Consumption of nitric oxide by endothelial cells: Evidence for the involvement of a NAD(P)H-, flavin- and heme-dependent dioxygenase reaction

Kurt Schmidt*, Bernd Mayer

Department of Pharmacology and Toxicology, Karl-Franzens-Universität Graz, Universitätsplatz 2, A-8010 Graz, Austria

Received 28 August 2004; revised 2 October 2004; accepted 4 October 2004

Available online 14 October 2004

Edited by Barry Halliwell

Abstract In the present study, we investigated the mechanism of nitric oxide (NO) inactivation by endothelial cells. All experiments were performed in the presence of superoxide dismutase to minimize the peroxynitrite reaction. Incubation of the NO donor diethylamine/NO adduct with increasing amounts of intact cells led to a progressive decrease of the NO concentration, demonstrating a cell-dependent consumption of NO. In cell homogenates, consumption of NO critically depended on the presence of NADPH or NADH and resulted in the formation of nitrate. Both NO consumption and nitrate formation were largely inhibited by the heme poisons NaCN and phenylhydrazine as well as the flavoenzyme inhibitor diphenylene iodonium. Further characterization of this NO consumption pathway suggests that endothelial cells express a unique membrane-associated enzyme or enzyme system analogous to the bacterial NO dioxygenase that converts NO to nitrate in a NAD(P)H-, flavin- and heme-dependent manner.

Keywords: Endothelial cell; Nitric oxide; Consumption; Nitrate; Nitric oxide dioxygenase

1. Introduction

Nitric oxide (NO) bioavailability is critically determined by both the rate of synthesis and decay. In aqueous aerobic solutions, NO decay is solely due to autoxidation yielding nitrite as end-product [1–3]. At physiologically relevant concentrations of NO, the autoxidation reaction is rather slow, but in hydrophobic compartments such as the cell membrane the reaction is ~300-fold accelerated because of increased partitioning of NO and oxygen within the membrane interior [4]. Nonetheless, the autoxidation reaction is still not fast enough to solely account for the extremely short biological half-life of NO (1–2 s [5,6]), indicating that additional, cell-dependent mechanisms are involved in the inactivation of NO. One well known pathway contributing to the rapid inactivation of NO in tissues is through its reaction with superoxide yielding peroxynitrite [7]. In the vascular system, formation of superoxide, mainly catalyzed by NAD(P)H oxidases [8], particularly contributes to the inactivation of NO under pathological conditions [9]. NO also reacts very rapidly with hemoglobin but this reaction is limited by the diffusion of NO into erythrocytes [10,11], questioning whether hemoglobin substantially contributes to the biological inactivation of NO [12]. In the last few years, several other NO consumption pathways have been identified, including lipoxygenases [13,14], prostaglandin H synthase [15], peroxidases [16], cytochrome *c* oxidase [17], and catalase [18]. While inactivation of NO by these enzymes ultimately results in the formation of nitrite, oxidation of NO to nitrate by dihydrolipoamide dehydrogenase [19], a flavohemoglobin-like NO dioxygenase [20,21] and a yet unidentified protein [22] has also been demonstrated.

In the course of our on-going studies on endothelial NO/cyclic GMP signaling, we have observed a pronounced enzymatic inactivation of NO that was insensitive to superoxide dismutase (SOD) and thus not attributable to the peroxynitrite reaction. The aim of this study was to elucidate the mechanism(s) underlying vascular endothelial NO consumption that may play a pivotal role in cardiovascular diseases associated with endothelial dysfunction.

2. Materials and methods

2.1. Materials

Cell culture media, antibiotics and fetal calf serum were purchased from PAA Laboratories GmbH (Linz, Austria). Diethylamine/NO (DEA/NO) was from Alexis (Grünberg, Germany); stock solutions were prepared in 10 mM NaOH. All other chemicals including SOD (from bovine erythrocytes, 2500–7000 units/mg protein, product number S 2515) were from Sigma (Vienna, Austria).

2.2. Cell culture

Porcine aortic endothelial cells were isolated as described previously [23] and cultured at 37 °C and 5% CO_2 up to 3 passages in Dulbecco's modified Eagle's medium, containing 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 1.25 μg/ml amphotericin B.

2.3. Preparation of cell suspensions, homogenates and subcellular fractions

For experiments with intact cells, endothelial cells from 5 to 10 petri dishes (~5×10^6 cells/per dish) were harvested, washed with phosphate-buffered saline and resuspended in incubation buffer (50 mM Tris/HCl, pH 7.4, containing 100 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 3 mM $CaCl_2$). For preparation of homogenates, endothelial cells

* Corresponding author. Fax: +43-316-380-9890.
E-mail address: kurt.schmidt@uni-graz.at (K. Schmidt).

Abbreviations: DEA/NO, diethylamine/NO adduct; DPI, diphenylene iodonium; DTPA, diethylenetriamine pentaacetic acid; ETYA, eicosatetraynoic acid; NO, nitric oxide; SOD, superoxide dismutase

0014-5793/$22.00
doi:10.1016/j.febslet.2004.10.010

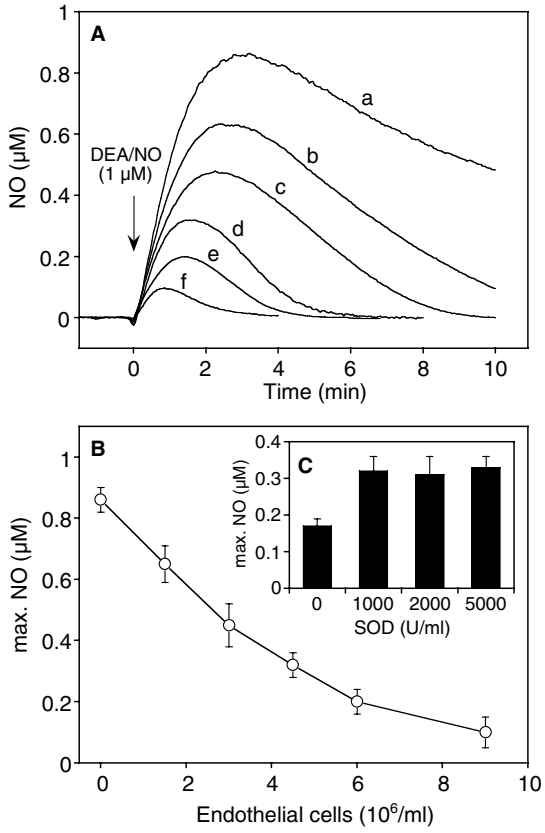

Fig. 1. NO consumption by intact endothelial cells. (A) Representative recordings of the NO concentration profiles obtained upon decomposition of 1 μM DEA/NO in buffer containing 1000 U/ml SOD in the absence (a) and presence of 1.5×10^6 (b), 3×10^6 (c), 4.5×10^6 (d), 6×10^6 (e), and 9×10^6 cells/ml (f). (B) Maximal concentrations of NO obtained following addition of 1 μM DEA/NO to buffer containing 1000 U/ml SOD and cells as indicated. (C) Maximal concentrations of NO obtained following addition of 1 μM DEA/NO to buffer containing 4.5×10^6 cells/ml and SOD as indicated. Data shown in (B) and (C) are mean values ± S.E.M. ($n = 3$).

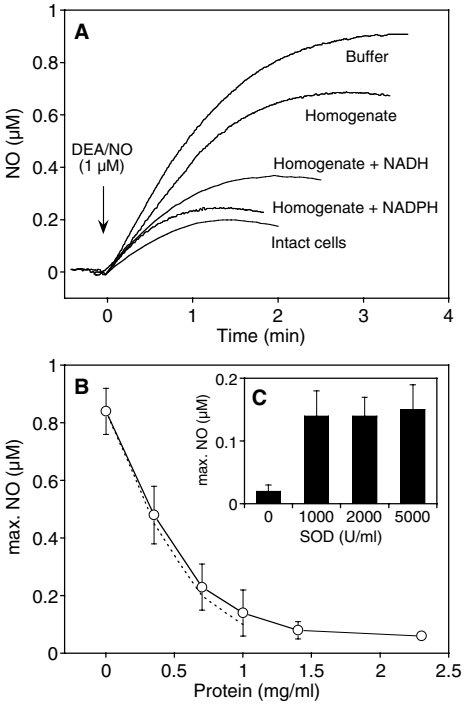

Fig. 2. NO consumption by endothelial cell homogenates. (A) Representative recordings of the NO concentration profiles obtained upon decomposition of 1 μM DEA/NO in buffer, homogenates (0.7 mg/ml protein) or cell suspensions (6×10^6 cells/ml). Experiments were performed in the presence of 1000 U/ml SOD and, where indicated, 0.2 mM NADPH or NADH. (B) Maximal concentrations of NO obtained following addition of 1 μM DEA/NO to buffer or homogenates (0.35–2.3 mg/ml protein) in the presence of 1000 U/ml SOD and 0.2 mM NADPH. Results obtained with intact cells (Fig. 1B) are shown as dotted line. (C) Maximal concentrations of NO obtained following addition of 1 μM DEA/NO to homogenates (1 mg/ml protein), containing 0.2 mM NADPH and SOD as indicated. Data shown in (B) and (C) are mean values ± S.E.M. ($n = 3$).

from 20 to 30 petri dishes were harvested, washed with phosphate-buffered saline, resuspended in 1 ml of ice-cold incubation buffer and disrupted by sonication. Where indicated, homogenates were centrifuged for 30 min at $30\,000 \times g$ to obtain particulate and cytosolic fractions. Protein was determined with the Bradford [24] method using bovine serum albumin as standard.

2.4. Electrochemical detection of NO

NO concentrations were measured with a Clark-type electrode (ISO-NO, World Precision Instruments, Mauer, Germany) under constant stirring in an open, thermostated vessel (37 °C) containing 1 ml of incubation buffer (see above) and cells or homogenate as indicated. Unless otherwise stated, all experiments were performed in the presence of 1000 U/ml of SOD. After equilibration or preincubation with the inhibitors (10 min), DEA/NO (final concentration 1 μM) was added and the concentration of NO monitored over time. Where indicated, experiments were performed in the presence of NADPH or NADH, which was added immediately before addition of DEA/NO.

2.5. Determination of nitrite and nitrate

The formation of nitrite and nitrate from DEA/NO (10 μM) was determined by allowing the donor (half-life = 2.1 min at pH 7.4 and 37 °C) to decompose for 1 h at 37 °C in 0.4 ml incubation buffer, containing 500 U/ml of SOD, 0.2 mM of NADPH, and homogenates as indicated, followed by determination of nitrite and nitrate using a spectrophotometric method adapted from Miranda et al. [25]. Where indicated, homogenates were preincubated with inhibitors for 10 min at 37 °C prior to the addition of NADPH and DEA/NO. For the determination of nitrite, 0.3 ml of the incubated samples was mixed with 0.3 ml of Griess reagent (20 mg naphthylethylenediamine and 200 mg sulfanilamide in 5% (v/v) HCl) and incubated for 15 min at 37 °C. Following centrifugation at $20{,}000 \times g$ (5 min), 0.2 ml of the supernatants was transferred into a 96-well plate for the photometrical determination of nitrite. Subsequently, 0.1 ml of a saturated VCl_3 solution (40 mg in 5 ml of 1 N HCl) was added to each well and the absorbance measured after a 90-min incubation at 37 °C to determine the concentration of nitrite plus nitrate, from which the nitrite concentration was subtracted to obtain the concentration of nitrate. Nitrite and nitrate standards were prepared in buffer containing SOD and NADPH and assayed as described above. Data obtained with homogenates were corrected for endogenous nitrite and nitrate determined in the absence of DEA/NO.

3. Results

3.1. NO consumption by intact cells

Consistent with the known rates of NO release from DEA/NO and autoxidation [26], decomposition of 1 μM DEA/NO in buffer resulted in a transient increase in the NO concentration that reached a peak of 0.85 μM after ~3 min and then declined to 0.48 μM within 10 min (Fig. 1A, curve a). In the presence of 1.5×10^6 cells/ml (curve b), the peak was smaller (0.63 μM) and followed by a more pronounced decline in NO

(0.1 μM at 10 min). Increasing the cell concentration progressively enhanced the consumption of NO such that only a marginal and short-lasting increase in NO (peak ~0.1 μM, duration <4 min) was obtained in the presence of 9×10^6 cells/ml (curve f). As evident from Fig. 1B, there was an inverse, almost linear correlation between the amount of endothelial cells and the peak concentration of NO, demonstrating that the decline in the NO peak can serve as a reliable measure for NO consumption. To minimize superoxide-mediated NO inactivation, these experiments were performed in the presence of 1000 U/ml SOD. As shown in Fig. 1C, the peak concentration of NO was not affected when SOD was increased to 2000 or 5000 U/ml, demonstrating that the concentration of SOD applied in this study (1000 U/ml) is sufficient to outcompete the peroxynitrite reaction.

3.2. NO consumption by homogenates

Consumption of NO was also observed with endothelial cell homogenates, but was less pronounced than with intact cells (Fig. 2A). However, addition of NADPH (0.2 mM) enhanced NO consumption by homogenates to a level comparable to that of intact cells without affecting NO concentrations measured in buffer or cell suspensions (data not shown). A similar, albeit less pronounced effect was observed when NADH (0.2 mM) was used instead of NADPH. Fig. 2B shows the concentration-dependent effect of endothelial cell homogenates on the peak concentrations of NO measured in the presence of 0.2 mM NADPH. For comparison, the results obtained with intact endothelial cells (cf. Fig. 1B), normalized to cellular protein, are shown as dotted line. At equivalent protein concentrations, intact cells and homogenates showed virtually identical NO consumption, indicating that the loss of NO consuming activity upon cell homogenization is due to dilution of an essential nucleotide cofactor. Therefore, all further experiments were routinely performed in the presence of 0.2 mM NADPH. As in intact cells, 1000 U/ml of SOD was sufficient to prevent superoxide-mediated NO inactivation (Fig. 2C).

As shown in Fig. 3, the effect of cell homogenates was virtually abolished after heat-denaturation, suggesting the involvement of a heat-labile factor, presumably a protein. The essential role of NAD(P)H and oxidation of NO to nitrate (see below) suggested the involvement of flavin and heme containing proteins. Indeed, preincubation of the homogenate for 10 min with the flavoenzyme inhibitor diphenylene iodonium (DPI) (0.1 mM) or the heme poisons phenylhydrazine (0.1 mM) and NaCN (1 mM) substantially reduced the consumption of NO (Fig. 3). None of the drugs affected the NO signal measured upon decomposition of DEA/NO in buffer (data not shown). Inhibition of the arachidonic acid cascade by pretreatment of homogenates with 0.1 mM mepacrine (peak concentration 0.09 ± 0.03 μM NO), 0.1 mM eicosatetraynoic acid (ETYA) (0.11 ± 0.05 μM NO) or 0.1 mM indomethacin (0.1 ± 0.04 μM NO) was ineffective, excluding an involvement of prostaglandin H synthase and lipoxygenases. Similarly, the soluble guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (0.1 mM, 0.1 ± 0.02 μM NO) and the Fe^{2+} chelator diethylenetriamine pentaacetic acid (DTPA) (0.1 mM, 0.1 ± 0.06 μM NO) had no effect, indicating that neither scavenging of NO by the ferrous heme bound to soluble guanylyl cyclase nor Fenton chemistry or lipid peroxidation

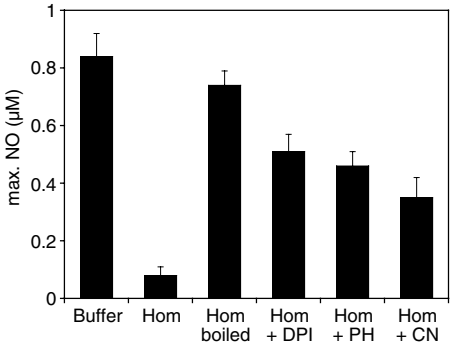

Fig. 3. Effects of inhibitors on NO consumption by cell homogenates. Homogenates (1.4 mg/ml protein) were preincubated for 10 min at 37 °C in the presence of 1000 U/ml SOD and, where indicated, 0.1 mM DPI, 0.1 mM phenylhydrazine (PH), or 1 mM NaCN (CN). Following addition of 0.2 mM NADPH and 1 μM DEA/NO, the maximal concentration of NO was determined. For heat-denaturation, the homogenates were boiled for 10 min prior to the addition of SOD, NADPH and DEA/NO. Data are mean values ± S.E.M. ($n = 3$–5).

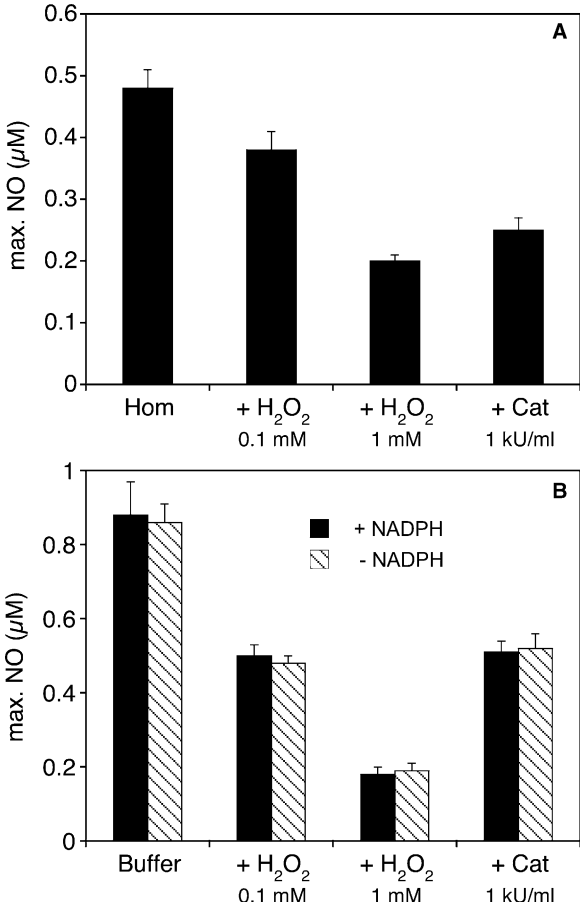

Fig. 4. Effects of H_2O_2 and catalase on NO consumption. (A) Maximal concentrations of NO obtained following addition of 1 μM DEA/NO to homogenates (0.35 mg/ml protein), containing 1000 U/ml SOD, 0.2 mM NADPH, and, where indicated, H_2O_2 (0.1–1 mM) or catalase (1000 U/ml). (B) Maximal concentrations of NO obtained following addition of 1 μM DEA/NO to buffer, containing 1000 U/ml SOD and, where indicated, H_2O_2 (0.1–1 mM) or catalase (1000 U/ml). Experiments were either performed in the absence (hatched columns) or presence of 0.2 mM NADPH (filled columns). Data are mean values ± S.E.M. ($n = 3$).

significantly contributes to the consumption of NO by endothelial cells.

Since peroxidases were reported to inactivate NO [16], we studied whether addition of H_2O_2 or catalase affects the endothelial consumption of NO. These experiments were performed at lower protein concentrations (0.35 mg/ml) to allow the detection of both increased and decreased rates of consumption. Addition of H_2O_2 (0.1–1 mM) to homogenates enhanced NO consumption (Fig. 4A), but the same effect was observed in buffer containing SOD (Fig. 4B). Omission of SOD from the buffer completely abolished the effect of H_2O_2 (data not shown), suggesting that the inactivation of NO was caused by SOD operating in reverse mode, i.e., catalyzing the formation of superoxide from H_2O_2 [27]. NO was also consumed by catalase in both homogenate (Fig. 4A) and buffer (Fig. 4B). Since the effects of catalase and SOD/H_2O_2 were already observed in the absence of NADPH, neither of these pathways appears to contribute to the NAD(P)H-dependent consumption of NO by endothelial cells.

3.3. NO consumption by subcellular fractions

After centrifugation of the homogenate at 30 000 × g, about 80% of the NO consuming activity was found in the particulate fraction (data not shown). At equivalent protein concentrations (0.35 mg/ml), NO consumption by the particulate fraction was substantially higher than by the homogenate, whereas the cytosolic fraction was virtually inactive (Fig. 5A). The NO consuming activity of the particulate fraction was markedly inhibited after preincubation with 0.1 mM DPI or 1 mM NaCN and virtually abolished in the absence of NADPH. As shown in Fig. 5B, maximal NO consumption was obtained with 0.2 mM NADPH and the EC_{50} was ~10 μM. NADH was slightly more potent than NADPH (EC_{50} ~ 2.5 μM), but the maximal effect was less pronounced (75% at 20 μM).

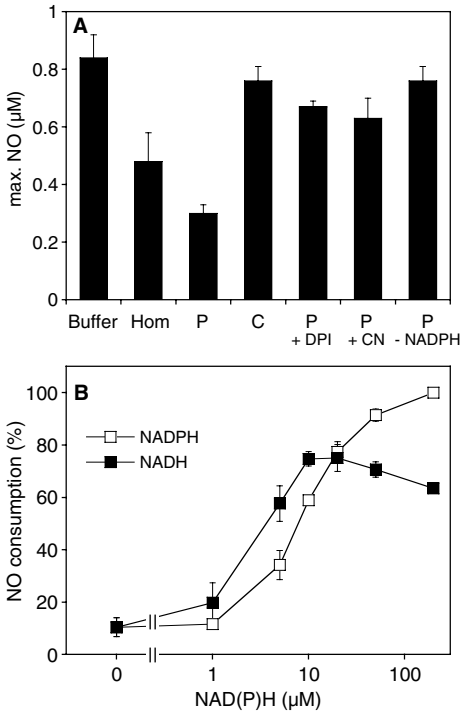

Fig. 5. NO consumption by subcellular fractions. (A) Maximal concentrations of NO obtained following addition of 1 μM DEA/NO to buffer, homogenates, particulate (P) and cytosolic fractions (C, 0.35 mg/ml protein, each), containing 1000 U/ml SOD and 0.2 mM NADPH. Where indicated, proteins were preincubated for 10 min at 37 °C in the presence of 0.1 mM DPI or 1 mM NaCN (CN) or experiments performed in the absence of NADPH. (B). Effects of NADPH and NADH on NO consumption by particulate fractions. Values are expressed as percentage of NO consumption measured in the presence of 0.2 mM NADPH. Data are mean values ± S.E.M. ($n = 3$).

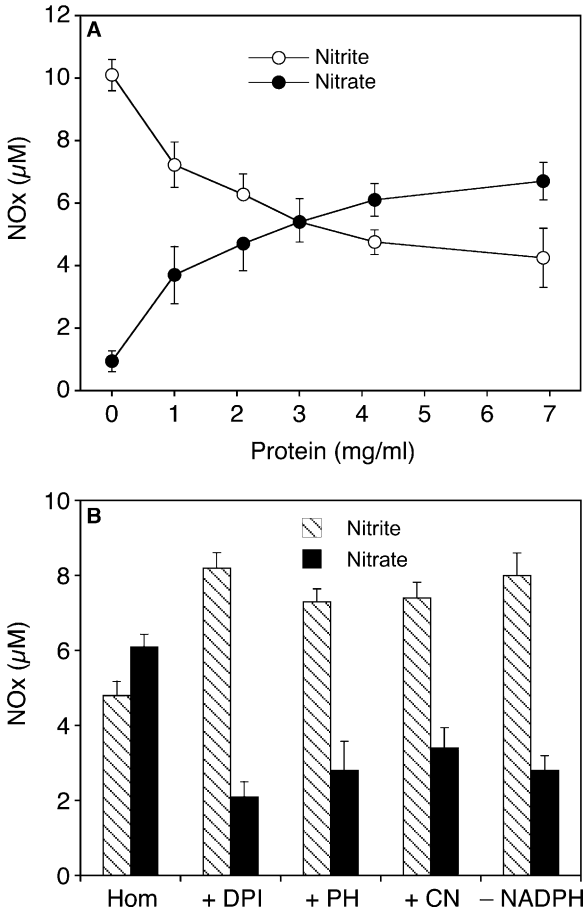

Fig. 6. Effect of homogenates on nitrite and nitrate formation. (A) Buffer or homogenates (1.0–6.9 mg/ml protein) were incubated for 60 min at 37 °C with 10 μM DEA/NO in the presence of 500 U/ml SOD and 0.2 mM NADPH, and then nitrite (open circles) and nitrate (filled circles) were determined as described in Section 2. (B) Homogenates (4.2 mg/ml protein) were preincubated for 10 min at 37 °C in the presence of 500 U/ml SOD and, where indicated, 0.1 mM DPI, 0.1 mM phenylhydrazine (PH), or 1 mM NaCN (CN). Then, 10 μM DEA/NO and 0.2 mM NADPH (if not otherwise indicated) were added, samples incubated for 60 min, and nitrite (hatched columns) and nitrate (filled columns) determined. Data are mean values ± S.E.M. ($n = 3$–5).

3.4. Nitrate formation by homogenates

To get more information on mechanism underlying the consumption of NO by endothelial cells, we measured the NO oxidation products, nitrite and nitrate, following decomposition of DEA/NO (10 μM) in buffer and cell homogenates (Fig. 5). Since the 1000 U/ml of SOD used in the other experiments interfered with the detection of nitrate, the amount of SOD was reduced to 500 U/ml. Consistent with the well known NO autoxidation reaction [1–3], the major end-product of NO degradation in buffer was nitrite (10.1 ± 0.5 μM), and nitrate was barely detectable (0.9 ± 0.3 μM, Fig. 6A). Addition

of increasing amounts of homogenate progressively increased the concentration of nitrate up to 6.7 ± 0.6 μM and, conversely, diminished the concentration of nitrite to 4.2 ± 1.0 μM. Nitrate was not detectable upon incubations of the homogenates with 10 mM nitrite, suggesting a direct oxidation of NO to nitrate. In accordance with the NO consumption data, DPI (0.1 mM), phenylhydrazine (0.1 mM), NaCN (1 mM) and omission of NADPH reduced the formation of nitrate by homogenates (Fig. 6B). Treatment of the homogenates with 0.1 mM ETYA (6.1 ± 0.8 μM nitrate), 0.1 mM indomethacin (6.6 ± 0.5 μM), 0.1 mM DTPA (5.8 ± 0.4 μM) or the peroxynitrite/superoxide scavenger Mn(III)tetrakis(4-benzoic acid)porphyrin (0.1 mM, 6.2 ± 0.7 μM nitrate) had no effect. Moreover, modulation of intracellular tetrahydrobiopterin levels by preincubation of the cells for 24 h with 0.1 mM sepiapterin (5.8 ± 0.5 μM nitrate) or 50 mM 2,4-diamino-6-hydroxypyrimidine (6.5 ± 0.6 μM) or preincubation of the homogenate with 0.1 mM miconazole (6.2 ± 0.4 μM), 0.1 mM econazole (6.1 ± 0.1 μM), or 1 mM N^G-nitro-L-arginine (6.3 ± 0.5 μM) was ineffective, excluding a role of dihydropteridine reductase, cytochrome P450 reductase or NO synthase in the conversion of NO to nitrate.

4. Discussion

In the present study, we demonstrate that endothelial cells express a considerable NO-consuming activity that is insensitive to SOD and thus not due to the reaction with superoxide. Consumption of NO was preserved in homogenates supplemented with NAD(P)H and resulted in the formation of nitrate. Lack of nitrate formation upon incubation of homogenates with nitrite suggests direct oxidation of NO by a dioxygenase. Together with the dependence on NADPH ($EC_{50} \sim 10$ μM) and NADH ($EC_{50} \sim 2.5$ μM), the sensitivity to DPI, phenylhydrazine and NaCN points to a combined action of a NAD(P)H:FAD oxidoreductase with a heme protein. Thus, NO consumption by endothelial cells appears remarkably similar to the reaction catalyzed by the bacterial flavohemoglobin, NO dioxygenase [28,29]. A BLASTP homology search using the protein sequence of *E. coli* flavohemoglobin as template yielded no promising mammalian analog. Only a minor homology (25%) was found for NADH cytochrome *b*5 reductase, but a role of this enzyme can be excluded, as it is highly specific for NADH [30,31]. Similarly, dihydrolipoamide dehydrogenase, which has been recently shown to catalyze the conversion of NO to nitrate in enzyme assays, is also specific for NADH [19]. Moreover, this enzyme is insensitive to DPI and cyanide, excluding its involvement in endothelial NO consumption. A role of cytochrome P450 reductase, which contributes to the oxidation of NO to nitrate by human intestinal Caco-2 cells [21], is also unlikely because of its very low affinity for NADH ($K_m \sim 15$ mM [32]). The lack of effect of miconazole and econazole also excludes classical P450 enzymes as the heme component of the NO oxidation pathway. Since NO consumption by Caco-2 cells is inhibited by imidazoles and barely stimulated by NADH [21], the NO metabolic pathway of endothelial cells appears to be different from that of intestinal cells.

Since the *E. coli* flavohemoglobin also functions as a dihydropteridine reductase [33,34], we tested for a role of this enzyme by modulating endothelial tetrahydrobiopterin levels with sepiapterin or 2,4-diamino-6-hydroxypyrimidine [35], but none of the treatments affected nitrate formation. Together with the lack of nitrite oxidation by endothelial homogenates, these data also argue against the involvement of a tetrahydrobiopterin-dependent conversion of nitrite to nitrate as shown for hepatocytes [36]. Recently, an oxidation of NO to nitrate has also been observed in cerebellar cells and brain homogenates [22]. The molecular mechanism underlying this reaction is unclear but apparently involves a protein, since the effect was virtually abolished by heat or protease treatment. However, in contrast to our results obtained with endothelial cells, NO consumption in the brain was insensitive to cyanide and DPI, suggesting a distinct mechanism for the inactivation of NO.

To further characterize the enzyme(s) involved in the endothelial consumption of NO, we tried to purify the protein(s). After centrifugation of the homogenate at $30\,000 \times g$, about 80% of the NO consuming activity was found in the particulate fraction, indicating that in contrast to the soluble *E. coli* NO dioxygenase, the mammalian analog is associated with the plasma membrane or intracellular organelles. For solubilization of the protein, several detergents including Triton X-100, CHAPS and octyl-β-D-glycopyranoside were tested, but found to be either ineffective or inapplicable due to inhibition of NO consumption by homogenates (data not shown). Further experiments are currently performed in our laboratory to establish an alternative approach for purification and identification of the endothelial NO consuming enzyme (system).

In summary, our data demonstrate that endothelial cells inactivate NO to nitrate in a unique NAD(P)H-, flavin- and heme-dependent reaction that may be catalyzed by a membrane-associated analog of the bacterial NO dioxygenase. This as yet unidentified enzyme (system) may contribute to the biological inactivation of NO in the vascular system. Since decreased NO bioavailability is a major cause of endothelial dysfunction [37], identification of the NO consumption pathway is essential for the development of new therapeutic strategies to interfere with pathological NO oxidation in cardiovascular disease.

Acknowledgements: We thank Margit Rehn for excellent technical assistance. This work was supported by the Fonds zur Förderung der Wissenschaftlichen Forschung in Österreich.

References

[1] Ignarro, L.J., Fukuto, J.M., Griscavage, J.M., Rogers, N.E. and Byrns, R.E. (1993) Proc. Natl. Acad. Sci. USA 90, 8103–8107.
[2] Lewis, R.S. and Deen, W.M. (1994) Chem. Res. Toxicol. 7, 568–574.
[3] Goldstein, S. and Czapski, G. (1995) J. Am. Chem. Soc. 117, 12078–12084.
[4] Liu, X.P., Miller, M.J.S., Joshi, M.S., Thomas, D.D. and Lancaster, J.R. (1998) Proc. Natl. Acad. Sci. USA 95, 2175–2179.
[5] Thomas, D.D., Liu, Z.P., Kantrow, S.P. and Lancaster, J.R. (2001) Proc. Natl. Acad. Sci. USA 98, 355–360.
[6] Kelm, M. and Schrader, J. (1990) Circ. Res. 66, 1561–1575.
[7] Beckman, J.S., Beckman, T.W., Chen, J., Marshall, P.A. and Freeman, B.A. (1990) Proc. Natl. Acad. Sci. USA 87, 1620–1624.
[8] Wolin, M.S., Gupte, S.A. and Oeckler, R.A. (2002) J. Vasc. Res. 39, 191–207.
[9] Kojda, G. and Harrison, D. (1999) Cardiovasc. Res. 43, 562–571.

[10] Liu, X.P., Miller, M.J.S., Joshi, M.S., Sadowska-Krowicka, H., Clark, D.A. and Lancaster, J.R. (1998) J. Biol. Chem. 273, 18709–18713.
[11] Vaughn, M.W., Huang, K.T., Kuo, L. and Liao, J.C. (2000) J. Biol. Chem. 275, 2342–2348.
[12] Liao, J.C., Hein, T.W., Vaughn, M.W., Huang, K.T. and Kuo, L. (1999) Proc. Natl. Acad. Sci. USA 96, 8757–8761.
[13] Coffey, M.J., Natarajan, R., Chumley, P.H., Coles, B., Thimmalapura, P.R., Nowell, M., Kühn, H., Lewis, M.J., Freeman, B.A. and O'Donnell, V.B. (2001) Proc. Natl. Acad. Sci. USA 98, 8006–8011.
[14] O'Donnell, V.B., Taylor, K.B., Parthasarathy, S., Kühn, H., Koesling, D., Friebe, A., Bloodsworth, A., Darley-Usmar, V.M. and Freeman, B.A. (1999) J. Biol. Chem. 274, 20083–20091.
[15] O'Donnell, V.B., Coles, B., Lewis, M.J., Crews, B.C., Marnett, L.J. and Freeman, B.A. (2000) J. Biol. Chem. 275, 38239–38244.
[16] Abu-Soud, H.M. and Hazen, S.L. (2000) J. Biol. Chem. 275, 37524–37532.
[17] Torres, J., Sharpe, M.A., Rosquist, A., Cooper, C.E. and Wilson, M.T. (2000) FEBS Lett. 475, 263–266.
[18] Brunelli, L., Yermilov, V. and Beckman, J. (2001) Free Radical Biol. Med. 30, 709–714.
[19] Igamberdiev, A.U., Bykova, N.V., Ens, W. and Hill, R.D. (2004) FEBS Lett. 568, 146–150.
[20] Gardner, P.R., Martin, L.A., Hall, D. and Gardner, A.M. (2001) Free Radical Biol. Med. 31, 191–204.
[21] Hallstrom, C.K., Gardner, A.M. and Gardner, P.R. (2004) Free Radical Biol. Med. 37, 216–228.
[22] Griffiths, C., Yamini, B., Hall, C. and Garthwaite, J. (2002) Biochem. J. 362, 459–464.
[23] Schmidt, K., Mayer, B. and Kukovetz, W.R. (1989) Eur. J. Pharmacol. 170, 157–166.
[24] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[25] Miranda, K.M., Espey, M.G. and Wink, D.A. (2001) Nitric Oxide 5, 62–71.
[26] Schmidt, K., Desch, W., Klatt, P., Kukovetz, W.R. and Mayer, B. (1997) Naunyn-Schmiedeberg's Arch. Pharmacol. 355, 457–462.
[27] McBride, A.G., Borutaite, V. and Brown, G.C. (1999) Biochim. Biophys. Acta 1454, 275–288.
[28] Gardner, P.R., Gardner, A.M., Martin, L.A. and Salzman, A.L. (1998) Proc. Natl. Acad. Sci. USA 95, 10378–10383.
[29] Gardner, P.R., Gardner, A.M., Martin, L.A., Dou, Y., Li, T., Olson, J.S., Zhu, H. and Riggs, A.F. (2000) J. Biol. Chem. 275, 31581–31587.
[30] Tauber, A.I., Wright, J., Higson, F.K., Edelman, S.A. and Waxman, D.J. (1985) Blood 66, 673–678.
[31] Marohnic, C.C., Bewley, M.C. and Barber, M.J. (2003) Biochemistry 42, 11170–11182.
[32] Hubbard, P.A., Shen, A.L., Paschke, R., Kasper, C.B. and Kim, J.P. (2001) J. Biol. Chem. 276, 29163–29170.
[33] Vasudevan, S.G., Shaw, D.C. and Armarego, W.L. (1988) Biochem. J. 255, 581–588.
[34] Vasudevan, S.G., Armarego, W.L., Shaw, D.C., Lilley, P.E., Dixon, N.E. and Poole, R.K. (1991) Mol. Gen. Genet. 226, 49–58.
[35] Schmidt, K., Werner, E.R., Mayer, B., Wachter, H. and Kukovetz, W.R. (1992) Biochem. J. 281, 297–300.
[36] Kim, Y.M. and Lancaster Jr., J.R. (1993) FEBS Lett. 332, 255–259.
[37] Kelm, M. and Rath, J. (2001) Basic Res. Cardiol. 96, 107–127.