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Tetrahydrobiopterin-dependent nitrite oxidation to nitrate in isolated rat hepatocytes

Young-Myeong Kim[a] and Jack R. Lancaster Jr.[a,b,c,*]

Departments of [a]Surgery, [b]Anesthesiology and Critical Care Medicine, and [c]Pharmacology, University of Pittsburgh School of Medicine, 497 Scaife Hall, Pittsburgh, PA 15261, USA

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We have found that isolated rat hepatocytes and cell extracts catalyze the stoichiometric conversion of nitrite (either as a product of the oxidation of endogenously synthesized nitric oxide or added as sodium nitrite) to nitrate, which in extracts requires the presence of tetrahydrobiopterin and is inhibited by cyanide but apparently not by carbon monoxide. The reaction is sensitive to heat denaturation and does not involve oxidation of NO_2^- by a peroxidative or radical oxygen mechanism. These results indicate the presence of a hitherto undescribed mammalian mechanism of inorganic nitrogen oxide oxidation that may be a protective mechanism against one potentially damaging effect of endogenous •NO production (NO_2^- formation), and also that assays of •NO formation based on NO_2^- determination alone may be an inaccurate measurement of this activity.

Nitric oxide; Nitrite; Tetrahydrobiopterin; Nitrogen oxide

1. INTRODUCTION

Nitric oxide (•NO) is a reactive radical molecule that is important in a remarkable variety of physiological and pathophysiological processes [1–3]. Its roles in inflammation and immune activation have been characterized as 'double-edged' because it participates in processes that are both protective and damaging towards the host [4,5]. It is presently unclear what is the mechanistic basis that determines the damaging vs. protective actions of •NO although it is likely that several factors are important, including the apparently limited diffusibility of •NO and consequently localized actions [6], the chemical conditions that dictate the nature of the reactions of •NO with oxygen species [7,8], the role of •NO as an endothelium-derived relaxing factor and thus control of localized vascular flow [9], and the possible existence of endogenous protective mechanisms against •NO toxicity [3,10,11].

Upon inflammatory stimulation either in vivo [12] or in vitro [13] •NO is synthesized in rodents by the liver in prodigious amounts. In vitro, hepatocyte •NO production results in several damaging effects, including inhibition of iron-containing enzyme function, specifically aconitase and mitochondrial electron transfer [14,15]. Nevertheless, •NO production by the hepatocyte appears to be less detrimental to this cell than other cell types, such as the activated macrophage and its tumor cell targets [3,16]. Especially in light of the host protective role that hepatic •NO production plays in in vivo inflammation-induced liver damage [17,18], it is reasonable to suggest that this cell may possess mechanisms for protection against the damaging effects of •NO.

Under biological conditions •NO is oxidized to produce nitrite and nitrate [19]. Although NO_3^- is fully oxidized and thus relatively unreactive, NO_2^- can be toxic to cells and its damaging effects are in fact similar to those on •NO, its one electron reduction product [20,21]. Here we demonstrate the existence of a unique mammalian enzymatic mechanism for the detoxification of NO_2^- in rat hepatocytes, a tetrahydrobiopterin-dependent oxidation of NO_2^- to NO_3^-.

2. MATERIALS AND METHODS

2.1. *Reagents*

Williams medium E, penicillin, and streptomycin were purchased from Grand Island Biological Co. (NY). Insulin was purchased from Eli Lilly Co. 5,6,7,8-tetrahydrobiopterin (BH_4) was obtained from Dr. B. Schirks Laboratories (Jona, Switzerland). Percoll was obtained from Pharmacia (Piscataway, NJ). All other chemicals and proteins were purchased from Sigma (St. Louis, MO), unless indicated otherwise.

2.2. *Isolation of hepatocytes*

Hepatocytes were isolated from male Sprague–Dawley rats (Harlan Sprague–Dawley, Indianapolis, IN) weighing 200–300 g, using a modification of the in situ collagenase (type IV; Sigma) perfusion technique previously published [12]. Highly purified hepatocytes were obtained

Corresponding author. Fax: (1) (412) 648-9551.

Abbreviations: BH_4, tetrahydrobiopterin; CM, cytokine mix, composed of a combination of rat recombinant interferon-γ, recombinant human interleukin-1β, and recombinant murine tumor necrosis factor-α; EPR, electron paramagnetic resonance spectroscopy; LPS, lipopolysaccharide; NMMA, N^G-monomethyl-L-arginine; NOS, nitric oxide synthase; NO_x^-, total nitrogen oxides ($NO_2^- + NO_3^-$).

from Percoll gradient centrifugation by repeated differential centrifugation at $50 \times g$ for 4 min to remove non-parenchymal cells.

2.3. *Cell culture and induction of •NO production*

Isolated cells were cultured in Williams medium E supplemented with 1 μM insulin, 2 mM L-glutamine, 10^5 U/l penicillin, 100 mg/l streptomycin and 10% low endotoxin fetal calf serum (Hyclone Laboratories, Logan, UT) in a 100×15 mm petri dish at a density of 5×10^6 cells per plate for 10 h. For induction of nitric oxide synthase (NOS) activity, the hepatocytes were subsequently treated for 13 h with 100 U/ml rat recombinant interferon-γ (Amgen), 5 U/ml recombinant human interleukin-1β (Cistron), 500 U/ml recombinant murine tumor necrosis factor-α (Genzyme), and 10 μg/ml lipopolysaccharide (LPS) (*E. coli* 0111:B4; Sigma) as described previously [10]. This mixture is referred to as cytokine mix (CM) plus LPS.

2.4. *Preparation of crude cytosol*

Harvested hepatocytes were washed twice with ice-cold phosphate buffered saline containing protease inhibitors, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml pepstatin A, and 1 μg/ml chymostatin. The cell suspension (5×10^7 cells/ml) was homogenized on ice in a glass homogenizer with a Teflon pestle for 3 min. The crude cytosol was obtained as the supernatant from centrifugation at $30{,}000 \times g$ for 30 min.

2.5. *Assays of enzyme activity*

2.5.1. •NO production by cultured hepatocytes pretreated with CM plus LPS

Hepatocytes were isolated and pretreated as described above. After the addition of fresh medium not containing CM or LPS, at the indicated time points a 200 μl sample of the culture medium was withdrawn and mixed with 0.5 N NaOH (400 μl) and 10% $ZnSO_4$ (400 μl), and centrifuged at maximum speed in a microcentrifuge to remove protein. The supernatant was used for measurement of NO_2^- and NO_3^-.

2.5.2. NOS assay in hepatocyte extract

The reaction mixture consisted of 1 mM NADPH, 20 μM FAD, 20 μM FMN, 0.5 mM BH_4, 4 mM L-arginine, and 5 mM glutathione, in a final volume of 200 μl in 40 mM Tris-HCl, pH 7.7. The reaction was initiated by addition of cell extract (30 μl) at 37°C and terminated at various times by addition of 400 μl of 0.5 N NaOH and 400 μl of 10% $ZnSO_4$. After centrifugation, NO_2^- and NO_3^- were measured in the supernatant.

2.5.3. Nitrite oxidation activity

For cultured hepatocyte assay, the cells were isolated and cultured as described above except 0.75 mM N^G-monomethyl-L-arginine (NMMA) was added during the 13 h incubation ± CM and LPS. At zero time, fresh medium without CM or LPS was added, containing 85 μM $NaNO_2$ and NMMA. Medium NO_2^- and NO_3^- was measured as described above. Nitrite oxidation activity in the hepatocyte extract was assayed as described above for NOS activity in the extract, except that 56 mM $NaNO_2$ replaced in the L-arginine and the reaction was initiated by the addition of cell extract as described in the figure legends. The omission or addition of cofactors and inhibitors were as described in the figure legends.

2.6. *Measurement of NO_2^- and NO_3^-*

NO_2^- and NO_3^- in the culture medium and in the nitrite oxidation assay were measured as described previously [10]. Total NO_x^- in the reaction mixture and culture media was measured using an automated procedure based on the Griess reaction [22]. NO_2^- alone was measured by adding an equal volume of Griess reagent and measuring absorbance at 550 nm. Protein concentration was measured by protein assay kit (P5656; Sigma) and all data were presented as the mean ± standard error of the mean (S.E.M.).

3. RESULTS

During the course of our on-going studies of the biochemical responses of isolated rat hepatocytes to the damaging effects of endogenous •NO formation, we have consistently noted variations in long-term cultures in the relative amounts of NO_2^- and NO_3^-, the products of •NO oxidation [23]. We thus investigated this systematically by pretreating cultured hepatocytes with a combination of cytokines (interferon-γ, interleukin-1β, and tumor necrosis factor-α) plus lipopolysaccharide (CM plus LPS) for 13 h to induce endogenous •NO synthesis [13], and then at zero time the medium was replaced with fresh medium not containing CM or LPS and at various times thereafter assayed for NO_2^- and NO_3^-. As shown in Fig. 1A, at early times (0–5 h) most of the product of •NO oxidation was NO_2^-, but at longer times (5–49 h) 95% of the NO_2^- was eventually converted to NO_3^-, even after net nitrogen oxide synthesis had ceased. Thus, it appears that the predominant product of •NO oxidation initially is NO_2^-, which is subsequently converted to NO_3^-. As shown in Fig. 1B, a similar conversion of NO_2^- to NO_3^- occurs in assays of nitric oxide synthase (NOS) activity in vitro using crude hepatocyte cell extract; oxidation of NO_2^- under these conditions is considerably more rapid than the conversion in intact cells. A similar result has recently been reported for extracts of rat cerebellum producing •NO from the constitutive isoform of NOS [24].

To directly determine the presence of this unique activity, we assayed the conversion of added NO_2^- to NO_3^- by extract of hepatocytes pretreated with CM plus LPS (Fig. 2). In this experiment, the substrate for NOS (L-arginine) was replaced with $NaNO_2$, precluding the enzymatic synthesis of •NO. Nitrite disappearance and stoichiometric conversion to NO_3^- (data not shown) indeed occurs, and it requires the simultaneous presence of both extract and the mixture of cofactors utilized for

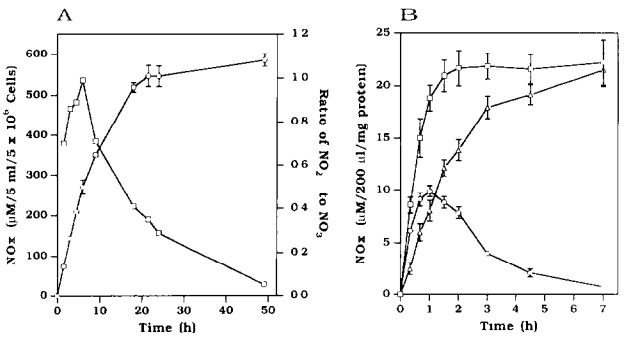

Fig. 1. Time–course of NO_2^- and NO_3^- formation in hepatocyte culture (A) and in cytosol (B). (A) Cultured hepatocytes pretreated with CM plus LPS. Hepatocytes were isolated, pretreated with CM plus LPS, and NO_2^- and NO_3^- were determined as described in section 2. (○) Accumulation of NO_2^- + NO_3^- (NO_x^-); (□) ratio of NO_2^--to- NO_3^-. (B) NOS assay in hepatocyte extract. NOS activity was assayed as described in section 2. (□), (○), and (△) indicate accumulation of NO_x^-, NO_2^-, and NO_3^-, respectively.

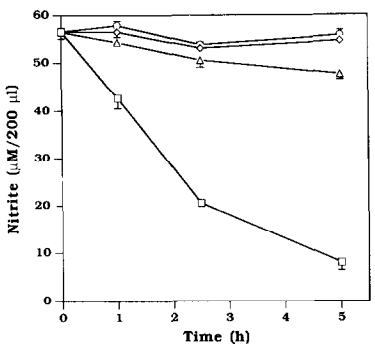

Fig. 2. Nitrite oxidation activity in the extract from hepatocytes stimulated with CM plus LPS. Nitrite oxidation activity was measured as described in section 2 in the presence (□) or absence (△) of the same cofactors as NOS assay in Fig. 1, except that L-arginine was replaced by 56 mM $NaNO_2$ and 20 μl extract (2.5 mg protein/ml) was added. (○) and (◇) denote NO_2^- alone (no cofactors or extract) and NO_2^- plus cofactors without extract, respectively.

maximal NOS activity (BH_4, NADPH, FAD, FMN, and glutathione).

Fig. 3A presents the effects of a single deletion of each component of the cofactor mixture on nitrite oxidation, assayed at a single time point (2.5 h). It is clear that the essential cofactor for this activity is BH_4. It is noteworthy that small but significant oxidation of nitrite occurs in the extract in the absence of cofactors (as is also observed in Fig. 2) and that this activity is completely prevented by NADPH. However, NADPH is not inhibitory when present along with the other cofactors (Fig. 3A). The explanation for this effect is presently unknown.

As also shown in this figure (Fig. 3B), the NO_2^- oxidation is not inhibited by N^G-monomethyl-L-arginine (NMMA), a potent inhibitor of NOS activity [25]. This demonstrates that the activity is probably not due to NOS, although the precise mechanism of inhibition of the enzyme by this compound has not been elucidated. Activity is inhibited by cyanide, an inhibitor of many heme-containing enzymes, but apparently not by carbon monoxide. Thus, the activity may involve a hemoprotein(s) (see section 4).

Since treatment of cells with cytokines induces a multitude of responses [26], we next examined whether this nitrite oxidation activity is induced in hepatocytes by CM plus LPS. As shown in Fig. 4, nitrite oxidation activity is present in hepatocytes (A) and extract (B) whether or not they have been pretreated with CM plus LPS. This rules out NOS or the induction of other responses of these inflammatory mediators (such as the acute phase response [27]) as responsible for this activity.

4. DISCUSSION

We demonstrate here the presence in isolated rat hepatocytes of a tetrahydrobiopterin-dependent activity that oxidizes NO_2^- to NO_3^-. The requirement for a reductant (BH_4) for an oxidative reaction (NO_2^- to NO_3^-) indicates the involvement of a mixed-function oxidation reaction, a class of reaction that is known to be abundantly present in the liver and is catalyzed principally by the cytochrome P450's [28]. However, to the best of our knowledge this is the first report of a BH_4- dependent oxidation of inorganic nitrogen oxides. The inhibition by cyanide indicates the involvement of a hemoprotein(s), although no inhibition was observed with carbon monoxide. This insensitivity to CO may indicate that the putative hemoprotein(s) is only transiently present in the reduced ferrous state during catalytic turnover that is required for sensitivity to this inhibitor. However, at this stage this result precludes the definitive assignment of the activity to a hemoprotein- catalyzed activity.

Is this reaction a result of a specific mammalian nitrite oxidase enzyme(s)? Two previous reports have described oxygen uptake upon addition of NO_2^- to cytochrome oxidase [29,30], indicating oxidation of the NO_2^-. However, this activity required very high concentrations of NO_2^- (0.5 M and higher). We find no detectable oxidation of nitrite under our conditions by cytochrome oxidase (50 U/ml) in either the presence or absence of BH_4. Hoppel and Porterfield demonstrated nitrite oxidation in rat liver homogenates with the addition of no further cofactors and attributed the activity to a coupled reaction requiring an unidentified peroxidative activity and catalase [31], which was verified subsequently by Chance [32]. A similar reaction is also catalyzed by certain other hemoproteins in the presence of H_2O_2, including horseradish peroxidase [33,34] and metmyoglobin [33]. Roediger and Radcliffe also demonstrated nitrite oxidation by isolated rat colonocytes

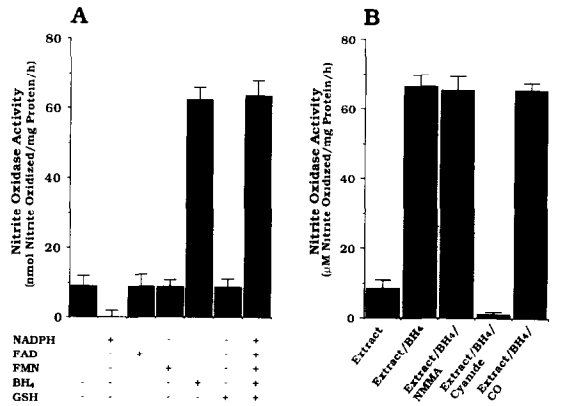

Fig. 3. Effects of single cofactor addition and NMMA and iron ligands on nitrite oxidation activity. (A) Cofactor specificity. Cofactor concentration and experimental conditions were as described in Fig. 2 except that incubation was for 2.5 h and 40 μl cell extract was added. (B) Ligand effects. The assay was performed as described above, except only extract and $NaNO_2$ were added, in the absence or presence of 0.5 mM BH_4, 0.5 mM NMMA, or 2 mM KCN. For CO treatment, the mixture was bubbled with pure CO gas for 5 s after 10 and 40 min of reaction at 37°C.

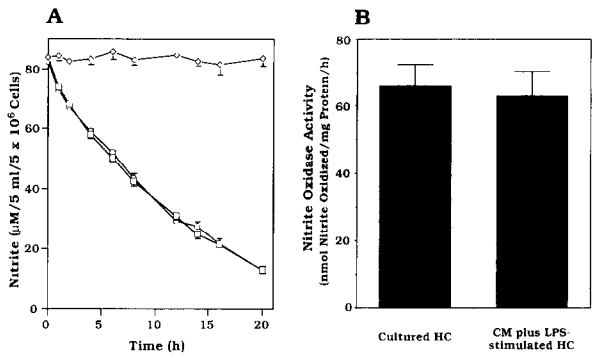

Fig. 4. Effect of pretreatment with CM + LPS on nitrite oxidation activity. (A) Cultured hepatocytes. Nitrite oxidation activity was determined as described in section 2 utilizing hepatocytes pretreated in the presence (□) or absence (○) of CM plus LPS. Also shown is medium alone without hepatocytes (◇). (B) Hepatocyte extract. Nitrite oxidation activity in the extract from hepatocytes (40 μl) pretreated in the presence or absence of CM plus LPS was assayed as described in section 2.

[35]. In light of the observation that, under some conditions, BH_4 can be oxidized to produce H_2O_2 [36], this raises the possibility that the activity we observe is due to such a mechanism. However, we find that H_2O_2 (0.5 mM) cannot replace BH_4 in the assay of hepatocyte extract nitrite oxidation activity, and that addition of catalase (400 U/200 μl) does not affect the reaction. In addition, BH_4 or H_2O_2 plus catalase (900 U/ml) in the absence of hepatocyte extract does not result in NO_2^- oxidation under our conditions. Other potential non-enzymatic mechanisms involving reactive oxygen intermediates (similar to the demonstration of the oxidation of ammonia to nitrate and nitrite [37–39]) are unlikely also, since the reaction catalyzed by hepatocyte extract is not inhibited by the addition of allopurinol (50 μM) or the combination of catalase (400 U/200 μl) and superoxide dismutase (150 mU/200 μl). NO_2^- is also not oxidized by xanthine oxidase plus hypoxanthine either in the absence or presence of iron-EDTA (10 mM) as a Fenton reagent, as reported previously [39]. These conditions have been shown previously to generate substantial amounts of strongly oxidizing species [40]. Finally, the activity is completely inactivated by heat denaturation (100°C for 5 min). These results indicate that the activity may be due to a novel enzyme, although such a conclusion must await further studies.

Nitrite is the one-electron oxidation product of $^{\bullet}NO$, and thermodynamically this couple (next to the oxidation of $^{\bullet}NO_2$ to NO_3^-) is the most readily reversible among the 8 oxidation steps of the nitrogen oxides [41]. Thus, in the heterogenous environment of the intracellular cell compartment containing both abundant reductants and oxidants (O_2,$^{\bullet}O_2^-$, H_2O_2), interconversion between $^{\bullet}NO$ and NO_2^- might be common in the absence of specific mechanisms for the removal of NO_2^-. Indeed, NO_2^- is damaging to several hepatic cell functions such as drug metabolism [42], and it has also been shown that under some conditions, NO_2^- is reduced to $^{\bullet}NO$ by mitochondria and cytochrome P450's, which can result in auto-inactivation of the cytochrome P450's and nitrosylation of essential heme-containing proteins such as cytochrome *c* [42,43]. In addition, abundant studies in the nutrition literature have demonstrated the reactions of NO_2^- with biomolecules [44], and using electron paramagnetic resonance (EPR) spectroscopy we have presented evidence that this effect may be a consequence of $^{\bullet}NO$ production in a reducing environment that results in destruction of iron-containing enzyme function [20]. Since we [45] and others [46,47] have described EPR evidence of a similar damage to iron- containing proteins during the endogenous production of $^{\bullet}NO$ by NOS (necessarily occurring in the presence of O_2 since this is a required substrate for NOS activity), this raises the possibility that a significant portion of the cytotoxic effector mechanism of NOS induction under inflammatory and immune activation conditions may be due to $^{\bullet}NO$ both newly produced by this enzyme and also by reduction of NO_2^-. As has been speculated previously by Stuehr and Nathan [48], it may be that this is a mechanism that contributes to the destruction of phagocytized intracellular pathogens by the macrophage since the internal pH of the phagolysosome is acidic (pH 5.8 [49]), a condition known to facilitate the non-enzymatic decomposition of HNO_2 to $^{\bullet}NO$ and $^{\bullet}NO_2$ with consequent cellular toxicity [50]. Indeed, Klebanoff [51] has recently demonstrated the bacteriostatic potential of NO_2^- under acidic conditions, and provided evidence under certain conditions for a potentiation of this toxic effect with H_2O_2, which is also a product of phagocytic activation. In addition, evidence also suggests that at least two pathogenic organisms (*Plasmodium falciparum* [52] and *Crytococcus neoformans* [53]) are sensitive to NO_2^-, under conditions that mimic those when phagocytyzed by the macrophage.

In a very recent report, Ignarro et al. [24] have demonstrated that the immediate product of the oxidation by O_2 of $^{\bullet}NO$ newly produced by cerebellar NOS is NO_2^-, which is also true for pure $^{\bullet}NO$ in aqueous solution [8]. Gradual conversion of NO_2^- to NO_3^- is catalyzed by cellular components in crude extracts (but not by preparations more purified for NOS activity), as well as by added ferrohemoproteins (as has been documented previously [54]). The results presented here may offer an enzymatic explanation for this result, namely, a BH_4-dependent nitrite oxidation activity that is removed upon further purification of NOS. These results and those presented here suggest that assay of NOS activity in intact cells and extracts by measuring only NO_2^- may give an incomplete indication of total $^{\bullet}NO$ production, especially under conditions where cellular BH_4 and ferrohemoproteins are variable. It is noteworthy that a similar nitrite oxidation was not observed by Ignarro et al. in an activated rat macrophage cell line, supporting the suggestion by Stuehr and Nathan that NO_2^-, as well

as •NO, is important to macrophage-mediated immune defense mechanisms. Thus, the presence or absence of this nitrite oxidation activity in specific cell types may correlate with the cellular toxic vs. messenger roles of •NO. Further characterization of this unique activity is under way.

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