Role of glutathione and nitric oxide in the energy metabolism of rat liver mitochondria

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Received 1 August 1997; revised version received 3 September 1997

Abstract Previous studies have suggested that nitric oxide (NO) inhibited mitochondrial respiration. NO and/or its intermediate(s) react with various molecules, such as hemeproteins and free SH groups. The inhibitory effect of NO on mitochondrial respiration was decreased by exogenously added glutathione (GSH). However, a decrease of intramitochondrial GSH by pretreating animals with L-buthionine sulfoximine had no appreciable effect on the inhibitory effect of isolated mitochondria. Furthermore, the effect of NO was not affected by depleting free SH residues in mitochondria by *N*-ethylmaleimide. These results suggest that cytosolic but not intramitochondrial GSH might be an important factor that determines the NO-dependent regulation of mitochondrial energy metabolism.

Key words: Mitochondria; Glutathione; Nitric oxide; Energy metabolism

1. Introduction

Nitric oxide (NO) is a reactive gaseous free radical which regulates the activity of various enzymes through posttranslational modification, such as nitrosylation of metal complexes and free cysteinyl residues [1]. Potential sites of NO-iron complex in cells include heme and non-heme iron groups in enzymes, such as cytochrome *c* oxidase [2–4] and other proteins in the mitochondrial electron transport system [5,6]. Some metabolites of NO, such as peroxynitrite, also inhibit various enzymes including aconitase [7,8].

Previous studies in this laboratory had suggested that low levels of NO reversibly inhibited the respiration and ATP synthesis of isolated mitochondria [9] and ascites tumor cells [10] particularly under physiologically low oxygen tensions. Kinetic analysis revealed that cytochrome *c* oxidase was the primary site for the inhibition by NO [10].

NO and/or its intermediary metabolite(s) also react with free sulfhydryl groups of various compounds to form *S*-nitrosothiols [11]. In mammalian cells, reduced glutatione (GSH), a major non-protein thiol, is synthesized exclusively in a cytosolic compartment and plays important roles in cellular functions, such as protection of cells and tissues from oxidative stress [12–14]. Because NO and/or its metabolite(s) react with thiols [15] and the resulting *S*-nitrosothiols function as a stable NO donor [16], cellular GSH might play important roles in regulating the fates of NO functions.

During the experiments with ascites hepatoma cells [10], we found that the activity of NO to inhibit mitochondrial respiration was increased by treating cells with digitonin, a membrane permeabilizing agent. The enhancing effect of digitonin was inhibited by adding thiol compounds in the incubation medium. Because GSH is the major low molecular weight thiol, cellular GSH might be an important factor that affects the NO-dependent inhibition of mitochondrial energy metabolism. The present work describes the effects of GSH and related thiols in and around mitochondria on the inhibitory action of NO.

2. Materials and methods

2.1. Chemicals

NO and argon gases were obtained from Kinkisanki Co. (Osaka, Japan). All other reagents used were of the highest purity and obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Preparation of NO solution

NO solution was prepared after bubbling NO gas through 50 mM HEPES-NaOH buffer, pH 7.4, as described previously [17]. Briefly, two small tubes were fitted with an air-tight septum with glass tubes inserted for delivery and escape of gases with the first tube containing 5 M KOH and the second containing the HEPES-NaOH buffer. Argon was delivered into two tubes at a flow rate of 100 ml/min. After 15 min, argon was replaced by NO (100 ml/min). After 15 min, the saturated NO solution (1.9 mM) was stored at 4°C and used for experiments within 3 h; NO concentration remained unchanged during the experiments. NO concentration was determined using electron spin resonance and the NO trapping agent, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (carboxy-PTIO) [18,19].

2.3. Preparation of S-nitrosoglutathione (GSNO)

GSNO was prepared according to the method of Hart [20] by incubating equimolar GSH and sodium nitrite in acidified water at 0°C. GSNO solutions were freshly prepared from frozen stored GSNO powder before each experiment. The concentration of GSNO was determined spectrophotometrically at 335 nm ($\varepsilon = 992\ M^{-1}\ cm^{-1}$) and at 545 nm ($\varepsilon = 15.9\ M^{-1}\ cm^{-1}$).

2.4. Isolation of mitochondria

Male Wistar rats (200–250 g) were fasted overnight. Liver mitochondria were isolated according to the method described previously [21] using a medium containing 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4), and 0.1 mM EDTA. EDTA was omitted in the final wash and the mitochondrial samples were suspended in 0.25 M sucrose containing 10 mM Tris-HCl, pH 7.4, at 10–20 mg protein/ml and stored at 4°C until use. Mitochondrial protein was determined by the method of Bradford.

2.5. Analysis of oxygen consumption

Oxygen consumption by isolated mitochondria was determined polarographically using a Clark type oxygen electrode fitted to a 2 ml water-jacketed closed chamber [22]. Isolated mitochondria (1 mg protein/2 ml) were suspended in the reaction medium consisting of 0.2 M

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Abbreviations: NO, nitric oxide; GSH, reduced glutathione; BSO, L-buthionine sulfoximine; NEM, *N*-ethylmaleimide; GSNO, *S*-nitrosoglutathione; NOS, nitric oxide synthase; GSSG, oxidized glutathione; Cys, cysteine; NAC, *N*-acetylcysteine

PII S0014-5793(97)01156-3

sucrose, 10 mM KCl, 1 mM $MgCl_2$, 2 mM sodium phosphate and 10 mM Tris-HCl (pH 7.4). Oxygen consumption was monitored in the presence of 5 mM succinate and 600 μM ADP. During the experiments, GSNO or NO-saturated solutions were added to the reaction mixture at varying oxygen tensions.

2.6. Decrease of mitochondrial GSH

L-Buthionine sulfoximine (BSO) (0.25 mmol/kg) was injected intraperitoneally to rats every 12 h for 3 days. At the indicated times, liver mitochondria were isolated as described above.

2.7. Depletion of free SH residues in mitochondria

Liver mitochondria were incubated with varying concentrations of *N*-ethylmaleimide (NEM) for 10 min at 4°C. Then, mitochondria were washed twice with 0.25 M sucrose buffer by repeated centrifugations and used for experiments.

2.8. Measurement of glutathione and free thiols

Freshly isolated mitochondria were immediately sonicated in 100 μl of ice-cold 5% sulfosalicylic acid solution at a concentration of 100 mg protein/ml. After centrifugation at 12000×*g* for 15 min, concentrations of free thiols and total glutathione (GSH and 2GSSG) in the acid-soluble fractions were determined. Total glutathione was measured by the method of Tietze [23]. Levels of free thiols were determined using Ellman's reagent [24].

3. Results

3.1. Effect of NO and GSNO on mitochondrial respiration

In the presence of succinate and inorganic phosphate, ADP increased the rate of oxygen consumption by rat liver mitochondria. Consistent with previous observations [9], the state 3 respiration was reversibly inhibited by a small amount of NO (Fig. 1). The inhibitory effect of NO was stronger at low oxygen tensions than at high tensions. On the other hand, the same concentration of GSNO exhibited no significant effect on mitochondrial respiration particularly at high oxygen tensions. When oxygen tensions became lower than 50 μM, GSNO inhibited the respiration only slightly. Similar inhibition was also observed in the dark.

As shown in Fig. 2, NO inhibited the respiration of mitochondria in a dose-dependent manner. To study the role of cytosolic thiols in the energy metabolism of mitochondria, the inhibitory effect of NO was also tested with mitochondria in the presence of a physiological concentration (5 mM) of GSH. The inhibitory effect of NO was markedly decreased by exogenously added GSH. The effect of GSH also depended on its concentration (Fig. 3).

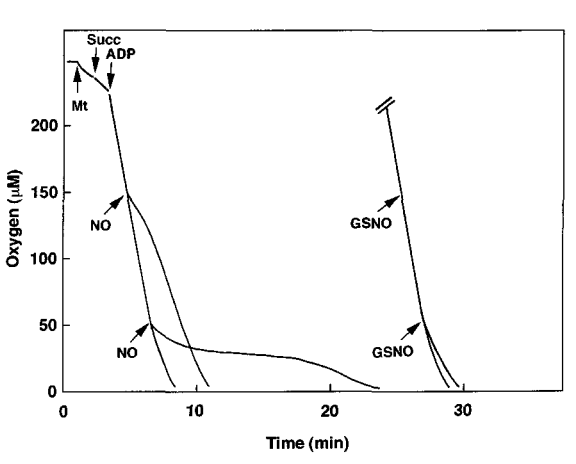

Fig. 1. Effect of NO on mitochondrial respiration. State 3 respiration of mitochondria (0.5 mg/ml) was initiated by adding 600 μM ADP in the presence of 5 mM succinate (Succ). Aliquots of the NO-saturated solution and GSNO were added to the reaction mixture to give a final concentration of 2 μM at the indicated times. Experiments were performed at least five times using different samples of mitochondria with similar results.

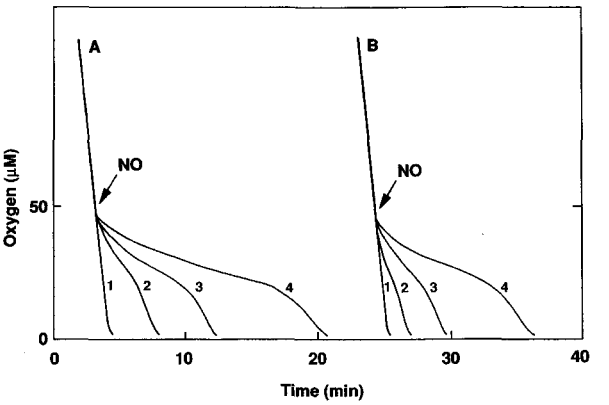

Fig. 2. Effect of GSH on mitochondrial respiration inhibited by NO. Mitochondrial respiration was monitored in the presence (B) or absence (A) of 5 mM GSH. At the indicated times (arrows), NO-saturated solution was added to the reaction mixture. Added NO concentrations of numbers 1, 2, 3 and 4 were 0, 0.5, 1 and 2 μM, respectively. Other conditions were the same as described in Fig. 1. Experiments were performed at least five times using different samples of mitochondria with similar results.

3.2. Effect of various thiols on inhibitory effect of NO

To test the specificity of GSH, the effect of related compounds on the NO action was also studied. The inhibitory effect of NO was also decreased by the presence of other thiols, such as cysteine and *N*-acetylcysteine (NAC) (Fig. 4). Among various thiols used, the inhibitory effect of cysteine was the most apparent. Oxidized glutathione (GSSG) had no appreciable effect on the inhibition by NO.

3.3. Effect of mitochondrial GSH on NO-dependent respiratory inhibition

Under physiological conditions, GSH levels in the mitochondrial matrix are fairly high (~10 mM). To determine the role of mitochondrial GSH in the inhibition by NO, its effect was also determined with mitochondria isolated from animals which were pretreated with BSO, a specific inhibitor of γ-glutamylcysteine synthetase. When animals were treated with BSO, the GSH level in mitochondria gradually decreased. After 3 days of BSO treatment, it decreased to about 50% of the control level (Fig. 5). The inhibitory effect of NO remained unaffected even when intramitochondrial GSH was decreased to 50%.

3.4. Effect of depletion of free SH residues in mitochondria

Because it was practically difficult to decrease mitochondrial GSH completely by pretreating animals with BSO, the inhibitory effect of NO might not be affected by its treatment. To test this possibility, the levels of free SH groups in mitochondria were depleted by treatment with varying concentrations of NEM. However, the inhibitory effect of NO on the respiration was not affected by depleting free SH groups in mitochondria (Fig. 6).

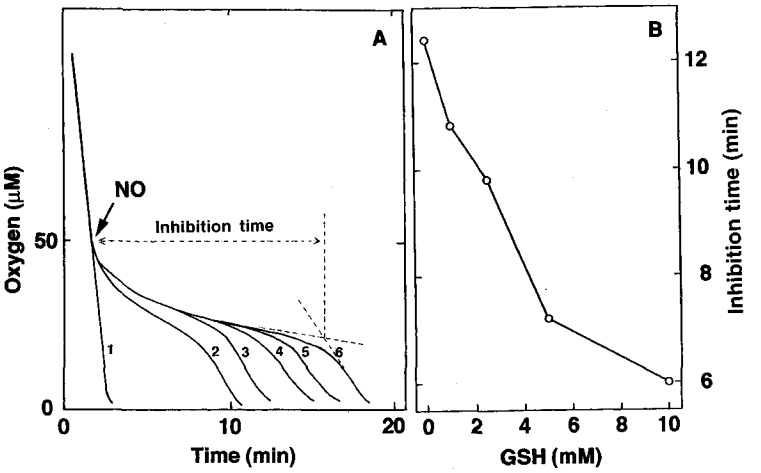

Fig. 3. Dose-dependent effect of GSH on mitochondrial respiration inhibited by NO. Oxygen consumption of isolated mitochondria was monitored in the presence of varying concentrations of GSH (A). Number 1 shows the control respiration. This respiration was not affected by the presence of GSH. At the indicated time, aliquots of NO-saturated solution were added to give a final concentration of 2 μM (numbers 2–6). Numbers 2–6 show the respiration in the presence of 10, 5, 2, 1 and 0 mM GSH, respectively. The time required for the disappearance of inhibitory action of NO (Inhibition time) in the presence of various concentrations of GSH was measured and plotted in (B). Other conditions were the same as described in Fig. 1. Experiments were performed at least five times using different samples of mitochondria with similar results.

4. Discussion

Previous studies in this and other laboratories have revealed that NO reversibly inhibits the respiration of isolated mitochondria [2–4,9,25] and tumor cells [10] particularly under physiologically low oxygen tensions. Some papers reported interactions of mitochondria with NO after GSH depletion [26], and effects of GSH on NO inhibition of mitochondrial function [27]. The present work demonstrates that the inhibitory effect of NO on mitochondrial respiration was decreased by the presence of a physiological concentration of extramitochondrial GSH and related thiols. Because the inhibitory effect on NO action was observed with GSH, cysteine and NAC but not GSSG, free thiol groups of these compounds might be essential for their inhibitory effect. Although cellular levels of free cysteinyl residues in proteins are also high, the rate constants for *S*-nitrosylation of NO with protein thiols are fairly low as compared with low molecular weight thiols [28]. In fact, under identical conditions, the presence of metallothioneine had no appreciable effect on the inhibitory action of NO (data not shown). It should be noted that the inhibitory effect of cysteine was stronger than those of GSH and NAC. However, as cellular levels of free cysteine are significantly lower than those of GSH (2–10 mM) [29], this tripeptide might predominantly be responsible for the suppression of NO action in cells and tissues.

Mitochondrial matrix contains relatively high concentrations of GSH. The importance of mitochondrial GSH for the maintenance of their functions has also been known for many years [30–32]. However, the decrease of mitochondrial GSH by BSO had no appreciable effect on the inhibitory action of NO. This experiment was carried out using mitochondria from BSO-treated animals. Because GSH levels of liver mitochondria were decreased only to about 50%, this decrease may not be sufficient for suppressing the anti-NO action of GSH. It was practically difficult to decrease mitochondrial GSH levels to lower than 50%. To overcome such frustrating situations, we used NEM to deplete intramitochondrial free SH groups. However, depletion of titrable free thiols in mitochondria failed to enhance the inhibitory effect of NO. Under identical conditions, NEM did not affect the activity of cytochrome *c* oxidase (data not shown). Thus, cytosolic GSH might be more important for determining the inhibitory effect of NO than intramitochondrial thiols. It should be noted that the active site of cytochrome *c* oxidase, a transmembranous target enzyme for NO, which reacts with substrate oxygen, is located inside the mitochondrial membrane [33]. NO has been shown to interact with its active site thereby inhibiting mitochondrial respiration. Because extramitochondrial GSH but not intramitochondrial SH inhibited the NO action, the primary site for the interaction between GSH and NO might be the extramitochondrial

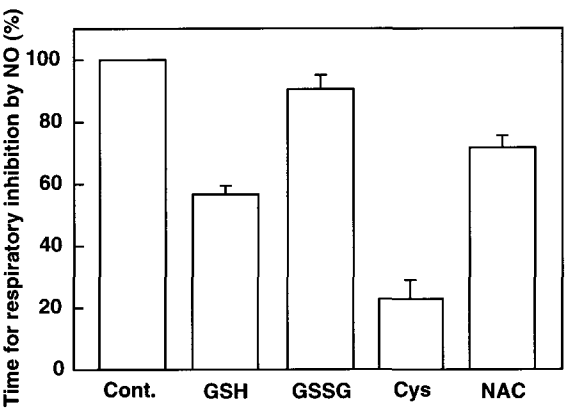

Fig. 4. Effect of various thiols on mitochondrial respiration inhibited by NO. Mitochondrial respiration was monitored in the presence or absence of various thiols, such as 5 mM GSH, cysteine (Cys), NAC and GSSG. Aliquots of NO-saturated solution were added to give a final concentration of 1 μM at an oxygen tension of 50 μM. Other conditions were the same as described in Fig. 1. The control experiments were performed without thiols. The time required for the disappearance of the inhibitory effect of NO was measured as in Fig. 3 and expressed as percent of control. Results are mean ± S.D. from five experiments.

compartment. The molecular mechanism for the inhibitory effect of GSH on the NO action should be studied further using mitoplasts and inside-out vesicles of mitochondria.

Under physiological conditions, NO and related metabolites react with various thiols, GSH, cysteine and albumin, and form *S*-nitrosothiols [29,34–36], which are more stable than NO. Thus, thiol compounds in and around cells might directly react with NO thereby decreasing effective concentrations of this gaseous radical. However, the chemical reactivity of NO with GSH is fairly low ($k = 6 \times 10^6$ M^{-2} s^{-1}) as compared with heme-containing proteins (diffusion limited reaction). In fact, at an oxygen concentration of 50 μM, the life time of NO (2 μM) was about 9.5 and 10 min in the presence and absence of 5 mM GSH, respectively, as determined by a NO-selective electrode (Intermedical Co., Tokyo, Japan). It should be noted that the action of NO on mitochondrial respiration of digitonin-permeabilized cells but not intact cells was decreased by adding 5 mM GSH [10]. Thus, the decrease in the effective concentration of NO by direct interaction with GSH may not account for the suppressive effect of this tripeptide. Alternatively, the chemical reaction between NO and GSH might possibly be enhanced by the presence of mitochondria. Unfortunately, in the presence of high concentrations of mitochondrial and other proteins, this electrode can not be used for low levels of NO. Because the inhibitory effect of NO is particularly apparent at low oxygen tensions presumably due to competition of the two gases for the binding to cytochrome *c* oxidase [2,9,10,25], GSH and related thiols may affect the mode of this competition. The mechanism by which GSH suppresses the inhibitory effect of NO should be studied further.

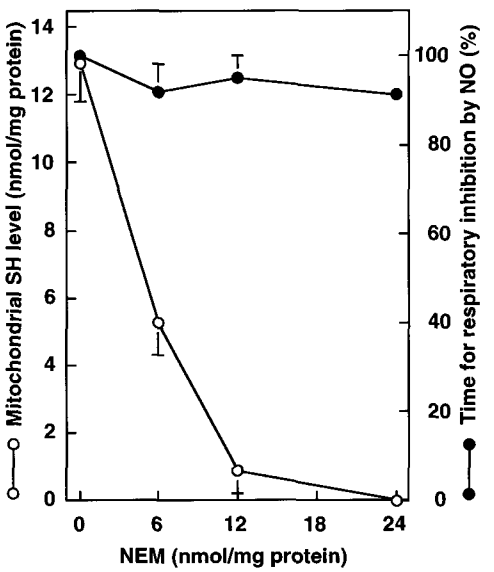

Fig. 6. Effect of depletion of SH residues in mitochondria. Isolated mitochondria were incubated with varying doses of NEM at 4°C for 10 min. After that, free thiol levels in mitochondria were measured and plotted (open circles). At the same time, as in Fig. 3, the time for the disappearance of the inhibitory effect of NO on these mitochondrial respiration was measured and plotted (% control; closed circles) by adding NO-saturated solution to the reaction mixture to give a concentration of 1 μM at an oxygen tension of 50 μM. The control experiments were performed without NEM treatments. Other conditions were the same as described in Fig. 1. Results are mean ± S.D. from five experiments.

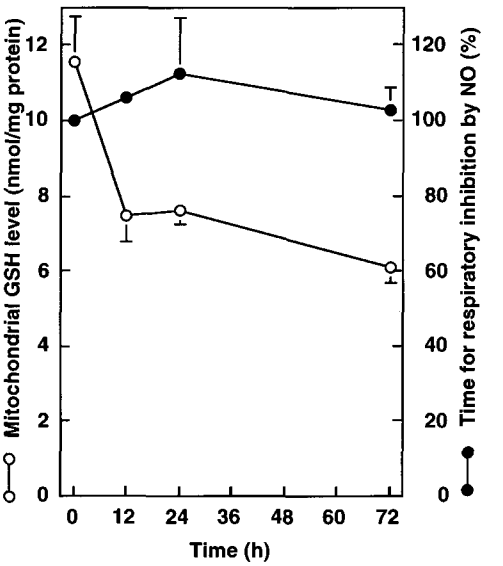

Fig. 5. Effect of decrease of GSH in mitochondria. After repeated intraperitoneal injections of BSO, a specific inhibitor of GSH synthesis, liver mitochondria were isolated and the GSH levels were measured (open circles). At the same time, as in Fig. 3, the time for the disappearance of the inhibitory effect of NO on these mitochondrial respiration was measured and plotted (% control; closed circles) by adding NO-saturated solution (final concentration 1 μM) to the reaction mixture at an oxygen tension of 50 μM. The control experiments were performed without BSO treatments. Other conditions were the same as described in Fig. 1. Results are mean ± S.D. from five experiments.

Although extracellular thiols, such as albumin, also react with NO, this interaction might occur minimally in the circulation because of the presence of erythrocytes which contain high levels of hemoglobin. Thus, cytosolic thiols including GSH might be more important for the inhibition of mitochondrial respiration by NO than those in the circulation and extracellular compartments. This notion is consistent with our previous report [10].

The metabolism of GSH occurs via inter- and intraorgan cycles, such as hepatic secretion of GSH, its degradation by tissues that have γ-glutamyltransferase and reabsorption of its constituent amino acids [29]. Hence, local concentrations of cysteine might be fairly high in tissues enriched with γ-glutamyltransferase, such as kidney and small intestine. Thus, the suppressing effect of cysteine on the inhibitory action of NO might also operate in these tissues.

It should be noted that iNOS is expressed in activated macrophages particularly at the site of inflammation. For example, iNOS activities in Kupffer cells and hepatocytes of septic animals are high. Hence, NO formed in these cells might interact with their mitochondria thereby inhibiting the energy metabolism. However, such an inhibitory effect would be decreased by the presence of extramitochondrial GSH (and cysteine). Hence, cytosolic but not intramitochondrial GSH might be an important factor for stabilizing the energy metabolism of mitochondria.

Acknowledgements: This work was supported by grants from the Ministry of Education, Science and Culture of Japan and from the Osaka City University Research Foundation.

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