Nitric oxide (NO) is both an endogenously generated species and the active species released from a variety of important drugs. Due to its endogenous generation and use as a therapeutic agent, the metabolism and fate of NO is of interest and concern. To date, most attention regarding the metabolism and fate of NO has been paid to its oxidized metabolites. Due to the reducing environment of cells, we considered that NO may also undergo reductive metabolism as well. Therefore, we have examined the reductive metabolism of NO by hepatocytes. Generation of nitrous oxide (N₂O) was used as an indicator of NO reduction. Indeed, we observed that NO could be reduced to N₂O by the cytosolic fraction of hepatocytes. The N₂O production was partially inhibited by the thiol modifying agent, N-ethylmaleimide and thiol consumption was observed during N₂O formation. Thus, our results indicate that NO reduction is feasible and likely occurs via a thiol-dependent process.

Experimental Procedures

Materials. EDTA, glycerol, NADPH, NADH, NAD⁺, glutathione (reduced), potassium cyanide, L-ascorbic acid, N-ethylmaleimide (NEM), sodium hydroxide, 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), desferrioxamine mesylate, and Trizma Base were purchased from Sigma Chemical Co. (St. Louis, MO). Sucrose, 1 N hydrogen chloride, 1 N sodium hydroxide, and methanol were purchased from Fisher Scientific (Pittsburgh, PA). NO gas was purchased from PRAXAIR (MO). Sucrose, 0.1 mM EDTA (10%) was added to the supernatant and it was used as cytosolic fraction. 1980; Zhao et al., 1995). Also, other groups have reported that the presence of mitochondria facilitated NO breakdown under anaerobic conditions, which was inhibited by cyanide (Clarkson et al., 1995; Borutaite and Brown, 1996). Using isolated rat hepatic mitochondria, we have confirmed that cytochrome c oxidase is capable of reducing NO (J.H. and J.M.F., unpublished data). Furthermore, we have found that reductive metabolism of NO can occur not only in mitochondria but also in other fractions of hepatic cells. Herein, we show that the cytosolic fraction of the rat hepatic cell is capable of reducing NO to produce nitrous oxide (N₂O) under anaerobic conditions, indicating that reductive NO metabolism is feasible.

Preparation of Cytosolic Fraction. Rat livers were minced and homogenized using a tissue grinder in buffer A (10 mM Tris HCl, pH 7.4, 0.25 M sucrose, 0.1 mM EDTA) at 4°C. Homogenate was centrifuged at 800g for 10 min at 4°C. The supernatant was taken and centrifuged at 8000g for 10 min. The supernatant again was centrifuged at 105,000g for 1 h at 4°C. Glycerol (10%) was added to the supernatant and it was used as cytosolic fraction. Cytosolic fraction was kept frozen at 80°C until use.
Protein Determination. Protein concentrations were determined using the Bradford, Coomassie blue method described by Bio-Rad Laboratories (Hercules, CA). BSA (Pierce, Rockford, IL) was used as a standard. Samples were diluted with 0.01 N NaOH to make a final concentration in the 0.1 to 10 mg/ml range. The Bio-Rad reagent was added to the samples and the optical density was measured at a wavelength of 595 nm using a Beckman DU 30 spectrophotometer (Beckman Instruments, Berkeley, CA). Sample protein concentrations were determined by comparison to a standard curve that was constructed using various concentrations of BSA.

N₂O Assay. The formation of N₂O was measured using a gas chromatographic method described previously (Fukuto et al., 1992). Thus, 3 ml of the cellular extract or buffer was placed into a 15-ml round-bottom flask equipped with a Teflon septum and stopcock. The solution was degassed on a vacuum line using several vacuum-argon purge cycles using a gas-tight needle fixed to the vacuum line and placed through the septum. After the final cycle, the sample was left under argon. NO gas was injected into the flask through the septum using a gas-tight syringe. After degassing and the completion of the appropriate additions to the flask, the stopcock was closed to seal the reaction mixture. The samples were incubated at 37°C for various times. For the experiment with NEM, the degassed sample was preincubated with NEM at 37°C for 30 to 40 min before adding NO gas. After completion of the reaction, 500 µl of headspace gas was drawn through the septum with the stopcock open and injected into a gas Hewlett-Packard model 5710A gas chromatograph equipped with a thermal conductivity detector, 6 ft × 1/8 inch Porapak Q column and operating with helium carrier gas (30 ml/min) and isothermally at 60°C. Under these conditions, the retention time for N₂O was 2 min and peak area was used for quantitation of N₂O. A standard curve for N₂O was made by injecting various amounts of authentic N₂O gas into the gas chromatograph and correlating the peak integration with the amount of N₂O injected.

Thiol Measurement. Three milliliters of cellular extract or buffer in the 15-ml flask with septum was degassed as described above and left under argon. NO gas was injected and the samples were incubated at 37°C for 30 min. After incubation, the samples were degassed again to remove any remaining NO gas. The amount of thiol in the sample was measured by a modified method of Sedlak and Lindsay (1968). Briefly, 200 µl of sample was mixed with 40 µl of 0.01 M 5,5′-dithio-bis-(2-nitrobenzoic acid) in methanol and 600 µl of 0.2 M Tris buffer (pH 8.2), and methanol was added to make a total volume of 4 ml. After 30 min, the samples were filtered with filter paper, and absorbance was measured at a wavelength of 412 nm using a UVIKON 810 spectrophotometer (UVIKON, San Diego, CA). A standard curve was prepared using various concentrations of reduced glutathione.

NAD(P)H Consumption. The quantitation of NAD(P)H consumption was performed by measuring the formation of NAD(P)⁺ using fluorometric analysis as described previously (Komori et al., 1994). Briefly, samples were diluted 10-fold and 100 µl of the diluted sample was mixed with 400 µl of 0.2 N HCl for longer than 10 min to destroy the reduced form of NAD(P)H. Then 100 µl of 6 N NaOH was added and incubated at 60°C for 10 to 15 min. This was followed by the addition of 1.4 ml of distilled water. Fluorescence was measured at λexcitation = 365, λemission = 460 nm using an Aminco-Bowman spectrophotofluorometer (Silver Spring, MD). The standard curve was made using solutions of NAD⁺ of known concentration.

Determination of Solution NO Concentration. The concentration of NO in the reaction solution was determined by the method previously used in our laboratory (Farias-Eisner et al., 1996).

Results

The reduction of NO can conceivably lead to a number of products. A single one-electron reduction of NO leads initially to a species referred to as nitrooxyl (HNO) (reaction 1). This is a metastable species, which can react with itself to generate hyponitrous acid (reaction 2), which then dehydrates to give N₂O (reaction 3; Bazylnski and Hollocher, 1985). HNO anion can also react sequentially with 2NO molecules to generate N₃O₃⁻ (reaction 4), which then will decompose to give N₂O₃ and NO₃⁻ (reaction 5; Bonner and Hughes, 1988). Regardless, the detection of N₂O is always indicative of NO reduction, possibly through the generation of free N̈O. It should be noted, however, that N̈O intermediacy is not absolutely required for N₂O formation (discussed later).

[1] NO + e⁻ → N̈O
[3] HONNOH → N₂O + H₂O
[4] N̈O + 2NO → N₂O₃⁻
[5] N₂O₃⁻ → N₂O + NO₃⁻

The cytosolic fraction from hepatocytes was capable of producing N₂O when incubated with NO at 37°C for 30 min under anaerobic conditions, as measured by headspace gas analysis. The generation of N₂O observed in the cytosolic fraction of the rat hepatic cells was proportional to added NO as shown in Fig. 1. N₂O was not detected without NO addition. In experiments where 1 ml of NO gas (45 µmol) was added to the headspace of the reaction mixture (15-ml flask containing 3 ml of rat hepatocyte cytosol) (prepared as indicated in the text), Values represent the mean ± S.D.

To study the mechanism of NO reduction, different reducing agents were added to the incubation flask and their effect on N₂O production was monitored. In the first 30 min of incubation, the addition of reducing factors such as ascorbate, NAPD, NADH, and GSH did not affect N₂O production significantly. The fact that additional reductants did not increase N₂O formation may mean that the tissue already possessed reducing capability enough for the first 30 min of NO...
reduction. Therefore, the sample was incubated with NO for 24 h to consume all endogenous reducing agents and then different reducing agents were added (Fig. 3). After a 24-h incubation, about 21 nmol N₂O/mg protein was generated in all samples. At that point, different reducing agents were added to each sample. For the control sample with no reducing agent added, there was no more N₂O produced after 24 h. The addition of 1 mM NADPH, 1 mM NADH, or 1 mM GSH was able to cause the tissue to continue the generation of N₂O between the 24- and 48-h incubation period. The amount of N₂O generated in the second 24-h incubation period (24 nmol/mg protein) was approximately the same as that generated in the first 24-h incubation period (21 nmol/mg protein). After the 48-h incubation, with the addition of NADPH, up to 5300 nmol of total N₂O was produced, which means that 23.7% of added NO was converted to N₂O. Under identical conditions except in the absence of tissue extract, less than 0.3% of the NO was converted to N₂O when NADH and NADPH were added as reducing cofactors. The addition of GSH to NO in the absence of tissue gave somewhat more N₂O than experiments with added NADH or NADPH, but was still only about 45% of that generated in the presence of the cytosolic fraction (data not shown).

Endogenously present reducing agents seem to be responsible for the reduction during the early period of incubation. Therefore, the decrease in reduced thiol and NADH levels in the cytosolic fraction was measured after incubation and compared with the sample incubated without added NO (Table 1). We find that the NAD(P)H level did not change significantly in the presence of NO over that in the control. However, thiol levels were significantly decreased.

Effects of desferrioxamine, NEM, and potassium cyanide (KCN) on N₂O generation from NO were examined (Fig. 4). The presence of 5 mM of the metal chelator desferrioxamine to the NO-cytosol incubation mixture did not significantly alter N₂O formation, indicating that a large portion of NO reduction was not metal-mediated. The addition of 1 mM KCN, a heme protein inhibitor, to the incubation mixture also did not significantly change the amount N₂O produced.

The thiol-modifying agent NEM inhibited N₂O formation by 88%, supporting the idea that thiols are somehow involved in the NO reduction process. The enhancement of N₂O production by NADH was also inhibited by NEM (data not shown).

Because cellular thiols appear to be the electron source for the reduction of NO, we tested whether the presence of GSH at a comparable concentration of cytosolic thiol would produce similar results. Thus, NO was incubated in buffer containing GSH only (Fig. 5). The amount of N₂O formed is divided by the thiol concentration of each sample for comparison. At early time points (30 min and 4 h), N₂O generation in GSH-containing buffer was about 22% of that generated with the addition of the cytosolic fraction. After 24 h, the amounts of N₂O formed in GSH-containing buffer and cytosolic fraction were not significantly different (data not shown).

To examine a pH dependence of the reaction between GSH and NO, 1 ml of NO gas was added to 1 mM GSH in buffer of pH 7, pH 8, and pH 9 (Fig. 6). As the pH increases, more N₂O was generated, indicating that NO reacts faster with thiolate than protonated thiol. Because pKₐ of GSH cysteine is 8.66, at pH 7, the thiolate would consist of 2.1% of the total GSH. At pH 8.0, the portion of thiolate would increase to 18.0 and 68.7% at pH 8 and 9, respectively.

Discussion

The reduction of NO in the cytosolic fraction of rat hepatic tissue was observed as evidenced by the formation of N₂O. The generation of N₂O from the reduction of NO in the incubation mixture was...
dose-dependent with respect to both NO and protein concentration and could be prolonged by the addition of the exogenous reducing agents NADH, NADPH, and GSH. After a 48-h incubation period and with the addition of NADH, up to 23.8% of added NO could be converted to $\text{N}_2\text{O}$ under the conditions of our experiments.

At early time points of the incubation, the addition of different reducing agents did not significantly increase the $\text{N}_2\text{O}$ production, implying the use of endogenous reducing factors. Significantly, intracellular reduced thiol levels were decreased during this time, indicating that thiols were somehow involved. Stoichiometric analysis indicates that the extent of thiol loss could have provided enough reducing equivalents to account for the amount of $\text{N}_2\text{O}$ generated. Although desferrioxamine failed to significantly decrease $\text{N}_2\text{O}$ formation, NEM had a significant inhibiting effect on $\text{N}_2\text{O}$ generation. Thus, it is clear that endogenous thiols are likely to be involved in NO reduction (as measured by $\text{N}_2\text{O}$ formation). Interestingly, we found that the mitochondrial fraction of hepatic cells also generated $\text{N}_2\text{O}$ from NO (J.H. and J.M.F., unpublished data), but the mechanism seems to be different from the cytosolic fraction because in mitochondria, $\text{N}_2\text{O}$ generation was partially inhibited by KCN.

After depleting all reductants in the cytosolic fraction by incubating for 24 h with NO, the addition of NADPH, NADH, or GSH supported further generation of $\text{N}_2\text{O}$. Due to the ability of either NADPH or NADH to regenerate GSH by the action of cytosolic glutathione reductase, it is probable that the actions of these two reducing agents was due to their ability to regenerate GSH. Therefore, the results remain consistent with the assumption that thiols are responsible for the observed NO reduction.

Interestingly, the direct reaction of GSH with NO in the absence of any cell components failed to produce the same amount of $\text{N}_2\text{O}$ as seen with cell cytosolic fractions, at least at early time points, indicating that tissue may have some device that can facilitate the process. The ability of the cytosolic fraction to catalyze the conversion of NO to $\text{N}_2\text{O}$ is significantly lost after heating, indicating a possible role for a thiol using protein. One possible explanation is that there may be certain proteins that contain thiols that are more reactive to NO than the thiol in GSH. For example, if a protein thiol exists predominately in the thiolate form (as opposed to the protonated thiol form), this would dramatically increase the reactivity of the protein thiol. In partial support of this idea, we found that an increase in pH increased the rate of $\text{N}_2\text{O}$ generation from NO by GSH. Interestingly, certain protein thiols have lower pK$_a$ values compared with typical free thiols (GSH has a pK$_a$ of 8.66) and would be expected to react more rapidly with NO to generate more $\text{N}_2\text{O}$. For example, Cys-149 in glyceraldehyde-3-phosphate dehydrogenase and Cys-14 and Cys-17 in thioredoxin have been shown to have reactive thiols with low pK$_a$ (<7) values (Stamler, 1994). Therefore, at least a part of the discrepancy between $\text{N}_2\text{O}$ formation in GSH-containing buffer versus the cytosolic fraction may be explained by the presence of these low pK$_a$ protein thiols.
Because the experiments described herein were performed in an anaerobic system, the physiological relevance remains to be determined. It is certain that NO reduction is possible in a physiological environment because N₂O detection is an unequivocal indication of NO reduction. However, whether N₂O would be expected to be generated in vivo from NO reduction remains speculative because other fates for NO-reduced species in an oxygen environment are possible (for example, see Fukuto et al., 1993). Moreover, because we are unaware of the exact mechanism of NO reduction (i.e., HNO intermediacy, direct N₂O generation, etc.), it is impossible at this time to speculate on the relative importance of reductive versus oxidative pathways for NO metabolism. The results of this study primarily point out the existence of NO-reductive pathways and do not a priori indicate that it occurs under normal physiological circumstances. However, if N₂O is generated in vivo, it may have some significant consequences. The formation of N₂O from NO will be a detoxification process because N₂O is orders of magnitude less toxic than NO (Marshall and Longnecker, 1990; Gillman and Lichtigfeld, 1994). In addition, other than being an anesthetic at high concentration, N₂O was suggested to have a direct influence on neurotransmission via acting on opioid receptors at a subanesthetic dose (Daras et al., 1983; Ori et al., 1989).

**References**

Bazyliński DA and Hollocher TC (1985) Evidence from the reaction between trioxodinitrate(II) and ¹⁷NO that trioxodinitrate(II) decomposes into nitrosyl hydrate and nitrite in neutral aqueous solution. *Inorg Chem* 24:2285.


