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Cellular hypomethylation is associated with impaired nitric oxide production by cultured human endothelial cells

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Abstract

Hyperhomocysteinemia (HHcy) is a risk factor for vascular disease, but the underlying mechanisms remain incompletely defined. Reduced bioavailability of nitric oxide (NO) is a principal manifestation of underlying endothelial dysfunction, which is an initial event in vascular disease. Inhibition of cellular methylation reactions by *S*-adenosylhomocysteine (AdoHcy), which accumulates during HHcy, has been suggested to contribute to vascular dysfunction. However, thus far, the effect of intracellular AdoHcy accumulation on NO bioavailability has not yet been fully substantiated by experimental evidence. The present study was carried out to evaluate whether disturbances in cellular methylation status affect NO production by cultured human endothelial cells. Here, we show that a hypomethylating environment, induced by the accumulation of AdoHcy, impairs NO production. Consistent with this finding, we observed decreased eNOS expression and activity, but, by contrast, enhanced *NOS3* transcription. Taken together, our data support the existence of regulatory post-transcriptional mechanisms modulated by cellular methylation potential leading to impaired NO production by cultured human endothelial cells. As such, our conclusions may have implications for the HHcy-mediated reductions in NO bioavailability and endothelial dysfunction.

Keywords

S-adenosylhomocysteine; Methylation; Endothelial dysfunction; Nitric oxide bioavailability; Endothelial nitric oxide synthase

Introduction

Hyperhomocysteinemia (HHcy) is a risk factor for vascular disease, but the underlying mechanisms remain incompletely defined (Castro et al. 2006).

Cellular methylation potential is determined by the concentrations of the homocysteine (Hcy) precursor, *S*-adenosylhomocysteine (AdoHcy). AdoHcy inhibits virtually all *S*-adenosylmethionine (AdoMet)-dependent-methyltransferases, which, in turn, catalyse the transfer of a methyl group from AdoMet to a wide variety of target molecules (including DNA and proteins), forming AdoHcy and the methylated substrate (Handy et al. 2011). AdoHcy is further converted into Hcy and adenosine by a reversible reaction catalysed by AdoHcy hydrolase, which favours AdoHcy production. When Hcy accumulates, its precursor, AdoHcy, will accumulate, as well. Elevated Hcy was shown to be directly associated with increased extracellular and intracellular AdoHcy concentrations in vivo (Castro et al. 2006). Increasing evidence indicates that Hcy may be regarded as a cellular demethylating agent, at least at the DNA level. In fact, we and others have observed an association between high levels of Hcy and AdoHcy, which, in turn, is correlated with global DNA hypomethylation status (Castro et al. 2006). In addition, several studies support the existence of epigenetic mechanisms in the context of Hcy-related endothelial dysfunction (Handy et al. 2011).

Vascular disease begins with endothelial dysfunction, a sensitive indicator of the atherosclerotic process, occurring even before the clinical manifestations of the pathology and predicting adverse clinical outcomes (Tai et al. 2004; Weiss et al. 2002). Reduced bioavailability of nitric oxide (NO) and consequent impairment of endothelium-dependent vasodilation result in endothelial dysfunction. Nitric oxide is synthesized by endothelial cells from the amino acid, L-arginine, via the enzymatic action of endothelial nitric oxide synthase (eNOS) and is an important mediator of intracellular signalling and a potent anti-

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atherogenic molecule responsible for the maintenance of vascular homeostasis (Luksha et al. 2009). Endothelial NOS expression is subject to significant degrees of regulation from transcriptional to post-translational levels (Tai et al. 2004; Weiss et al. 2002). Asymmetric dimethylarginine (ADMA) is an endogenous competitive inhibitor of eNOS and is derived from the proteolysis of methylated arginine residues in proteins (Cardounel et al. 2007). Methylation of arginine in proteins is carried out by protein-arginine methyltransferases type 1 (PRMTs 1), transferring one methyl group from AdoMet to various proteins and forming, in addition, AdoHcy (Boger 2006). AdoHcy is further hydrolysed to Hcy. Because of the metabolic link between ADMA and Hcy, ADMA has been suggested as a potential mediator of endothelial dysfunction in HHcy (Lentz et al. 2003; Dayal and Lentz 2005; Stuhlinger and Stanger 2005; Boger 2006). Evidence from animal models and clinical studies have suggested that accumulation of ADMA contributes to impaired NO generation and disease pathogenesis (Teerlink et al. 2009).

Several in vivo studies, either in animal models (Dayal et al. 2001; Devlin et al. 2004; Celotto et al. 2010) or in humans with severe (Celermajer et al. 1993), mild (Tawakol et al. 1997; Woo et al. 1997), or transient (Chambers et al. 1999; Chao et al. 2000) HHcy, showed that endothelial dysfunction is a key vascular phenotype occurring in the setting of HHcy; however, the mechanistic understanding of the association between HHcy and impaired NO bioavailability remains to be completely defined. The present study was designed to evaluate whether a hypomethylating environment, induced by accumulation of the Hcy precursor, AdoHcy, perturbs NO production by cultured human endothelial cells.

Materials and methods

Cell culture

Human umbilical vein endothelial cells (HUVEC) were obtained from umbilical cords of healthy foetuses from uncomplicated pregnancies and vaginal deliveries from healthy mothers, as described (Castro et al. 2005), or commercially obtained from Lonza (Walkersville, MD, USA). The cord was collected in buffered solution [4 mmol/L KCl, 140 mmol/L NaCl, 11 mmol/L D-glucose (all from Merck, Darmstadt, Germany), 10 mmol/L HEPES (Gibco, Grand Island, NY, USA), and 1% antibiotic–antimycotic solution (Sigma, St. Louis, MO, USA)], and stored at −4°C. Within 4 days, cells were isolated by collagenase (Gibco, Grand Island, NY, USA) treatment and were cultured essentially as previously described (Castro et al. 2005). Cells were grown in coated six-well plates (6×10 cm^2), 75 cm^2 flasks, or 100 cm^2 disks depending on subsequent analysis.

All experiments were performed at 80% confluence and between the fourth and sixth passage. After the removal of the culture medium, fresh medium, either without (control) or with increasing concentrations of an AdoHcy hydrolase inhibitor (adenosine dialdehyde, ADA) (5, 10, and 20 μmol/L) was added. After 24 h of incubation, lactate dehydrogenase (LDH) release was evaluated in the cell culture medium using the Cytotoxicity Detection Kit (Roche Diagnostic GmbH, Mannheim, Germany).

Metabolite analysis

Aliquots of the medium were collected from cells grown in six-well plates after 24 h of incubation and stored at −20°C for further Hcy and ADMA analysis. HUVEC were then extensively washed with ice-cold phosphate-buffered saline (PBS) and exposed to denaturation buffer for 15 min at 4°C. Denaturation buffer consisted of 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 1 mmol/L DTT (all from Merck, Darmstadt, Germany), 10 mmol/L HEPES (Gibco, Grand Island, NY, USA), and 1.0 mmol/L PMSF (Sigma, St Louis, MO, USA) (pH 7.9), containing 1% (v/v) Triton X-100 (from Merck, Darmstadt, Germany). The cell lysate

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was centrifuged at 4°C, 1,200 rpm, for 10 min. The supernatant (cytosol) was collected, and two aliquots were taken. One was promptly deproteinized with an equal volume of 10% perchloric acid and stored at −20°C for further AdoMet and AdoHcy evaluation, and the remainder was immediately frozen and kept at −20°C until protein determination.

Extracellular total Hcy (tHcy) levels, defined as the total concentration of Hcy after reductive cleavage of all disulfide bonds, were determined by HPLC analysis according to Araki and Sako (1987), with minor modifications. Extracellular ADMA concentrations were measured by HPLC with fluorescence detection as described previously (Teerlink et al. 2002), using modified chromatographic separation conditions (de Jong and Teerlink 2006). Intracellular AdoHcy and AdoMet concentrations were analysed by LC–MS/MS (Struys et al. 2000).

NO levels

Nitric oxide production in response to intracellular Ado-Hcy accumulation was measured by the Griess reaction (Guevara et al. 1998). For this purpose, cells were grown in six-well plates. Culture medium was replaced 24 h before incubations, which were conducted in 0.8 mL of basal endothelial cell culture medium without phenol red. After 12 and 24 h of incubation, aliquots of culture medium were collected for NO_x quantification. In addition, incubations for 18 h with 1 mmol/L of L-N^G-nitroarginine (L-NNA, from Cayman Chemicals, Ann Arbor, MI, USA), an eNOS inhibitor, in the presence and in the absence of 20 μmol/L of ADA, were also performed. Freshly collected (200 μL) cell culture supernatants were mixed with 200 μL Griess reagent (1:1 mixture of 1% sulphanilamide in 5% H_3PO_4 and 0.1% naphthylethylenediamine dihydrochloride in water) and incubated in 96-well plates for 10 min at room temperature. Absorbance at 543 nm was recorded. Cells were lysed and protein concentrations were determined. Nitrite concentration was calculated using sodium nitrite standards and normalized by cellular protein concentration (μmoles of NO_2^-/mg of cell protein).

Western blotting

Western blot was performed for analysis of eNOS levels, using four independent cultures. 30 cm^2 of 80% confluent HUVEC was used for each sample. Cells were washed three times with ice-cold PBS, directly lysed in cell lysis buffer containing protease inhibitors (Sigma, St Louis, MO, USA), collected with a cell scraper, and sonicated. After centrifugation, the obtained supernatant was used for total protein determination and Western blot analysis. Protein concentrations were quantified using the DC Protein Assay (Biorad, Hercules, CA, USA).

Protein samples (20–30 μg) were separated on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Hybond ECLTM, Amersham, GE Healthcare, Chalfont St. Giles, UK). The membranes were incubated with anti-eNOS (at a 1:500 dilution; Cell Signalling, Danvers, MA, USA) and anti-β-actin (at a 1:2000 dilution; Sigma, St. Louis, MO, USA) antibodies. A secondary anti-rabbit IgG HRP secondary antibody (Cell Signaling, Danvers, MA, USA) at a 1:2,000 dilution was used. Primary antibody incubation was performed overnight at 4°C, and secondary antibody incubation was performed for 1–1.5 h at room temperature. An ECL Plus Western Blotting Detection System was used for protein detection (GE Healthcare, Chalfont St. Giles, UK), membranes were exposed to Amersham Hyperfilm HCl (GE Healthcare, Chalfont St. Giles, UK), and a VersaDoc scanning system (BioRad, Hercules, CA, USA) was used for densitometric analysis.

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eNOS activity

eNOS activity was determined by measuring the efficacy of conversion of [^{3}H]-Arg to [^{3}H]-Citrulline in protein extracts from cultured cells using a commercial kit (Cayman Chemicals, Ann Arbor, MI, USA), according to the manufacturer's instructions. Four independent cultures were used.

Briefly, cells were grown to confluence in 100 cm^2 disks, treated with 20 μmol/L of ADA for 24 h, washed with PBS, harvested in PBS containing 1 mmol/L of EDTA, and centrifuged. The obtained pellet was lysed in 100 μL of lysis buffer, centrifuged, and 10 μL of the obtained supernatant were incubated with 40 μL of reaction buffer containing [^{3}H]-Arg for 60 min at 37°C. Reactions were terminated by addition of 400 μL of Stop Buffer from the kit. Cell lysates were applied to a cation exchange resin column from the kit. The citrulline was eluted, radioactivity determined, and normalized via protein concentration.

Reverse transcription and quantitative PCR

Total RNA was extracted from three independent cultures using the RNeasy Minikit (Qiagen, Valencia, CA, USA) and was reverse transcribed (2 μg) into cDNA using oligo(dT) SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Specific primers for *NOS3* gene (NM_000603.3) F:5′-CGTCCCTGTGGAAAGACAAG-3′ and R: 5′-CACGATGGTGACTTTGGCTA-3′ were designed with the Universal Probe Library Assay Design Center (Roche Applied Science). EIF4A2 gene (NM_001967.3) served as an internal control gene with F:5′-GTGTGAACT GGACCCTGTTG-3′ and R:5′-TATTTAACATTCAAACT TCATTAAGACATG-3′ primers. Amplification reaction assays were performed in triplicate and contained 1× SYBR Green PCR Mastermix (Applied Biosystems, Foster City, CA, USA) and primers at optimal concentration. A hot start at 50°C for 2 min was followed by an extension period at 95°C for 10 min and then for 40 cycles at 95°C for 15 s and 60°C for 1 min, using the 7300 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). Fluorescence emission was detected for each PCR cycle and the Ct (threshold cycle) values were determined.

Statistics

Data are presented as means ± SD. Statistical evaluation of the data was performed by paired Student's t test for comparisons between two groups, and by one-way analysis of variance followed by Dunnett's test for comparisons between more than two groups. A value of $p < 0.05$ was regarded as significant.

Results

Intracellular AdoHcy and AdoMet levels

As shown in Fig. 1a, ADA was effective in promoting a significant AdoHcy intracellular accumulation, confirming its inhibitory effect on AdoHcy hydrolase. AdoMet levels remained unaltered (189 ± 50, 216 ± 22, 169 ± 50 and 203 ± 30 pmol/mg protein for ADA 0, 5, 10, and 20 μmol/L, respectively, p = NS for trend). A significant ($p < 0.01$ vs. control) decrease in intracellular AdoMet/AdoHcy ratio was observed (5.8 ± 1.6, 1.8 ± 0.2, 1.0 ± 0.3 and 0.8 ± 0.1 for ADA 0, 5, 10, and 20 μmol/L, respectively), which was exclusively defined by the increasing levels of AdoHcy.

Hcy and ADMA export, the effect of AdoHcy hydrolase inhibition

In the presence of increasing concentrations of ADA, there was a significant decrease in both tHcy and ADMA extracellular concentrations, showing that both export levels were reduced in an ADA dose-dependent manner (Fig. 1b, c).

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Lactate dehydrogenase

Cell LDH release (%, average ± SD, $n = 3$), an indicator of cytotoxicity, after 24 h of incubation, in the absence (control 100 ± 8) or presence of the tested ADA concentrations (111 ± 16; 110 ± 17 and 105 ± 12, for ADA = 5, 10 or 20 μmol/L, respectively) was similar. Therefore, a possible cytotoxic effect for the ADA was ruled out in the present study.

NO levels under intracellular AdoHcy accumulation

We measured nitrite, a metabolite of NO, as an index of endothelial NO production. We observed a decrease in extracellular nitrite concentrations, after 12 and 24 h of incubation, which, for the highest ADA concentration tested, reached statistical significance (Fig. 2). The observed difference of extracellular nitrite concentrations, observed in the absence and the presence of 20 μmol/L of ADA, was abolished by co-incubation with 1 mmol/L of L-NNA, an eNOS inhibitor (Fig. 3).

eNOS: transcriptional and translational levels and enzymatic activity under intracellular AdoHcy accumulation

eNOS protein and activity—The expression of eNOS protein was studied using Western blot analysis. A typical band with a molecular mass of approximately 140 kDa was detected with an anti-eNOS antibody, with *β*-actin used as reference. Relative expression of eNOS was significantly reduced by intracellular AdoHcy accumulation (Fig. 4a1).

The functionality of the eNOS protein was assessed by monitoring the efficacy of L-arginine to L-citrulline conversion in cell lysates. Compared with controls, cells incubated with 20 μmol/L ADA for 24 h showed a significant decrease in eNOS activity ($p < 0.05$) (Fig. 4a2).

NOS3 mRNA—*NOS3* mRNA levels were quantified by RT-qPCR. As opposed to the ADA-induced decrease of eNOS protein and activity, a significant increase of *NOS3* gene transcription was found directly related to the intracellular accumulation of AdoHcy (Fig. 4b).

Discussion

The major finding of the present study is that intracellular accumulation of AdoHcy resulted in impaired NO production by cultured human endothelial cells. Furthermore, decreased eNOS protein expression and corresponding enzymatic activity were observed, but increased *NOS3* mRNA levels were found.

Hcy metabolism is biochemically linked to the principal epigenetic tag (5-methylcytosine) found in DNA (Handy et al. 2011). Inhibition of cellular methylation reactions by AdoHcy, which accumulates in the setting of HHcy, has been suggested to contribute to vascular dysfunction. Accordingly, several studies support the existence of epigenetic mechanisms in the context of Hcy-related endothelial dysfunction (Handy et al. 2011). However, thus far, the effect of intracellular AdoHcy accumulation on NO bioavailability has not yet been fully examined. The present study was designed to evaluate whether a hypomethylating environment, caused by intracellular AdoHcy accumulation, contributes to the impairment of endothelium-dependent NO production in cultured human endothelial cells.

Because AdoHcy hardly crosses the cell membrane (Ueland 1982; Castro et al. 2005), the intracellular accumulation of the Hcy precursor, AdoHcy, was achieved through the pharmacological inhibition of AdoHcy hydrolase by an adenosine analogue, ADA. As shown in Fig. 1, increasing concentrations of ADA were effective in increasing intracellular AdoHcy levels. Supporting our previous observations, the levels of AdoHcy exclusively

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determined the decrease in AdoMet/AdoHcy ratio since AdoMet levels remained constant over the range of ADA concentrations used (Castro et al. 2005). Hcy export from the HUVEC reflects an imbalance between its intracellular production and metabolism. Therefore, and as expected, a decrease in the concentration of extracellular Hcy was observed (Fig. 1b), confirming the inhibitory effect of ADA upon AdoHcy hydrolase.

The rapid metabolism and short half-life of NO brings additional difficulties to its analytical assessment (Guevara et al. 1998). However, the measurement of nitrite, a metabolite of NO, is currently used as an index of endothelial NO production (Guevara et al. 1998). As shown in Fig. 2, with increasing concentrations of ADA, a dose-dependent decrease in extracellular nitrite levels was observed in our system. This effect was abolished in the presence of the eNOS inhibitor, the L-NNA (Fig. 3). This finding confirmed that the decreased nitrite formation caused by intracellular AdoHcy accumulation was due to impaired NO synthesis by eNOS. Previous studies also found that NO release from cultured endothelial cells was impaired in the presence of elevated Hcy (Upchurch et al. 1997). In addition, in HHcy mice with endothelial dysfunction, elevated AdoHcy and decreased AdoMet/AdoHcy ratio were observed (Dayal et al. 2001). ADMA is an endogenous and potent inhibitor of eNOS that was found to accumulate in different study models including in humans with HHcy and vascular disease (Teerlink et al. 2009). Consequently, the accumulation of ADMA was suggested to contribute to reduced NO generation in Hcy-dependent endothelial dysfunction (Teerlink et al. 2009). Our results suggest that the accumulation of the Hcy precursor, Ado-Hcy, is not responsible for the ADMA accumulation observed in the setting of HHcy. In fact, an AdoHcy intracellular accumulation (Fig. 1a) was associated with decreased extracellular ADMA levels (Fig. 1c) in our cellular model. In addition, a significant impaired NO production was observed (Figs. 2, 3, 4). Therefore, our results suggest the existence of an ADMA-independent mechanism triggered by AdoHcy accumulation that may contribute to the HHcy-related impaired NO bioavailability. Interestingly, it has been recently suggested that the increase in ADMA levels, seen in humans with HHcy and vascular disease, is probably due to the decline of renal function and is not directly related to HHcy-dependent metabolic changes, nor does it contribute to endothelial dysfunction (Wilcken et al. 2006; van Guldener et al. 2007).

Nitric oxide is generated in endothelial cells from conversion of L-arginine to L-citrulline by the enzymatic action of eNOS, which in turn is encoded by the *NOS3* gene. To investigate whether the observed impairment in endothelial NO bioavailability was caused by decreased eNOS expression, we focused on its translational and transcriptional expression. As shown in Fig. 4a1, decreased eNOS protein levels were observed, suggesting eNOS down-regulation by intracellular AdoHcy accumulation. In agreement with these observations, an Hcy-induced decrease in eNOS protein levels was seen using human aortic endothelial cells in a prior study (Jiang et al. 2005).

We also examined the functionality of eNOS protein, monitoring the efficacy of L-arginine to L-citrulline conversion by cell lysates. Compared with controls, cells incubated with 20 μmol/L of ADA for 24 h showed a significant decrease in eNOS activity (Fig. 4a2), which is consistent with our findings of decreased nitrite levels (Fig. 2) and decreased eNOS levels (Fig. 4a1). Previous reports concerning Hcy-induced disturbances in eNOS activity are controversial. In fact, no effect (Heydrick et al. 2004) or down-regulation (Dayal et al. 2008) by Hcy upon eNOS activity had been reported previously in cultured endothelial cells.

Surprisingly, we found that relative *NOS3* mRNA levels were significantly increased by intracellular AdoHcy accumulation (Fig. 4b). We, and others, have already shown that global DNA hypomethylation is induced by AdoHcy accumulation (Castro et al. 2005, 2006). It is well known that DNA methylation is an epigenetic mechanism of gene

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regulation and that decreased methylation patterns usually are related to increased transcription of target genes by several mechanisms (Handy et al. 2011). However, it has been previously shown that in eNOS-expressing cells the core promoter CpG dinucleotides are fully demethylated (Chan et al. 2004). Accordingly, it was reported that treatment of human endothelial cells with a DNA methyltransferase inhibitor did not alter the levels of *NOS3* mRNA, as it did in eNOS non-expressing cells (Chan et al. 2004). These observations led us to exclude DNA hypomethylation as causing the increase in *NOS3* mRNA levels observed in our model. However, DNA is not the only target for the AdoHcy-mediated inhibition of AdoMet-dependent methyltransferases, and multiple targets may be affected, including proteins implicated in eNOS expression in human endothelial cells. An example of this point is the forkhead box O 1 (FOXO1) transcription factor, which acts as transcriptional repressor of *NOS3* expression. Recent data showed that protein arginine methyltransferase 1 (PRMT1) methylates FOXO1, which in turn hinders its phosphorylation and export to cytoplasm, thus augmenting the expression of FOXO1 target genes (Yamagata et al. 2008). The same authors showed that, in a HUVEC model, the knockdown of PRMT1 induced *NOS3* expression (Yamagata et al. 2008). Moreover, our data show that intracellular AdoHcy accumulation was associated with decreased extracellular ADMA concentrations, as well as increased relative *NOS3* mRNA levels. Recent observations by our group showed that protein-incorporated ADMA is strongly decreased by AdoHcy intracellular accumulation in HUVEC (Esse et al. 2010), indicating that PRMT1 is a target for AdoHcy-mediated inhibition and suggesting that FOXO1-methylation may be subsequently reduced by AdoHcy. Taken together, these findings suggest that PRMT1 was subjected to AdoHcy-mediated inhibition, causing FOXO1 hypomethylation and the observed Ado-Hcy-dependent enhanced *NOS3* transcription (Fig. 4b).

Regulation of eNOS protein levels is a complex process that is mediated at several levels, including post-transcriptionally (Fish and Marsden 2006). In fact, our paradoxical observations concerning eNOS transcriptional and translational levels disclose the existence of post-transcriptional events to be ascertained. Similar observations have been reported in different contexts. For example, in livers from patients with alcoholic hepatitis, *NOS3* mRNA was found to be increased, but eNOS activity decreased despite no differences in eNOS protein expression compared with non-alcoholic livers (Mookerjee et al. 2007). In addition, exercise training of diabetic animals was shown to lead to an increase in left ventricular eNOS protein and a concomitant decrease in *NOS3* mRNA (Grijalva et al. 2008).

In conclusion, we observed that a hypomethylating environment, due to increased intracellular levels of AdoHcy, impairs NO production by cultured human endothelial cells. This finding may have implications in the HHcy-mediated reduction in NO bioavailability and endothelial dysfunction. Taken together, our results clearly suggest that post-transcriptional events may be crucial mechanisms in the regulation of NO synthesis, and their modulation by the cellular methylation potential demands further investigation.

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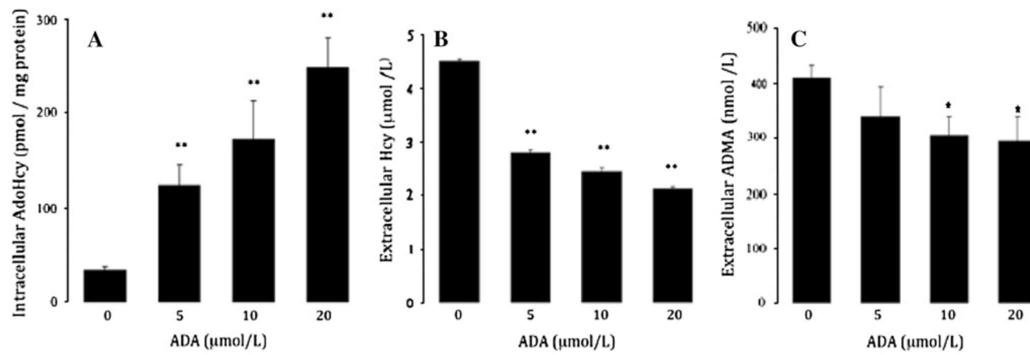

Fig. 1.

Intracellular AdoHcy (**a**), and extracellular Hcy (**b**) and ADMA (**c**) concentrations in HUVEC incubated in culture medium supplemented with increasing concentrations of ADA for 24 h. Data are presented as means ± SD. (***p* <0.01 vs. control, *n* 3; **p* <0.05 vs. control, *n* = 6)

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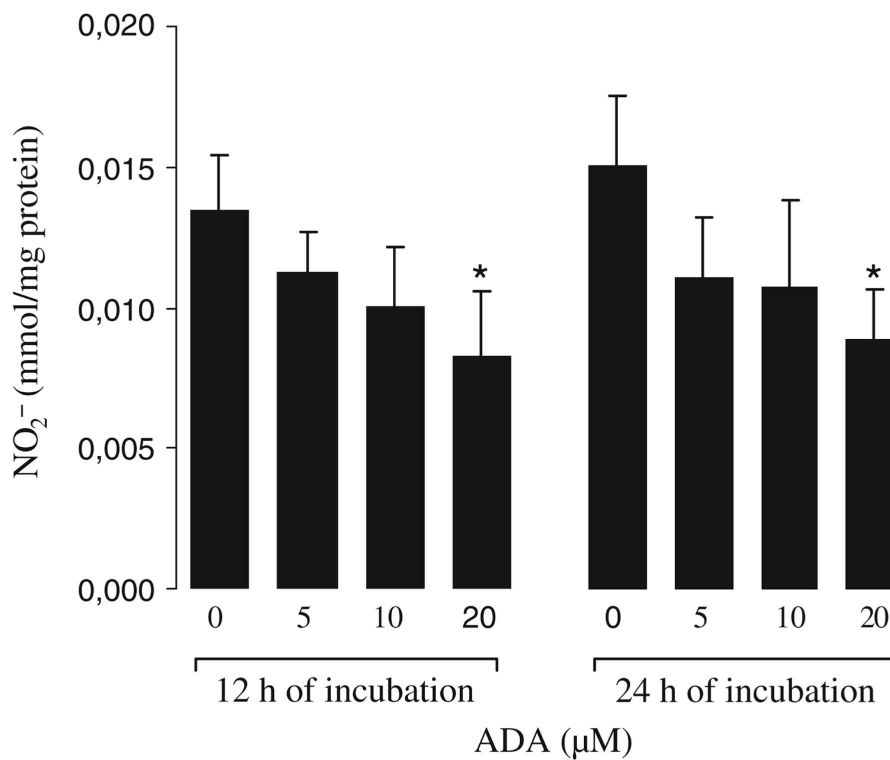

Fig. 2.
Nitrite levels in HUVEC culture medium measured by the Griess reaction. HUVEC were incubated in culture medium supplemented with increasing concentrations of ADA for 12 or 24 h. Data are presented as means ± SD, and are representative of three different cell lines (*p <0.05 vs. control)

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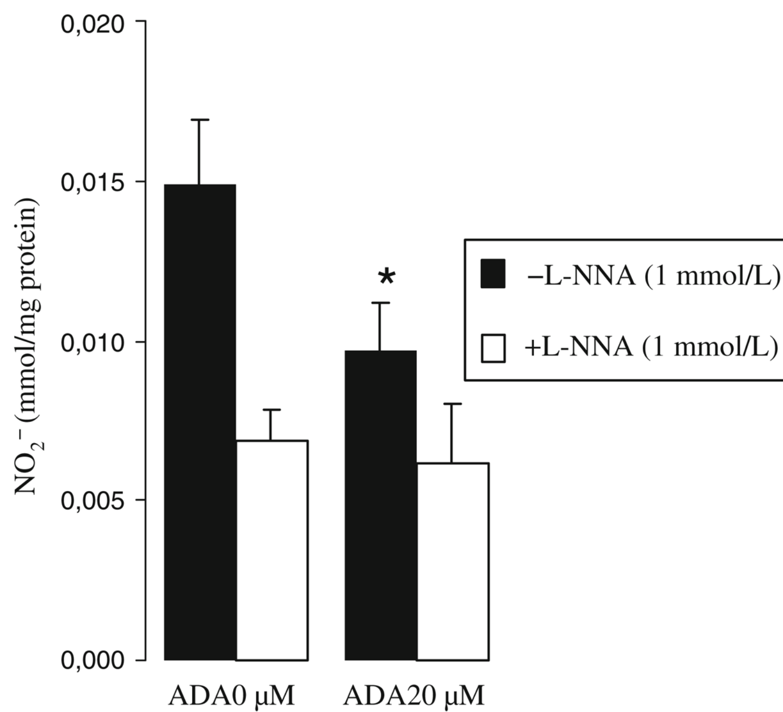

Fig. 3.
Nitrite levels in HUVEC culture medium supplemented with 0 and 20 μmol/L concentrations of ADA in the absence and in the presence of L-NNA (1 mmol/L) after 18 h of incubation. Data are presented as means ± SD (* p <0.05 vs. control, $n = 3$)

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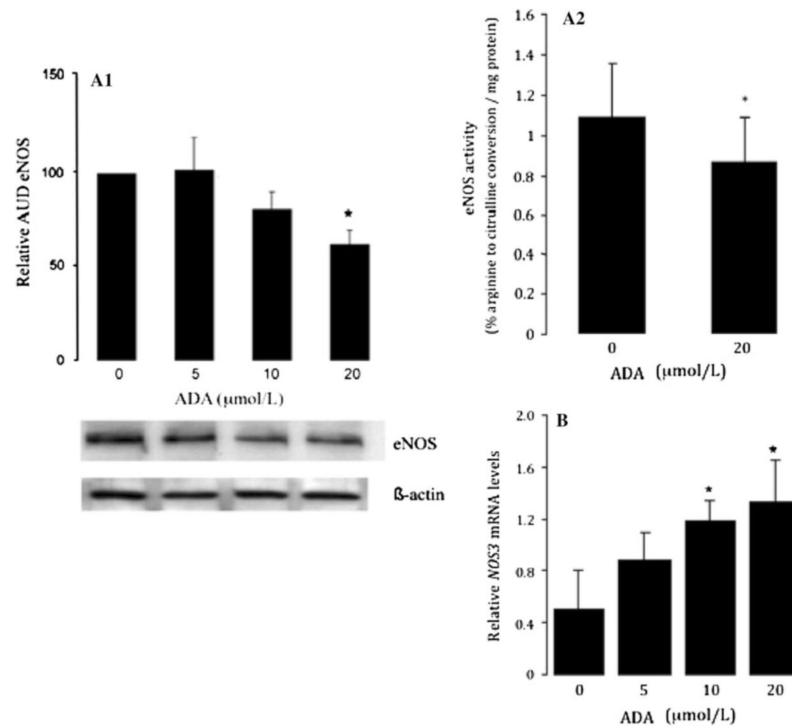

Fig. 4.

eNOS expression in HUVEC cultured with increasing concentrations of ADA after 24 h of incubation. Data are means ± SD. **a1** relative eNOS expression was determined by Western blotting with antibodies against eNOS and *β*-actin. Densitometry was performed on four blots. A representative blot is shown. (*$p<0.05$ vs. control). **a2** eNOS activity was measured in HUVEC incubated in the absence or presence of 20 μmol/L of ADA after 24 h by measuring the conversion of L-arginine to L-citrulline in cell lysates. Four different experiments were performed each in duplicate. (*$p<0.05$ paired Student's *t* test vs. control). **b** Real-time quantitative RT-PCR of relative *NOS3* mRNA levels of three or more independently prepared cDNA pools representing independent RNA isolations. (*$p<0.05$ vs. control)