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Effects of NO-Generating Compounds on Synaptosomal Energy Metabolism

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Abstract: The effects of nitroprusside and *S*-nitrosocysteine, compounds that generate nitric oxide (NO), on synaptosomal energy-producing pathways and energy level were investigated. The decrease in respiration was much faster and more pronounced with *S*-nitrosocysteine than with nitroprusside. *S*-Nitrosocysteine, at 10 μM, inhibited by 80% respiration with glucose and succinate (plus rotenone) in intact synaptosomes and with ascorbate/cytochrome *c* in broken preparations. Oxygenated hemoglobin reversed and/or prevented the inhibition, whereas glutathione (GSH) prolonged it. Under aerobic conditions, the synaptosomal energy level (creatine phosphate/creatine and ATP/ADP ratios) was reduced by the presence of *S*-nitrosocysteine, whereas lactate generation was enhanced. The effects on energy parameters were greater at 5 min than at 15 min of incubation and were more pronounced in the presence of GSH. Under strictly anaerobic conditions, lactate production was reduced by the NO-generating compounds in a concentration-dependent manner. It is concluded that (a) inhibition of oxidative phosphorylation by NO leads to a fall in the synaptosomal energy level, which in turn stimulates glycolysis; (b) glycolysis can be inhibited by higher concentrations of the radical; and (c) inhibitory effects on the energy-generating pathway and ATP level could contribute to NO toxicity under some in vivo situations. **Key Words:** Nitric oxide—Synaptosomal metabolism—Energy level—Oxidative phosphorylation—Glycolysis.
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It has become clear during the past few years that the nitric oxide radical ($NO^{\bullet}$) is an important intracellular messenger and an effector of a variety of processes (Moncada et al., 1991; Nathan and Hibbs, 1991; Bredt and Snyder, 1992). This very reactive gaseous molecule not only is involved in bactericidal and tumoricidal actions of macrophages (Nathan and Hibbs, 1991) but also may be responsible, at least in part, for tissue damage that occurs in postischemic reperfusion injury and neurodegenerative diseases (Oury et al., 1992; Buisson et al., 1993; Izumi et al., 1993). NO has been shown to inhibit DNA (Hibbs et al., 1988) and protein synthesis (Curran et al., 1991), induce intracellular loss of iron (Drapier and Hibbs, 1986), and cause inhibition of aconitase (Drapier and Hibbs, 1986; Welsh and Sandler, 1992), the mitochondrial respiratory chain (Drapier and Hibbs, 1988; Hibbs et al., 1988; Stadler et al., 1991; Bolaños et al., 1994; Brown and Cooper, 1994; Cleeter et al., 1994), and a glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (Dimmeler et al., 1992; Molina y Vedia et al., 1992; Zhang and Snyder, 1992; McDonald and Moss, 1993; Vaidyanathan et al., 1993).

Inhibition of proteins of the tricarboxylic acid cycle, the respiratory chain, and glycolysis should result in a reduction of ATP generation and a fall in the nucleotide content of the cell. Consistent with this prediction are studies that show that prolonged (hours/days) treatments with compounds that either release NO or increase its intracellular production lead to a decline in the intracellular ATP level, although the observed effect is usually rather modest (Corbett et al., 1992; Stefanovic-Racic et al., 1994). There is, however, no information on the short-term (minutes) effects of NO on cellular energetics. Moreover, instead of a *fall* in lactate generation expected from an inhibition of glyceraldehyde-3-phosphate dehydrogenase, a *rise* is commonly observed (Albina and Mastrofrancesco, 1993; Bolaños et al., 1994; Stefanovic-Racic et al., 1994), which suggests stimulation rather than blockade of glycolysis.

Brain tissue contains NO synthase (Bredt et al., 1990) and produces NO, and it has been reported that in hypoxic/ischemic CNS the concentration of the radical can rise to higher than 10 μM (Tominaga et al., 1994). As inhibition of anaerobic energy production may be one of the mechanisms responsible for the NO toxicity, the current study was designed to elucidate the apparently contradictory reports in the literature

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Abbreviations used: GSH, glutathione; NO, nitric oxide; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

and to answer the following pertinent questions: (a) Is glycolysis blocked by NO and in what situations? (b) Is the inhibition reversible and under what conditions? and (c) Is the energy level affected by NO and at what time scale and effector concentration? The experiments were performed using isolated nerve endings, a model system for neurons.

MATERIALS AND METHODS

Glutathione (GSH), potassium ferri- and ferrocyanide, cysteine, and bovine hemoglobin were purchased from Sigma (St. Louis, MO, U.S.A.). Sodium nitrite was from Aldrich (Milwaukee, WI, U.S.A.), and superoxide dismutase from Boehringer–Mannheim (Indianapolis, IN, U.S.A.).

Preparation of *S*-nitrosocysteine

A stock solution of *S*-nitrosocysteine was prepared under acidic conditions from cysteine and sodium nitrite (Lei et al., 1992) immediately before each experiment and used within 2 h. When required, the stock solution was diluted five- or 10-fold in 1 *M* HCl. Oxygenated hemoglobin was obtained from rat blood, as described by Randad et al. (1991).

Preparation of synaptosomes

Synaptosomes were isolated from the forebrains and midbrains of male Sprague–Dawley rats (220–250 g), as described by Booth and Clark (1978). In most experiments, the synaptosomal pellet was washed and suspended in a modified Krebs–Henseleit buffer (130 m*M* NaCl, 5 m*M* KCl, 1 m*M* NaH_2PO_4, 1.3 m*M* $MgSO_4$) containing 20 m*M* HEPES, pH 7.4. Unless stated otherwise, the synaptosomes were supplemented with 1.27 m*M* calcium and 10 m*M* glucose.

Incubation conditions

Measurements of respiration, aerobic lactate generation, and nucleotide levels. Synaptosomes were preincubated for 10 min at 37°C in a shaking water bath at 2–3 mg of protein/ml and then either transferred to the oxygen electrode-equipped chamber for measurements of respiration or used for determinations of lactate and nucleotide levels. At the end of the preincubation period, a sample was withdrawn and quenched with cold perchloric acid (0.6 *M* final concentration) for evaluation of the content of metabolites at time zero. Various amounts of stock solutions of the NO-producing agents were then added as demanded by the experimental protocol and the incubations continued. At the desired time (specified in the text or the tables), samples were quenched with perchloric acid (as above), kept on ice for 15–20 min, and centrifuged, and the clear extracts were neutralized with 2.5 *M* $KHCO_3$.

Measurements of lactate under anaerobic conditions. Aliquots of a concentrated suspension of synaptosomes (12–14 mg of protein/ml) were apportioned to vials containing three volumes of Krebs–HEPES buffer that had been preequilibrated for 45 min with argon. The buffer also contained 15 m*M* glucose, 10 n*M* glucose oxidase, and 5 n*M* catalase to ensure complete exhaustion of oxygen (Calhoun et al., 1988). The vials were fitted with caps incorporating thin Teflon septa that allowed additions to be made with microsyringes during the course of the experiment. Before the caps were put in place, the air above the liquid was flushed with argon gas; the vessels were then placed in a shaking water bath at 37°C. It was determined in separate experiments that after addition of synaptosomes (or small volumes of reagents of interest) the mixtures became anaerobic within a few seconds and that the oxygen-consuming system (glucose/glucose oxidase/catalase) did not interfere with glycolytic activity of synaptosomes. After a 5-min preincubation, duplicate samples were withdrawn, quenched with cold perchloric acid (as above), and used for determination of lactate at time zero. The remaining vessels were supplemented with the required test reagents (which were introduced in small volumes, usually not larger than 20 μl/ml of incubation mixture) and the incubation was continued for 30 min. At the end, samples were quenched and treated as described above for measurements of metabolites.

Measurement of cytochrome oxidase activity. Cytochrome *c* oxidase activity was measured in a medium containing 0.05 m*M* potassium phosphate, pH 7.4, 10 m*M* ascorbate, 20 μ*M* cytochrome *c*, and 0.23 m*M* *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD). Integrity of synaptosomes used in these experiments was destroyed by one cycle of freezing/thawing and subsequent addition of Triton X-100 (0.1% final concentration).

Measurements of metabolites

Lactate, creatine phosphate, and creatine were measured by a standard enzymatic procedure (Bergmeyer, 1974). ATP and ADP were determined by HPLC (Meglasson et al., 1989).

Other procedures

Oxygen consumption was measured at 37°C using an oxygen electrode as described previously (Erecińska and Dagani, 1990). Protein content was determined by the biuret reaction using bovine serum albumin as the standard.

Statistical evaluation of the data was performed using either Student's *t* test or *t* statistics with correction for multiple comparisons.

RESULTS

Effect of sodium nitroprusside and *S*-nitrosocysteine on respiration

Addition of 1 m*M* nitroprusside had no immediate effect on respiration, whereas a stimulation was observed with 1 m*M* ferrocyanide and a marked inhibition even with a low concentration of cyanide (0.1 m*M*, Fig. 1). However, closer inspection of the oxygen consumption records showed that whereas in a control incubation the transition between aerobiosis and anaerobiosis was very abrupt (i.e., the trace remained linear down to a very low oxygen tension), the transition in the presence of nitroprusside was more gradual and resembled the behavior with *S*-nitrosocysteine (Fig. 2IB and C). The response to nitroprusside was neither altered when experiments were carried out in the dark, nor affected by addition of 100 μ*M* GSH. Ferricyanide (1 m*M*) had no influence on respiration of synaptosomes.

Addition of *S*-nitrosocysteine inhibited oxygen consumption in intact synaptosomes respiring with either glucose (Fig. 2IB) or succinate (in the presence of rotenone, Fig. 2IC) and in broken synaptosomes supplemented with ascorbate, cytochrome *c*, and TMPD

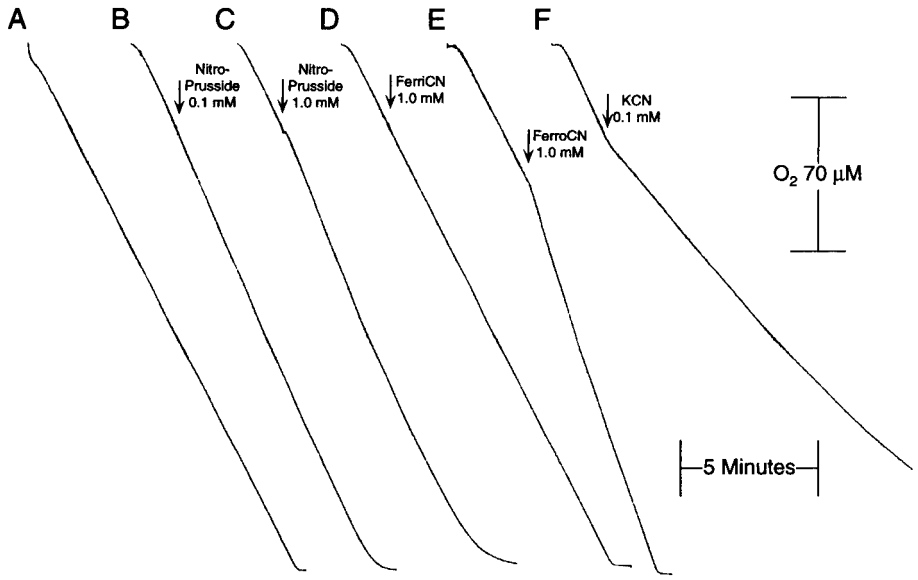

FIG. 1. Effect of nitroprusside and products of its decomposition on synaptosomal respiration. Oxygen uptake was measured at 37°C after a 10-min preincubation with glucose and calcium. Protein concentration was 3.1 mg/ml. FerroCN, ferrocyanide; FerriCN, ferricyanide.

(Fig. 3). The inhibition by *S*-nitrosocysteine usually required a few seconds to manifest itself fully and at 5–10 μM reached 80% or greater. With the same concentration of the compound, the kinetic characteristics of inhibition were dependent on protein content of the incubation mixture; with higher protein, a slower and more gradual response was observed. Moreover, longer inhibitions were seen when *S*-nitrosocysteine was introduced at a lower ambient oxygen tension in the chamber. The inhibitory action of the compound was prevented, or reversed, by oxygenated hemoglobin (Fig. 2IIA and B).

The inhibition of oxygen consumption by *S*-nitrosocysteine was prolonged by GSH (Fig. 3). Superoxide dismutase (150 U/ml) added to the suspension of intact synaptosomes also increased, somewhat, the length of inhibition of respiration by *S*-nitrosocysteine (data not shown).

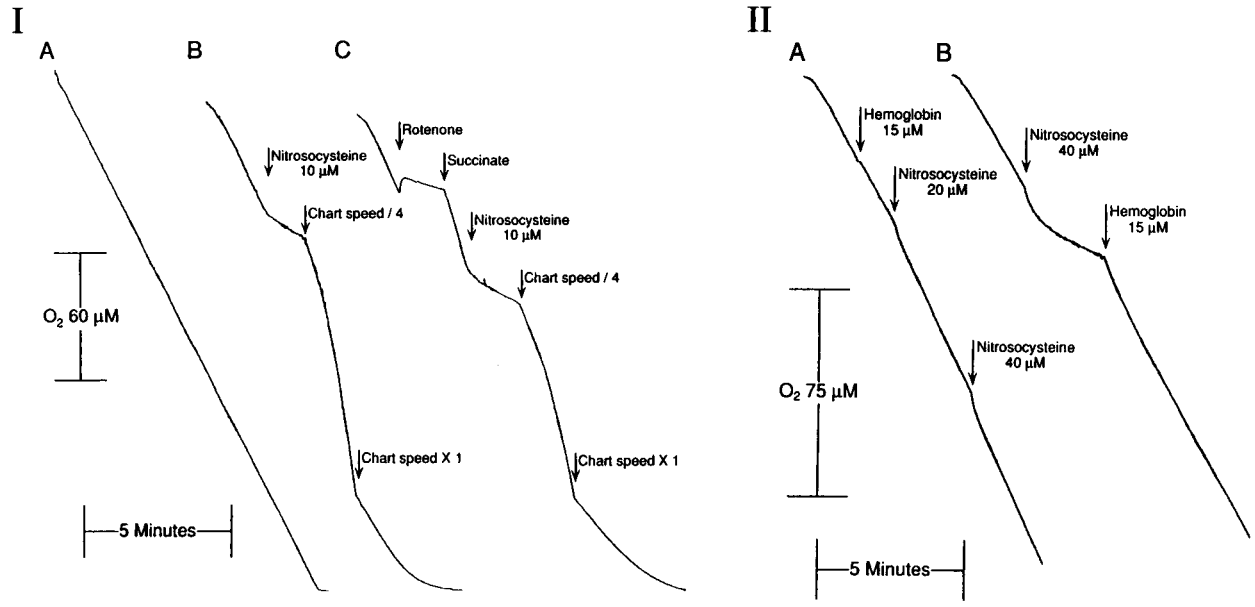

FIG. 2. Effect of *S*-nitrosocysteine in the presence and absence of hemoglobin on oxygen uptake by synaptosomes supplemented with different respiratory substrates. **I:** Oxygen uptake was measured at 37°C after a 10-min preincubation with glucose and calcium. Protein concentration was 2.6 mg/ml, succinate was added at 5 m*M*, and rotenone at 10 μM. A shows control rate of respiration with glucose. Note a decrease in the chart speed where indicated. **II:** Conditions are the same as in I. Protein concentration was 2.2 mg/ml. Oxygenated hemoglobin was added where indicated.

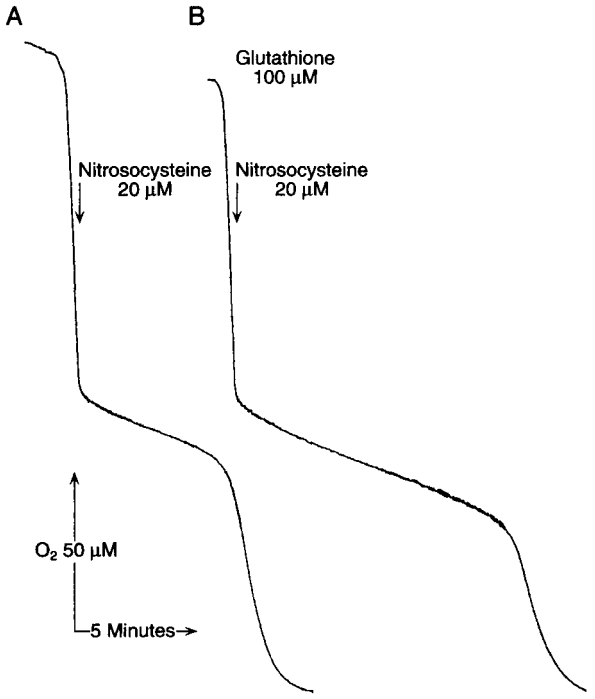

FIG. 3. Oxidation of reduced cytochrome *c* by broken synaptosomes in the absence (**A**) and presence (**B**) of GSH. Synaptosomes (0.2 mg of protein/ml) disrupted by freezing/thawing and addition of Triton X-100 were preincubated in 50 m*M* potassium phosphate buffer, pH 7.4, for 10 min at 37°C and then transferred to the oxygen electrode chamber. Ascorbate, cytochrome *c*, and TMPD were added in rapid succession using microsyringes (top of each trace) at final concentrations given in Materials and Methods. In B, 100 μM GSH was present.

Compounds that were present in the reagent solution, the solvent (HCl), and products of *S*-nitrosocysteine decomposition were also tested for their influence on synaptosomal respiration. HCl in concentrations equivalent to those added with the inhibitor, sodium nitrite, as well as nitrate (up to 5 m*M*) and cystine (100 μM) were without effect. By contrast, cysteine (0.1–1 m*M*) stimulated oxygen consumption almost twofold.

Synaptosomal glycolytic activity in the presence of NO-generating compounds

The rate of lactate generation from synaptosomes incubated for 30 min with glucose (after a 10-min preincubation) was 1.09 ± 0.09 nmol/min/mg of protein and was linear over this time interval; addition of either 1 m*M* nitroprusside or 100 μM *S*-nitrosocysteine caused a 20–30% increase. After a 15-min incubation with 100 μM *S*-nitrosocysteine, the amount of lactate produced was 69 ± 5% above the control. Addition of 10 μM rotenone increased the glycolytic rate to 11.8 ± 0.3 nmol/min/mg of protein, and this was unaltered by either 1 m*M* nitroprusside or up to 200 μM *S*-nitrosocysteine. However, when synaptosomes were incubated under strictly anaerobic conditions, the NO-generating compounds decreased lactate production (Table 1). The effect was small (10% inhibition) with 1 m*M* nitroprusside, whereas a dose-dependent relationship was observed with *S*-nitrosocysteine, with almost 90% inhibition at 200 μM.

The extent of inhibition of glycolysis by either nitroprusside or *S*-nitrosocysteine was not potentiated by 100 μM GSH (data not shown), but was reversed and/or prevented by reduced hemoglobin (Table 1).

Synaptosomal energy level in the presence of NO-generating compounds

Table 2 shows that addition of *S*-nitrosocysteine resulted in a fall in the synaptosomal energy level ([ATP]/[ADP] ratio), as manifested by a decrease in the concentration of ATP and, at a higher concentration of the compound, a rise in that of ADP. The effect was concentration and time dependent. Larger amounts of the NO-generating compound gave larger falls in the [ATP]/[ADP] ratio. On the other hand, larger decreases in the energy level were seen at shorter incubation times, and after 15 min, a restitution toward the control was observed. GSH, at very low concentrations (10–50 μM), markedly potentiated the effect of *S*-nitrosocysteine, and a substantial decline in [ATP]/[ADP] ratio was still seen at 15 min of incubation. Hemoglobin, on the other hand, had an opposite effect and only relatively minor changes in the nucleotides were seen in its presence.

In two experiments, phosphocreatine and creatine were measured after 5 min of incubation with and without 100 μM *S*-nitrosocysteine. In controls, the creatine phosphate level was 5.25 ± 0.4 nmol/mg of protein and the creatine level was 9.98 ± 0.91 nmol/mg of protein; the corresponding values with the NO-generating compound were 1.36 ± 0.3 nmol/mg of protein and 14.5 ± 1.2 nmol/mg of protein for creatine

TABLE 1. *Effect of NO-generating compounds on lactate production by synaptosomes incubated under anaerobic conditions*

	Lactate (nmol/mg of protein/min)	%
Control	11.4 ± 0.3 (9)	100
SNP (1 m*M*)	10.3 ± 0.2[a] (4)	90
S-NOC (20 μM)	7.22 ± 0.3[a] (4)	63
S-NOC (100 μM)	4.58 ± 0.3[a] (3)	40
S-NOC (200 μM)	1.25 ± 0.1[a] (3)	11
SNP (1 m*M*) + Hb (15 μM)	11.3 ± 0.6 (2)	100
S-NOC (20 μM) + Hb (25 μM)	11.8 ± 0.5 (3)	104
S-NOC (100 μM) + Hb (25 μM)	9.36 ± 0.8[a,b] (3)	82

Conditions of incubations are described in Materials and Methods. Lactate was measured by a standard enzymatic technique. Values represent means ± SEM for the number of experiments indicated in parentheses. Hb, hemoglobin; *S*-NOC, *S*-nitrosocysteine; SNP, sodium nitroprusside.

[a] Significantly different from control ($p < 0.05$).

[b] Significantly different from the same *S*-NOC concentration ($p < 0.05$).

TABLE 2. *Effects of S-nitrosocysteine (S-NOC), GSH, and hemoglobin (Hb) on synaptosomal energy level*

Conditions	ADP	ATP	ATP/ADP	n
5-min incubation				
Control	0.66 ± 0.06	3.33 ± 0.16	5.17 ± 0.19	6
S-NOC (20 μM)	0.58 ± 0.02	2.84 ± 0.11	4.90 ± 0.05	3
S-NOC (100 μM)	0.76 ± 0.04	2.77 ± 0.13	3.47 ± 0.11[a]	5
Hb (25 μM)	0.60 ± 0.01	2.93 ± 0.12	4.85 ± 0.11	2
GSH (500 μM)	0.72	3.66	5.06	1
S-NOC (100 μM) + GSH (10 μM)	0.73	2.02	2.79	1
S-NOC (100 μM) + GSH (50–500 μM)	1.05 ± 0.05	1.75 ± 0.01	1.72 ± 0.16[a]	7
S-NOC (100 μM) + Hb (25 μM)	0.66 ± 0.03	2.82 ± 0.03	4.32 ± 0.21[a,b]	4
15-min incubation				
Control	0.62 ± 0.02	3.19 ± 0.07	5.20 ± 0.13	6
S-NOC (100 μM)	0.66 ± 0.02	2.81 ± 0.10	4.25 ± 0.20[a]	6
Hb (25 μM)	0.58 ± 0.02	3.08 ± 0.15	5.32 ± 0.08	2
GSH (500 μM)	0.78	4.02	5.16	1
S-NOC (100 μM) + GSH (10 μM)	0.54	2.26	4.17	1
S-NOC (100 μM) + GSH (50–500 μM)	0.66 ± 0.03	2.25 ± 0.08	3.45 ± 0.22[a]	7
S-NOC (100 μM) + Hb (25 μM)	0.58 ± 0.02	2.87 ± 0.03	4.94 ± 0.12[b]	4

The incubations were carried out as described in Materials and Methods. GSH and Hb were added after a 10-min preincubation (time zero) immediately before *S*-NOC. Aliquots were quenched at the times indicated, the extracts neutralized, and the nucleotides measured by HPLC. Values are nanomoles per milligram of protein (means ± SEM) for the number of experiments indicated.

[a] Significantly different from control ($p < 0.05$).

[b] Significantly different from the same *S*-NOC concentration ($p < 0.05$).

phosphate and creatine, respectively. Consequently, the creatine phosphate/creatine ratio declined from 0.53 to 0.09.

Nitroprusside at 1 mM gave no alteration in the [ATP]/[ADP] ratio after 5 min but decreased it from 5.2 ± 0.2 to 4.81 ± 0.1 after 15 min of incubation; the difference was eliminated by 20 μM oxygenated hemoglobin.

DISCUSSION

The results of the current study lead to the following conclusions: (a) In rat brain synaptosomes, the NO-producing compounds decrease mitochondrial respiration, consistent with earlier studies in the literature (Drapier and Hibbs, 1988; Hibbs et al., 1988; Stadler et al., 1991; Bolaños et al., 1994; Brown and Cooper, 1994; Cleeter et al., 1994). A large reduction in oxygen consumption is seen at 5–10 μM *S*-nitrosocysteine. (b) Inhibition of mitochondrial respiration leads to a decrease in the synaptosomal energy level (i.e., lowered [ATP]/[ADP] ratio). (c) In the presence of oxygen, nitroprusside and *S*-nitrosocysteine enhance glycolysis, whereas in the absence of oxygen they inhibit it. (d) Effects of NO on synaptosomal energy production represent a fine balance among several variables: sensitivity of various reactions to NO, ambient oxygen tension, local NO level, and the concentration of the NO "buffering" sites.

It is well known that one of the products of nitroprusside breakdown is NO. However, the overall chemistry of nitroprusside decomposition is very complex (Bates et al., 1991; Rao et al., 1991) and the process produces, among other products, cyanide; the latter could also block the mitochondrial respiratory chain (e.g., see Brown and Cooper, 1994, where it is not clear which is the true inhibitor of cytochrome oxidase). The effects of both cyanide and NO are sensitive to the addition of hemoglobin; however, there is no known mechanism whereby cyanide would inhibit glycolysis. Our observation that nitroprusside reduced anaerobic lactate generation indicates that at least some of its actions are likely to be due to NO. The situation with *S*-nitrosocysteine is much more straightforward. We have shown previously (Vanderkooi et al., 1994) that in the absence of oxygen, addition of this compound to a buffer at neutral pH generates NO quantitatively. The other product of the *S*-nitrosocysteine breakdown, cystine, did not influence the energy-producing pathways. Thus is can be concluded that the effects of *S*-nitrosocysteine discussed in the present work are due to NO. Their reversal by hemoglobin is consistent with this conclusion. The observation that superoxide dismutase did not prevent the inhibition of respiration by *S*-nitrosocysteine indicates that peroxynitrite is not involved in any of the phenomena described here.

In synaptosomes incubated in the presence of oxygen, glycolysis is enhanced by the NO-generating compounds, consistent with some observations in the literature (Albina and Mastrofrancesco, 1993; Bolaños et al., 1994; Stefanovic-Racic et al., 1994). The rise in lactate generation is caused, most likely, by the decrease in cellular energy level (Table 2). A stimulation of phosphofructokinase by a decline in ATP and a rise in ADP and AMP would account for this effect. It should be noted that in aerobic experiments involving

measurements of metabolites, incubations are carried out in open flasks and that the tension of oxygen in the solution is probably only slightly lower than that in the atmosphere. This situation promotes elimination of NO via its reactions with oxygen and favors binding of NO, rather than O_2, to cytochrome oxidase. For these reasons, the action of *S*-nitrosocysteine, which produces a bolus of NO, is relatively short-lived and synaptosomes recover from the insult at longer incubation times.

Our data demonstrate (Table 1) that in anaerobiosis, glycolysis is suppressed by NO, although at concentrations larger than those (10–100 μM) that inhibit the mitochondrial respiratory chain (<5–10 μM). The most likely target for the action of NO is glyceraldehyde-3-phosphate dehydrogenase via a mechanism that involves either ADP-ribosylation (Dimmeler et al., 1992; Molina y Vedia et al., 1992; Zhang and Snyder, 1992) or SH-nitrosylation (McDonald and Moss, 1993; Vaidyanathan et al., 1993; Mohr et al., 1994). Another possible target is aldolase, which in the purified state has been shown to be markedly sensitive to low concentrations of nitrite (Yarbrough et al., 1980). A comparison of the effects on glycolysis under aerobic and anaerobic conditions leads to a conclusion that in the presence of oxygen, the final rate of lactate production is the product of two opposing effects of NO, an indirect stimulation via action on phosphofructokinase and a direct inhibition of the glycolytic enzymes. Because inhibition of glyceraldehyde-3-phosphate dehydrogenase and/or aldolase requires higher concentrations of NO than the blockade of mitochondrial proteins, the stimulatory effect predominates.

It is evident from the results in Table 2 that inhibition of mitochondrial respiratory chain leads to a decrease in cellular energy production and thus a decline in the energy state of synaptosomes. Under aerobic conditions, because of the differential sensitivity to NO of the energy-producing pathways discussed above, reduction in glycolytic rate is unlikely and hence an effect on this pathway cannot contribute to the *fall* in [ATP]. However, in the absence of oxygen, when glucose is the only energy source, the inhibitory effect of NO on glucose catabolism may exacerbate the fall in tissue ATP content.

The final issue concerns the extent to which the results obtained in a synaptosomal model apply to in vivo situations. The concentration of NO in whole brain is not known with certainty because the results currently available in the literature differ substantially. Under normoxic conditions, Balcioglu and Maher (1993) provide a figure of 2 nM, Malinski et al. (1993) of ~10 nM, and Tominaga et al. (1994) of 1.6 μM. In ischemia, the concentration was reported to rise to 4 μM (Malinski et al., 1993) or even 10 μM (Tominaga et al., 1994) and to remain elevated in the postischemic reperfusion period when brain oxygen tension is high. In macrophages and in their vicinity, the [NO] is likely to be in the micromolar range because these cells have a very powerful NO synthase. This means that in conditions when the ambient level of the radical is high, inhibition of cellular energy production is a plausible event that should be considered as one of the mechanisms that contribute to the toxicity of nitric oxide.

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