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Research Article

Nitric oxide synthase reduces nitrite to NO under anoxia

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Abstract. Cultured bEND.3 endothelial cells show a marked increase in NO production when subjected to anoxia, even though the normal arginine pathway of NO formation is blocked due to absence of oxygen. The rate of anoxic NO production exceeds basal unstimulated NO synthesis in normoxic cells. The anoxic release of NO is mediated by endothelial nitric oxide synthase (eNOS), can be abolished by inhibitors of NOS and is accompanied by consumption of intracellular nitrite. The anoxic NO release is unaffected by the xanthine oxidase inhibitor oxypurinol. The phenomenon is attributed to anoxic reduction of intracellular nitrite by eNOS, and its magnitude and duration suggests that the nitrite reductase activity of eNOS is relevant for fast NO delivery in hypoxic vascular tissues.

Keywords. Nitric oxide, nitrite, nitrite reductase, endothelial NOS, anoxia, ischemia.

Introduction

Nitrite has recognized beneficial effects when living tissue is subjected to conditions of low oxygen tension as may arise in ischemia, hypoxia or anoxia [1–3], but the mechanism of this phenomenon has remained controversial so far. Nitrite anions (NO_2^-) are metastable intermediates in the oxidation cascade [4], leading from nitric oxide radicals to the stable metabolite nitrate (NO_3^-). Nitrite levels in the vasculature are clearly positively correlated with endothelial NO synthase (eNOS) activity [5–8]. Typical nitrite levels [1, 4, 5, 9] are 0.1–1.0 μM in plasma, 0.5–2.0 μM in normoxic tissues with still higher levels of up to 20 μM reported [9, 10] in vascular tissues. Interestingly, the nitrite concentrations in oxygen-rich arterial blood were found to be higher than in venous blood [5, 11]. This arterial-venous gradient was interpreted as a manifestation of nitrite delivery to the perivascular tissues, and it was suggested that nitrite actually plays an active physiological role in control of the vascular flow. However, infusion studies did not show vasodilatory capacity of nitrite as such in humans under normoxia [8]. Rather, low oxygen tension seemed a crucial aspect of *in vivo* studies of vasodilation with direct infusion of nitrite [6, 12].

The undisputed beneficial effects of nitrite under ischemia are attributed to the reduction of nitrite back to NO, but the dominant mechanism for this reduction has been controversial. Direct uncatalyzed reduction is very slow [13] except at extreme acidic conditions as may arise in the stomach [14, 15], urine [16] or ischemic tissue [17, 18]. Therefore, enzymatic mediators for this nitrite reduction have been proposed. Dedicated nitrite reductases are known in bacteria but are lacking in mammals. However, certain mammalian enzymes show some nitrite reductase capacity supplementary to their normal physiological function. Examples are glutathione-S-transferase [19], xanthine oxido-reductase (XOR) [20–22], deoxy-hemo-

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globin [1, 5, 23, 24] and cytochrome P_{450} [25]. Finally, nitrite reductase activity has been reported for mammalian mitochondria. Mitochondrial cytochrome *c* oxidase may release NO under hypoxia [26], and another mechanism has been reported [27] involving ubiquinol and complex III of the respiratory chain. Although these various reaction pathways have been documented, their physiological relevance remains invalidated *in vivo*. Not all of them necessarily release NO in the form of a free radical. For example, the vasodilation activity of nitrite and deoxyhemoglobin was reported [5, 24] to be mediated by protein bound S-nitrosothiols.

Recently, we reported that eNOS has the capacity to reduce nitrite to NO under anoxia *in vitro* [28]. eNOS is the endothelial enzyme that catalyzes the synthesis of NO from L-arginine via an oxygen-consuming pathway. Since the conventional arginine pathway is blocked under conditions of low oxygen tension [29], we proposed that the newly discovered anoxic nitrite reductase pathway of eNOS might provide a significant alternative source of NO for tissues under acute hypoxia. In this work we demonstrate that the absence of oxygen causes a significant enhancement rather than a suppression of the NO release from endothelial cell cultures. The release of NO is enzymatically catalyzed by NOS, is accompanied by consumption of intracellular nitrite; and may be modulated by exogenous membrane-penetrating agents.

Materials and methods

Diethyldithiocarbamate (DETC) was purchased from Sigma (St Louis, USA), ferrous sulfate from Fluka (Buchs, Switzerland), cell culture materials from Life Technologies (Burlington, ON, Canada). The Ca-ionophore A23187 (CaI), N_ω-nitro-L-arginine (NLA) and N_ω-nitro-L-arginine-methyl ester (L-NAME) were from Sigma. Oxypurinol and the heme-inhibitor imidazole were from Sigma. The argon was purchased as compressed gas (product no. 14302, with purity >99.998% and [O_2] <3 ppb) from Air Products (Amsterdam, The Netherlands) and used without further processing.

The line of immortalized murine microvascular brain endothelial (bEND.3) cells [30] was kindly provided by Dr. Alan Schwartz (University of Washington, St. Louis, MO, USA). This cell line has high expression of eNOS but does not express inducible or neuronal NOS isoforms [31]. The cells were cultivated to confluence in 75 cm^2 flasks at 37 °C under a controlled atmosphere containing 5% CO_2 and 20% O_2. The DMEM growth medium contained 10% fetal calf serum, 2 mM L-glutamine, 10 IU/ml penicillin and 100 µg/l streptomycin. At confluence, the bottom of the flask was covered by a cellular monolayer consisting of $\sim 7.5 \pm 0.5 \times 10^6$ endothelial cells. Confluence and cell count are important parameters for NO synthesis [32] and were always verified by optical inspection via a stereomicroscope.

NO trapping in the cell cultures was initiated by replacing the medium with 10 ml fresh DMEM of 37 °C containing 2.5 mM DETC and adding ferrous sulfate (10 µM final). It should be noted that no stimulus for NO production was given to the cell cultures, so that the trapping experiment detected the basal NO production in the confluent endothelial cells. The trapping proceeded either in a normoxic atmosphere with 5% CO_2 and 20% O_2 or in an anoxic argon atmosphere.

Anoxia was initiated by replacing the medium with argon-bubbled DMEM of 37 °C containing 2.5 mM DETC, adding 10 µM ferrous sulfate and flushing the flask with argon before closing it with an airtight top. It was verified that flushing with argon did not change the temperature of the medium significantly. No chemical stimulus for NO production was administered. After 20 min of NO trapping at 37 °C, the flask was placed on ice for 2 min to terminate the enzymatic activity. Keeping the flask on ice throughout, the flask was opened, the cells scraped loose and harvested in ~4 ml of the incubation medium. The cellular fraction with the Fe-DETC complexes was separated by ultracentrifugation (1500 rpm for 10 min at 4 °C), resuspended in 350 ml incubation medium, pipetted into a syringe (id 4.8 mm) and snap frozen in liquid nitrogen until the frozen column was assayed with electron paramagnetic resonance (EPR).

The supplements were administered by preincubation at 37 °C in an atmosphere with 5% CO_2 and 20% O_2 for a certain time before the start of NO trapping. The preincubation times varied with supplement: preincubation with supplements like NLA, oxypurinol and nitrite was 20 min. The general heme inhibitor imidazole was administered only 1 min prior to initialization of NO trapping. Preincubation with L-NAME was for 2 or 20 min. All supplements were also present throughout the time of trapping.

EPR spectra were recorded at 77 K on a modified X-band ESP 300 radiospectrometer (Bruker BioSpin, Karlsruhe, Germany) operating near 9.54 GHz with 20 mW power. The frozen samples were placed in a quartz liquid finger dewar at the center of a Bruker ER4103TM cavity. The magnetic field was modulated at 100 kHz with an amplitude of 0.5 mT. The lock-in detector operated with gain 2×10^5, time constant and ADC conversion time 82 ms. Four scans were accumulated to reduce instrumental noise. The yields of NO-Fe^{2+}-DETC complexes in cell cultures were quantified by comparing the spin densities with frozen reference solutions of paramagnetic NO-Fe^{2+}-$(MGD)_2$ in PBS buffer.

Intracellular nitrite concentrations were determined with the nitrite colorimetric Griess reagent (Cayman Chemical, ITK diagnostics, Uithoorn, The Netherlands) on

0.5 ml cell lysate containing the cellular fraction of a single 75-cm^2 flask with 7.5×10^6 endothelial cells. The detection limit of this assay was ~0.2 μM nitrite in 0.5 ml lysate. This corresponds to a total quantity of ~100 pmol intracellular nitrite.

Cell viability after anoxia was verified by microscopic counting of dead cells after trypan blue staining of the cell culture. The staining and counting were done within 15 min after readmission of ambient air.

Results

NO trapping in oxygenated or anoxic cell cultures. Upon normoxic incubation for 20 min with 2.5 mM DETC and 10 μM ferrous sulfate, the cellular fraction from ~$7.5 \pm 0.5 \times 10^6$ endothelial cells had acquired a yield of 110 ± 8 pmol paramagnetic NO-Fe^{2+}-DETC mononitrosyl-iron complexes (MNIC) as detected by EPR. This basal yield was obtained without any stimulus and keeping the cells under controlled temperature and atmosphere with 5% CO_2 and 20% O_2. The yield was reproducible within 10% from flask to flask and day to day. A typical EPR spectrum (Fig. 1a–d) showed a clear triplet hyperfine structure centered at g = 2.035 as expected for MNIC. As expected for biological samples [33], a small contribution from paramagnetic Cu^{2+}-DETC complexes was superposed. The most intense central hyperfine line of these Cu^{2+}-DETC complexes was visible near g = 2.01 (Fig. 1). Preincubation for 20 min with 57 μM NLA reduced the MNIC yield to below the detection limit of ~10 pmol at the given spectrometer settings. At 5 μM NLA, a small yield of ~25 ± 2 pmol MNIC was detected (Fig. 1b).

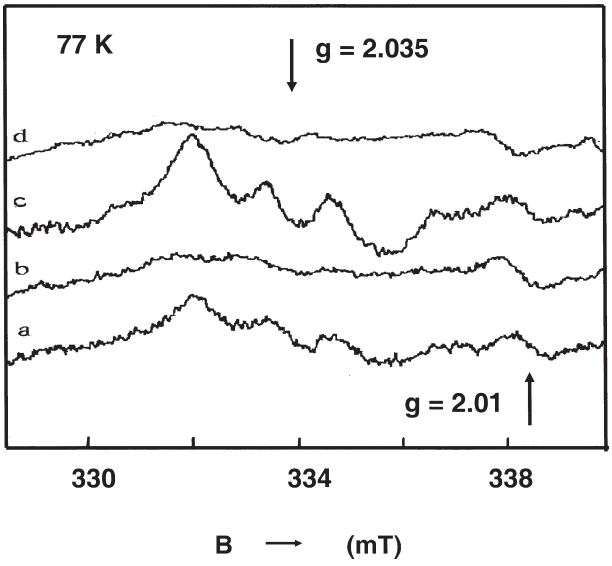

Figure 1. Electron paramagnetic resonance spectra at 77 K from the cellular fraction of ~$7.5 \pm 0.5 \times 10^6$ endothelial cells after NO trapping at 37 °C with iron-dithiocarbamate complexes. The triplet near g = 2.035 is identified as the mononitrosyl-iron complex (MNIC) adduct. The absorption line near g = 2.01 is the most intense hyperfine line of paramagnetic Cu^{2+}-diethyldithiocarbamate (DETC) complexes. (*a*) Under a controlled atmosphere containing 5% CO_2 and 20% O_2, 110 pmol MNIC formed in 20 min; (*b*) ~25 pmol MNIC formed in 20 min under the controlled atmosphere after preincubation with 5 μM NOS inhibitor NLA; (*c*) 160 pmol MNIC formed after 20 min anoxia. (d) Approximately 33 pmol MNIC formed during 20 min of anoxia after preincubation with 5 μM NOS inhibitor NLA.

This basal unstimulated yield of 110 ± 8 pmol compares favorably with a total MNIC yield of 400 ± 30 pmol as obtained when the cellular NOS production was stimulated by administration of 5 μM CaI A23187.

When anoxia was applied to the cells by argon, MNIC yields increased significantly over basal normoxic yields. In the absence of inhibitors, the anoxic yield from ~$7.5 \pm 0.5 \times 10^6$ endothelial cells increased to ~160 ± 10 pmol MNIC. These anoxic yields were typically ~50% higher than basal in the presence of oxygen (Fig. 1c).

The degree of anoxia was verified electrochemically by mounting a Clark type oxygen electrode in a 75-cm^2 culture flask. The electrode current at zero oxygen was calibrated at 37 °C by loading the flask with 10 ml borax (10 mM) and adding 100 mg sodium sulfite to remove the oxygen. The imposition of anoxia as described above was tested five times for the flask containing 10 ml medium. The imposition of anoxia consistently reduced the oxygen levels inside the medium to below the detection limit for dioxygen of ~2 μM.

In all cases considered (with and without oxygen, with and without supplements), the supernatant liquids were free of any EPR signals from MNIC or Cu^{2+}-DETC, attesting to the complete recovery of the paramagnetic complexes by separation of the cellular fraction via ultracentrifugation.

Inhibition of anoxic NO production by preincubation with NOS inhibitors. The MNIC yield under anoxia was completely abolished by preincubation for 20 min with 57 μM NLA. At 5 μM NLA, a small anoxic yield of ~33 ± 2 pmol MNIC was detected (Fig. 1d), slightly higher than observed before in the presence of oxygen. The intensity of the EPR absorption from paramagnetic Cu^{2+}-DETC complexes was not affected by anoxia.

Similarly, preincubation for 20 min with 5 μM L-NAME diminished the anoxic MNIC yield to ~85 ± 8 pmol. The same yield of 87 pmol was obtained if the cells were preincubated with 5 μM L-NAME for only 2 min prior to anoxia instead of 20 min. The anoxic yields were further reduced to 61 ± 5 pmol by reinforcing L-NAME to 50 μM. Generally, the anoxic yields were ~50% higher than the corresponding normoxic yields.

Preincubation with 10 mM of the heme-binding inhibitor imidazole diminished both normoxic and anoxic yields

Table 1. Yields of mononitrosyl-iron complex (MNIC) adducts (in pmol) in the cellular fractions of a single 75-cm^2 flask of cultured endothelial cells. Trapping proceeded for 20 min at 37 °C. The second row gives the preincubation times τ_{inc} of the supplements. During preincubation, the cells were kept at 37 °C in an atmosphere with 5% CO_2 and 20% O_2. 'Normoxia' indicates yields obtained under the atmosphere with 5% CO_2 and 20% O_2. 'Anoxia' indicates yields obtained under the argon atmosphere.

	Basal unstimulated	CaI (5 μM)	NLA (5 μM)	NLA (57 μM)	L-NAME (5 μM)	L-NAME (50 μM)	Imidazole (10 mM)	Oxypurinol (100 μM)	$NaNO_2$ (250 μM)
τ_{inc}	–	–	20 min	20 min	2 min	2 min	1 min	20 min	20 min
Normoxia	110 ± 8	400 ± 30	25 ± 2	[a]	93 ± 10	51 ± 8	85 ± 10	n.d.	116 ± 8
Anoxia	160 ± 10	n.d.	33 ± 2	[a]	87 ± 8 [b]	61 ± 8	80 ± 10	170 ± 10	154 ± 8

[a] Below EPR detection limit of ca 10 pmol. [b] 85 ± 8 pmol with τ_{inc} = 20 min.

to ~80 pmol. In contrast, preincubation for 20 min with 100 μM xanthine oxidase inhibitor oxypurinol did not affect the anoxic MNIC yields. The MNIC yields are collected in Table 1.

Extracellular nitrite does not affect the NO trapping yields. Alternatively, 250 μM exogenous sodium nitrite was administered starting from 20 min prior to NO trapping. This extracellular nitrite did not affect the MNIC yields, either in cultures that had been kept under controlled atmosphere, or in anoxic cultures. However, if these cellular fractions with exogenous nitrite were reduced with 20 mM sodium dithionite for 10 min, the MNIC yields were enhanced more than an order of magnitude to ~4 nmol. This experiment proves that the addition of 100 nmol ferrous iron per flask induces the formation of at least 4 nmol Fe-DETC traps in the cellular fraction of the culture.

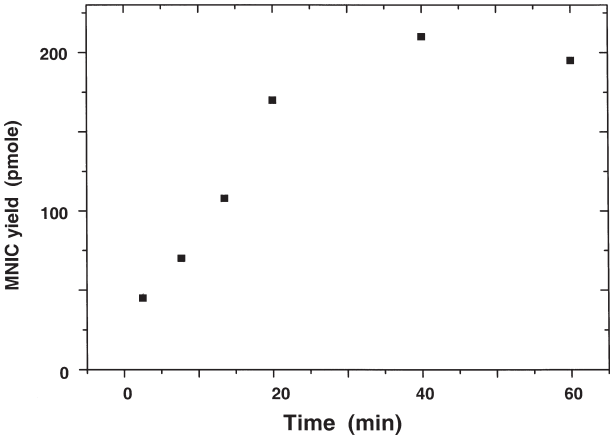

Figure 2. Kinetics of the formation of MNIC adducts in ~7.5 ± 0.5 × 10^6 endothelial cells. Anoxia was applied at 0 min. The EPR detection limit is ~10 pmol MNIC, and the experimental error ~10%.

Consumption of intracellular nitrite and viability of endothelial cells after anoxia. Intracellular nitrite levels were determined photospectrometrically by Griess coloration of cell lysates. The total nitrite content of a normoxic lysate from 7.5 ± 0.5 × 10^6 cells was 1.1 ± 0.2 nmol nitrite. The imposition of anoxia was seen to deplete the intracellular nitrite rapidly: After 10 min of anoxia, the quantity of nitrite had fallen eightfold to 0.15 ± 0.07 nmol. After 20 min of anoxia or longer, the intracellular nitrite had fallen below the detection limit of our assay (~0.1 nmol). Trypan blue staining of the cell cultures did not show any signs of enhanced mortality of cells that had been subject to anoxia. In all cases considered (normoxia, 10-min anoxia and 30-min anoxia) the percentage of dead cells was less than 0.1%.

Kinetics of the anoxic release of free NO. Finally, the kinetics of MNIC formation under anoxia was studied as a function of time (Fig. 2). The MNIC yield showed an approximately linear increase with time up to ~25–30 min after imposition of anoxia. At later times the signal intensity saturated at an asymptotic value of ~200 ± 20 pmol MNIC. The samples could be kept at 37 °C for more than 1 h without significant loss of MNIC adducts. When frozen in liquid nitrogen, the samples could be stored for several months.

The pH of the supernatant DMEM medium was seen to be affected by the imposition of anoxia. The pH of the DMEM medium was 7.5 ± 0.1 immediately after removal from the controlled atmosphere with 5% CO_2 and 20% O_2. The pH increased to 8.4 ± 0.1 by equilibration with ambient air, attesting to the small buffering capacity of DMEM medium with respect to the presence of CO_2 in the controlled atmosphere. In contrast, the cellular supernatants reached pH 9.2 ± 0.2 after exposure to anoxia for 20 min. Under anoxia, the pH increased with time for up to 30 min (data not shown). This rise in pH after anoxia was unaffected by the presence of the inhibitors NLA, L-NAME, oxypurinol and imidazole.

Discussion

The main and most prominent result from our experiments is the acute and large increase of MNIC yield in the endothelial cell cultures upon introduction of anoxia. Two observations show that the anoxic NO is released by eNOS: The effect of NLA and L-NAME shows that a

NOS isoform is involved, and Western blotting has shown that eNOS is the only isoform expressed by the bEND3 cell line [31].

The magnitude of the anoxic NO production is surprising, as the regular enzymatic pathway for NO production from arginine is blocked due to lack of oxygen. When discussing the physiological relevance of this new reaction pathway, we should clearly distinguish between three different regimes of oxygenation for vascular tissues: normoxia, hypoxia and real anoxia. Normoxic blood has a $pO_2 \sim 80–100$ Torr. As boundary between the hypoxic and anoxic regimes, we take the oxygen concentration below which the consumption of arginine via the normal enzymatic pathway is significantly slowed by lack of oxygen [29]. We define (see Fig. 11 in [29]) anoxia as $[O_2] < 15$ μM, corresponding to $pO_2 \sim 12$ Torr. Hypoxia refers to the intermediate interval 12–80 Torr where oxygen levels are sufficient to allow normal function of the eNOS enzyme.

Enhanced NO release from cultured endothelial cells under hypoxia has been reported before [34, 35]. The hypoxic enhancement was attributed to up-regulation of the arginine pathway by increases in the cytosolic calcium levels. Under the conditions chosen in these studies, the oxygen levels remained well above the critical level of 15 μM so that the arginine pathway was not blocked. In our study we consider the truly anoxic regime where oxygen is carefully excluded from the reaction vessels, culture media and from the atmospheres in the headspace and eNOS does not have sufficient residual oxygen for normal enzymatic function. Our study shows that the NO release from eNOS is enhanced even under these conditions. The eNOS starts to function as a nitrite reductase.

Our earlier *in vitro* experiments [28] documented the release of free NO from anoxic reduction of nitrite by eNOS. The NO was detected by three independent methods (optical spectroscopy, EPR spectroscopy and NO electrode) and isotopic labeling showed that nitrite was the source of the NO released by the eNOS. The release of NO was concomitant with consumption of NADPH and nitrite, and could be abolished by preincubation with the NOS inhibitors L-NAME and NLA. We attribute the anoxic NO release in the endothelial cell cultures to the same mechanism for two reasons. First, the consumption of intracellular nitrite and second, the inhibition by specific NOS inhibitors. In the discussion of the observed effect, we postpone consideration of the chemical source of NO and first consider the enzymatic mediator of the reaction. The significant suppression of the MNIC yields by imidazole suggests that a hemeprotein is involved in the observed effect. The dose-dependent inhibition by NLA and L-NAME inhibitors is specific for NOS. We did not attempt to estimate the IC_{50} dosages as these depend on the (unknown) intracellular L-arginine concentration, but we noted that the inhibitory doses for the anoxic pathway were comparable with those found for the regular arginine pathway. It suggests that, in absence of oxygen, nitro-arginine inhibitors act via direct competition with arginine for the arginine-binding site of NOS. This is analogous to the inhibition of the normal arginine pathway under normoxia. The observation that the MNIC yield is completely abolished by the NOS inhibitors shows that NOS is the dominant mediator in our cell cultures.

For the cultured endothelial cells considered here, the XOR enzyme was ruled out explicitly as a significant source of NO under hypoxia. Although this flavoenzyme is expressed in endothelial cells and has proven nitrite reductase activity *in vitro* under anoxic conditions [20–22], the anoxic NO yields in our cell cultures were not affected by addition of the XOR inhibitor oxypurinol.

Alternative non-enzymatic reaction pathways should be considered as well. Intracellular acidification may arise under anoxia [18]. In our assay, acidification did not release significant quantities of NO from intracellular nitrite since the observed NO release was completely cancelled by the specific NOS inhibitors NLA and L-NAME.

Next, the chemical source of the NO should be considered. Five possible sources come to mind: intracellular nitrite, extracellular nitrite, nitrate, arginine and endogenous S-nitrosothiols. Extracellular nitrite is ruled out as the source of anoxic NO since the yield did not increase upon administration of massive 250 μM extracellular nitrite prior to anoxia. It is likely that the timescale for equilibration of intra- and extracellular nitrite is long compared to our experiments since nitrite anions do not spontaneously cross the membrane barrier, and nitrite has a low degree of protonation at physiological pH ($pK_a = 3.2$). Reduction of nitrate *in vitro* requires strong reductors or catalyzing heavy metal ions that were lacking in our assay. Alternatively, hypoxic reduction of nitrate to nitrite and subsequently to NO by XOR [22] was ruled out because the formation of MNIC adducts was not affected by the presence of oxypurinol.

Arginine can also be ruled out as a direct source, since arginine oxidation requires the presence of dioxygen. Although some residual spurious oxygen may still be present in our system just after the imposition of anoxia, it is unimaginable that the arginine pathway remains active for up to 30 min, let alone enhances its activity by 50%. From the literature [29], it is known that the NO production via the arginine pathway collapses for dioxygen concentrations below 15 μM. We verified that the oxygen levels in our assay had fallen far below this critical threshold.

In vitro studies have shown that S-nitrosothiols may release free NO in the presence of reduced transition metal ions and may even directly nitrosylate iron complexes like the Fe-DETC traps used in this work. However, intracellular nitrosothiols can be ruled out as the source of NO since the anoxic NO burst could be suppressed

by NOS inhibitors. Since observed NO yields did not depend on extracellular nitrite levels, only intracellular nitrite remains as a plausible source of the NO released under hypoxia. Our previous data [26] have shown that eNOS is capable of nitrite reduction under hypoxia *in vitro*, and the Griess assay has confirmed the consumption of intracellular nitrite under anoxia. For a single flask with 7.5×10^6 cells we estimate a total intracellular volume of ~50 µl. (A confluent monolayer of bEND.3 cells is ~10 µm thick and covers ~70 ± 10% of the area of the 75-cm^2 culture flask. Therefore, the combined volume of all cells in a flask is estimated at 0.7×10 µm $\times$ 75 cm^2, = 52 ± 7 µl.) The Griess assay showed that this volume contained a total of ~1.1 nmol nitrite. This allows us to estimate the basal intracellular nitrite concentration as ~22 µM, a reasonable value in agreement with nitrite concentrations found in aortic tissues [9, 10].

The kinetics of anoxic NO release (Fig. 2) showed that the MNIC yield increased with time for up to about 25–30 min. The formation rate was ~160 pmol/20 min, *i.e.* 8 pmol MNIC/min. At any given time, the MNIC levels are a balance between formation and decay of NO-Fe^{2+}-DETC. Under our conditions, the decay of MNIC was negligible since these adducts were stable at 37 °C on a time scale of 1 h. Therefore, the kinetics of MNIC formation suggests that NO was released at a fairly constant rate for about 30 min, after which the release ceased. After 30 min, a total of ~200 pmol had been trapped by the Fe-DETC complexes, but the total NO production must have been higher since some NO will be bound in the form of EPR-silent diamagnetic NO-Fe^{3+}-DETC complexes [36], and some will be lost via other reaction pathways. The total anoxic NO release in a 75-cm^2 flask should be around 20–50 pmol NO/min. (EPR spectroscopy shows that the anoxic cell cultures form paramagnetic NO-Fe^{2+}-DETC complexes with a rate of 160 pmol/20 min, *i.e.* 8 pmol/min. The actual rate of NO formation is significantly higher: NO radicals may form ferric and ferrous mononitrosyl complexes with Fe-DETC traps. Only the ferrous fraction is paramagnetic and observed with EPR spectroscopy, whereas the ferric fraction remains unobserved in a standard trapping experiment. In biological systems, the fraction of ferric nitrosyl complexes is usually dominant. In [36] we have shown that the sum of ferric and ferrous nitrosyl adducts in bEND.3 cultures is four- to fivefold larger than the ferrous yield as observed in a standard EPR experiment as used in this work. Additionally, a small quantity of NO will have been lost to ambient atmosphere or reaction channels other than trapping by Fe-DETC complexes.) With the intracellular volume of ~50 µl (see above), this is equivalent to 0.4–1.0 pmol NO/min/mg endothelial cells. Under normoxic unstimulated conditions, we observed formation of MNIC at a rate of ~110 pmol/20 min (~5 pmol/min) corresponding to a release of 0.25–0.6 pmol NO/min/mg endothelial cells. This rough estimate is in reasonable agreement with estimates of 0.8 pmol NO/min/mg endothelial cells as a basal yield in humans [4, 37].

After ~30 min, the MNIC yield became stationary at ~200 pmol per flask. This yield is far smaller than the 4-nmol traps present in a flask and proves that only a small fraction of traps actually binds NO under the conditions used. Since enough Fe-DETC traps remain available, the observed saturation of the MNIC yield proves that the formation of NO under anoxia really ceases after ~30 min. The reason for this cessation should be considered. First, 30 min of anoxia might cause irreversible damage to the cells and changes in the chemical composition of the interior compartment. Endothelial cells react to oxygen deficiency by release of hypoxia-inducible factors (HIFs) [38] that initiate the transcription of a wide range of genes and vascular endothelial growth factors (VEGFs) [39] for neovascularization and homeostasis. Irreversible ischemic damage has been well documented for vascular tissues and endothelial cells in particular, and is manifest from the massive apoptosis observed in the first 12 h following reoxygenation [40, 41]. Our experiments here are concerned with much shorter time scales of up to 1 h. We observed that imposition of up to 30 min of anoxia did not enhance cell death, as determined by staining with trypan blue immediately after the reoxygenation. Alternatively, hemeproteins, including eNOS itself, are potential targets for inhibition by free NO through nitrosylation of the heme moiety [29]. Such self-poisoning of eNOS was previously observed by us [28] in experiments *in vitro* with anoxic reduction of nitrite. In our trapping experiments described here, however, significant nitrosylation of the heme of eNOS is unlikely since the abundance of Fe-DETC traps acts as an efficient NO sink and should keep the concentration of free NO radicals at a low value. The third, and in our opinion most plausible, explanation is depletion of the intracellular nitrite. The Griess assay showed that confluent cells in a oxygenated 75-cm^2 flask contain a total intracellular nitrite pool of ~1.1 nmol nitrite, and that this quantity is consumed after imposition of hypoxia. The magnitude of the anoxic NO release (20–50 pmol/min) suggests that nitrite should indeed be depleted after some 20–50 min of anoxia. These estimates make it plausible that nitrite depletion is the reason that MNIC yields become stationary after ~30 min of anoxia. We did not further investigate the reason for the saturation behavior since we expect that 30 min of anoxia cause many changes inside the cells, thereby making their physiology less and less representative for actual tissue endothelium as time proceeds. Instead, the magnitude of the anoxic NO release and, in particular, its extended duration indicate that the nitrite reductase capacity of eNOS be a remarkably robust reaction mechanism in endothelial cells.

In conclusion, our previous work has shown that eNOS may release NO from nitrite under anoxia. Significantly, the NO was released as a freely diffusing radical. Here, the work was extended to study the magnitude and duration of the NO release from cultured endothelial cells under anoxia. The anoxic release of NO exceeded basal NO production and showed concomitant depletion of the intracellular nitrite stores. Significantly, magnitude of the anoxic release was intermediate between basal and that seen with stimulation with CaI. Therefore, it falls in the dynamic range of normoxic physiological regulation, and does not reach the cytotoxic levels as encountered in, say, inflammation, rejection of tissue transplants or septic shock. The benign magnitude and duration of the release suggest that the eNOS-mediated nitrite reduction have physiological relevance for NO levels near the endothelium under acute hypoxia.

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