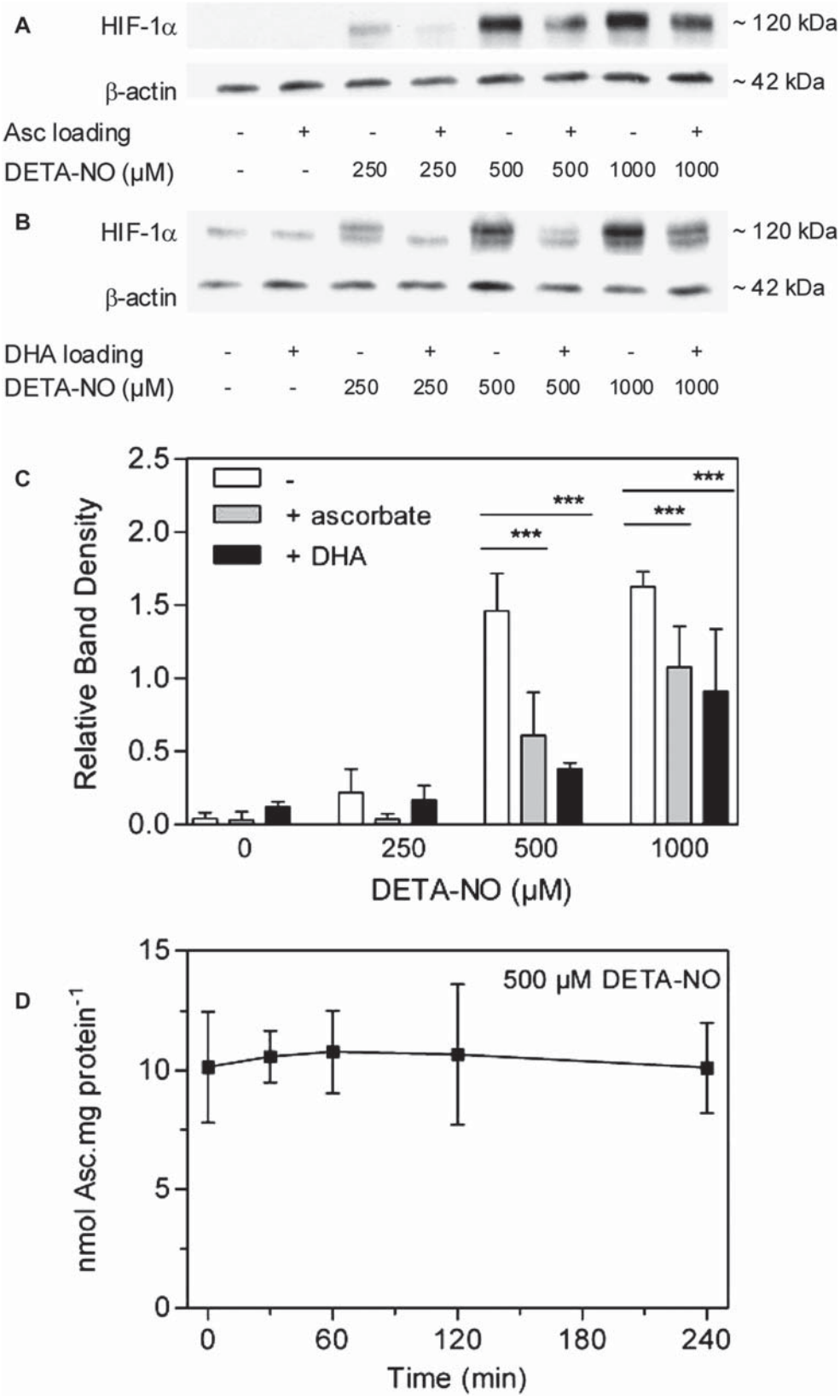

Figure 2. Effect of vitamin C on NO-induced stabilization of HIF-1α protein. HUVECs were incubated with either 200 μM ascorbate for 24 h (A) or 200 μM DHA for 30 min (B) before increasing concentrations of DETA-NO were added for 4 h. Whole cell extracts were separated by SDS-PAGE and HIF-1α was detected by Western blot analysis. (C) Combined densitometry of Western blots. Results are means ± SD for three experiments, $^{***}p < 0.001$. Effect of DETA-NO on intracellular ascorbate content (D). HUVECs were loaded with 200 μM DHA for 30 min and 500 μM DETA-NO was added for 4 h. Concentrations of intracellular ascorbate were measured by HPLC. Results are means ± SD for four experiments.

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This induction of HIF-1α protein accumulation was antagonized by pre-incubation of the cells with either 200 μM ascorbate for 24 h (Figure 2A) or 200 μM DHA for 30 min (Figure 2B). Irrespective of whether cells were loaded with reduced vitamin C for 24 h or oxidized vitamin C for 30 min, approximately the same intracellular concentrations of ascorbate were measured and very similar inhibitory effects were found and were significant ($p < 0.001$) in the presence of 500 μM and 1 mM DETA-NO (Figure 2C). Incubation of HUVECs with ascorbate or DHA alone showed no alteration in HIF-1α protein. To verify that treatment of the cells with DETA-NO does not deplete intracellular ascorbate and hence inhibit HIF-1α degradation, we loaded HUVECs with 200 μM DHA for 30 min, incubated them with 500 μM DETA-NO for 4 h and quantified vitamin C content by HPLC. Figure 2D shows that intracellular ascorbate content was not altered by treatment with the NO donor.

Effect of NO on 20S proteasomal activity

Reactive nitrogen species are known for post-translational protein modification and the 20S proteasome was suggested as a potential target. Therefore, we investigated whether inhibition of functional activity of the 20S proteasome by NO might be involved in reduced HIF-1α degradation. Cell lysates were incubated with increasing concentrations of DETA-NO and the fluorogenic substrate S-LLVY-AMC at 37°C under light-protected conditions and chymotryptic activity of the 20S proteasome was followed by AMC cleavage in an automated plate reader. The slopes of the time-course data were calculated, and Figure 3 depicts that 20S proteasomal activity was significantly inhibited by 1 mM DETA-NO ($p=0.021$) and was down to 44% with 5 mM DETA-NO compared to untreated controls. Although DETA-NO at concentrations of 250 μM and 500 μM increased HIF-1α protein significantly, these concentrations were without effect on proteasomal activity.

Ascorbate counteracted the effect of DETA-NO and restored 20S chymotryptic activity from 58.7 ± 18.1% due to 1 mM DETA-NO to 96.5 ± 21.3% in the presence of 1 mM DETA-NO together with 1 mM ascorbate (Figure 4).

Influence of high concentrations of NO on cell integrity

The neutral red assay was utilized to investigate the consequences of the incubation with DETA-NO on cell integrity. No changes in cell viability were observed after 4 h treatment of HUVECs with the NO donor at concentrations of up to 10 mM DETA-NO (Figure 5).

Discussion

Vitamin C is one of the major antioxidants in the human body and was suggested to protect the vascular system against endothelial dysfunction [9,11,14]. Cultured endothelial cells from human umbilical cords (HUVECs) accumulated vitamin C in reduced (ascorbate) and oxidized form (DHA). The uptake rates for DHA were ~ 10-fold higher than the rates observed with ascorbate. To estimate the significance of the two modes of vitamin C uptake, one has to consider the concentration ratio of DHA and ascorbate under physiological conditions and the apparent affinities. DHA may not be found at all in human plasma or only at very low concentrations [36]. This means that endothelial cells preferentially acquire ascorbate by uptake of the reduced form. The apparent affinity is 28.3 μM for HUVECs and within the physiological range of plasma ascorbate, which is between 25–100 μM [34]. However, whenever ascorbate is oxidized by reaction with

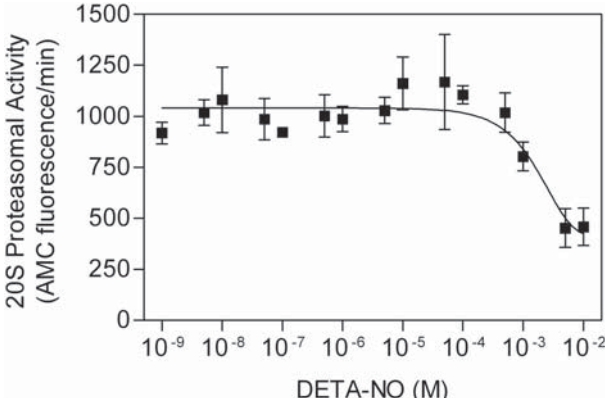

Figure 3. Effect of DETA-NO on 20S proteasomal activity. Proteasome was isolated from HUVECs as described in the Methods sections and lysates were incubated with various concentrations of DETA-NO over 100 min at 37°C. Chymotryptic activity of 20S proteasome was followed by AMC cleavage. Results are means ± SD for four experiments.

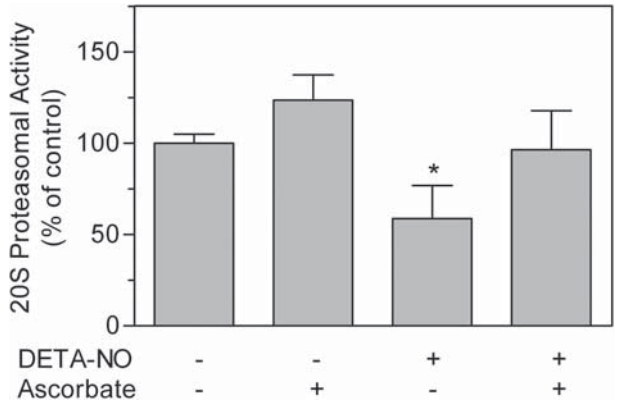

Figure 4. Effect of ascorbate on NO-mediated inhibition of 20S proteasomal activity. Lysates from HUVECs were incubated with 1 mM DETA-NO with or without 1 mM ascorbate for 100 min at 37°C. Results are means ± SD for three experiments, $^{*}p < 0.05$ vs untreated control.

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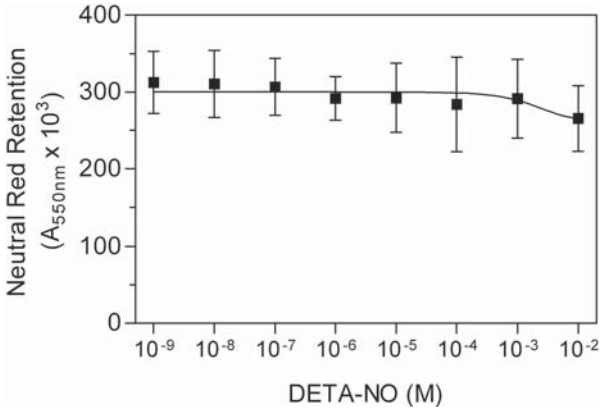

Figure 5. Effect of DETA-NO on viability of HUVECs. Cells were incubated with up to 10 mM DETA-NO for 4 h and cell integrity was monitored by the neutral red assay. Results are means ± SD for three experiments.

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oxidants, endothelial cells accumulate the oxidized vitamin and recycling of ascorbate from DHA accounts for prevention of irreversible loss. Considering a cell water content of 4 µl/mg protein for microvascular cells [37] and an intracellular amount of 10 nmol ascorbate/mg protein after incubation with either 200 µM ascorbate for 24 h or 200 µM DHA for 30 min, HUVECs accumulated ascorbate at concentrations ~ 2.5 mM. According to the transport of vitamin C into HUVECs, our data are consistent with previous findings [8,38].

Knowles et al. [7] first reported that ascorbate is involved in the hypoxic response by the transcription factor HIF-1 in cancer cells. When we used HUVECs, basal HIF-1α was present at a very low amount during normoxic conditions (20% O_2) due to fast degradation by the proteasome system. However, treatment of the cells with the NO donor DETA-NO inhibited HIF-1α degradation. We used up to 1 mM DETA-NO and found that NO was roughly a thousandth of the initial concentration of the releasing compound, reflecting steady state levels of NO that activated platelets can produce locally [39]. NO increased HIF-1α protein levels dependent on its concentration and was ~ 40-fold following incubations with 1 mM DETA-NO. From these data one might presume that massive NO generated during inflammatory conditions can induce HIF-1α signalling and upregulation of HIF-1α responsive target genes may lead to short-time adaptions such as enhanced glycolysis to meet an increased energy demand.

Highly significant reduction of NO-induced HIF-1α protein accumulation was found by loading HUVECs with vitamin C. It is well established that ascorbate rather preserves NO than it reacts with it. However, one may speculate that reactive products of NO, like the potent oxidant peroxynitrite, generated from NO and superoxide released by endothelial cells, might be responsible for HIF-1α stabilization and that ascorbate diminishes HIF-1α by scavenging reactive NO derivates. Although intracellular ascorbate content was not altered during incubation with DETA-NO, the changes in intracellular oxidation status could be rather small or ascorbate could be recovered by other antioxidants. Therefore, we can not fully exclude that vitamin C-mediated HIF-1α reduction was not solely due to its radical scavenging properties.

Several authors have suggested that NO and NO-derived reactive species are involved in post-translational protein modification and cross-linked and aggregated proteins can bind to the 20S proteasome and act as irreversible inhibitors [28,29,30]. Although the 20S proteasome is non-ATP-dependent and more resistant to oxidative stress than the ATP- and ubiquitin-dependent 26S proteasome [40,41], it seems to be the 20S proteasomal core with its chymotrypsin-like activity that is crucial for removing nitrated proteins in plasma [42]. Therefore, we hypothesized that NO-mediated stabilization of HIF-1α might occur through interaction of NO with the 20S proteasome. Even though this work documents inhibition of the 20S proteasomal activity by high but physiologically relevant concentrations of NO, we could not prove our hypothesis. NO concentrations about one order of magnitude higher than that for HIF-1α accumulation were required for inhibition of the 20S proteasomal activity. Based on these findings, we conclude that the suppressive effect of NO on HIF-1α protein degradation is not due to inhibition of the proteasomal activity.

Metzen et al. [25] have proposed that stabilization of HIF-1α by NO and ROS occurs through binding to the non-heme iron of prolyl hydroxylases (PHDs) to block their activity and inhibit HIF-1α ubiquitination. In our study, intracellular ascorbate was ~ 2.5 mM and thus was one order of magnitude higher than the K_m values reported for purified PHD reaction [43]. Therefore, our data might provide further evidence that vitamin C is essential for maintaining iron of PHDs in its active Fe(II) form, hence improving PHD activity and possibly preventing binding of NO. On the other hand, S-nitrosylation of cysteine in the oxygen-dependent degradation (ODD) domain was identified as a key mechanism for NO-mediated HIF-1α stabilization independent of the PHD pathway [44]. Based on these observations, one might speculate that reductants like ascorbate could convert S-nitrosylated proteins back to the reduced and active forms and this could be one of several effects of ascorbate on HIF-1α degradation. Even though, we are aware that atmospheric oxygen levels (20 % O2) in cell culture incubators do not exactly reflect in vivo oxygen tension, which has been reported to range between 10 and 13 % in human blood [45], our findings underline the role of vitamin C as a relevant modulator of NO-mediated signalling in the vascular endothelium. However, further studies are necessary to elucidate the underlying mechanisms by which intracellular ascorbate actually prevents NO-induced HIF-1α accumulation.

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