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Nitric Oxide-Independent Effects of Nitric Oxide Donors on Energy Metabolism in Erythrocytes

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ABSTRACT. In order to study the roles of nitric oxide (NO) in various biological events, several types of NO-releasing agents have been extensively used. Although both NO and its donors and/or their decomposed products may have biological activities, most of the cellular responses to these donors have been postulated to reflect NO-dependent events. Among the various NO donors, 1-hydroxy-2-oxo-3-(*N*-methyl-aminopropyl)-3-methyl-1-triazene (NOC7), 3-morpholinosydnonimine *N*-ethylcarbamide (SIN-1), *S*-nitrosoglutathione, *S*-nitrosocysteine (CysNO), and related nitrosothiols are commonly used agents. To investigate the biological activities of these donors and their decomposed products, we tested their effects on energy metabolism in erythrocytes. When incubated with freshly prepared erythrocytes, NOC7, Cys-NO, and their decomposed products, but not NO and its oxidized metabolites, nitrite and nitrate, decreased cellular ATP levels. Although SIN-1 generates both NO and superoxide radical thereby forming peroxynitrite ($ONOO^-$), this donor had no appreciable effect on cellular ATP levels, even in the presence of superoxide dismutase. These results indicate that NOC7 and CysNO and/or their decomposed product(s), but not NO and its oxidized metabolites, are responsible for the decrease in cellular ATP levels. Thus, the effects of not only NO and its oxidized metabolites (NO_2^-, NO_3^-), but also NO donors and their decomposed products, should be taken into account when attempting to understand the mechanism of biological responses induced by NO donors. BIOCHEM PHARMACOL **59**;5:557–561, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. nitric oxide; nitric oxide donors; ATP; erythrocytes; glycolysis; organic cation

NO§ has attracted much interest not only as an endothelium-derived relaxing factor (EDRF) [1], but also as a multifunctional molecule that triggers various biological processes. Because the lifetime of NO is fairy short (~several seconds), various types of synthetic NO donors which slowly release NO have been used for the analysis of NO-dependent biological events. NO reversibly binds to mitochondrial complex I, II, and IV [2], thereby inhibiting their electron transfer [3–5]. As a result, NO reversibly inhibits ATP synthesis in mitochondria and decreases its cellular levels [5]. It has also been reported that glyconeogenesis and cellular utilization of glucose are affected by NO [6–9] and its donors [8–11]. To analyze the mechanism of NO-dependent changes in energy metabolism, various types of NO donors with long half-lives have been extensively used. In the presence of free metals and reducing agents, *S*-nitrosoglutathione (GS-NO), CysNO, and *S*-nitroso-*N*-acetylpenicillamine release NO. In the absence of metals and reducing agents, their half-lives *in vitro* are fairly long (>10 hr). Some of these *S*-nitrosothiols also occur in healthy human plasma [12]. NOC7 has been used as a rapid and spontaneous releaser of NO (half-life of 5 min). Another NO donor commonly used is SIN-1, which spontaneously releases NO and superoxide, thereby forming peroxynitrite ($ONOO^-$). The physico-chemical properties of these NO donors and their decomposed products differ significantly from one compound to another. Judging from their chemical structure, the decomposed product(s) of some NO donors seems to affect cellular metabolism and transport, including synthesis and/or utilization of ATP. Erythrocytes have high levels of hemoglobin, which rapidly traps NO and oxidizes it to biologically inactive metabolites such as NO_3^-. Thus, erythrocytes might permit studies on the effects of NO donors and their decomposed products on the synthesis and/or utilization of ATP without being affected by mitochondrial energy metabolism. The present work establishes that the decomposed products of some NO donors, but not NO, markedly decreased ATP levels in erythrocytes.

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§ *Abbreviations:* NO, nitric oxide; NOC7, 1-hydroxy-2-oxo-3-(*N*-methyl-aminopropyl)-3-methyl-1-triazene; SIN-1, 3-morpholinosydnonimine *N*-ethylcarbamide; CysNO, *S*-nitrosocysteine; SOD, superoxide dismutase; and KRP, Krebs–Ringer phosphate buffer.

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MATERIALS AND METHODS

Materials

NOC7 and the glucose assay kit were obtained from Wako Pure Chemical. SIN-1 and the ATP assay kit were obtained from Funakoshi and Sigma, respectively. Human recombinant Cu, Zn-SOD was obtained as described previously [13]. CysNO was synthesized under acidic conditions by the method of Saville [14]. NO solution was prepared by infusing NO gas into 50 mM HEPES buffer (pH 7.4) as reported previously [15]. Decomposed products of NOC7 were prepared by incubating NOC7 at 37° in 10 mM phosphate buffer (pH 7.4) containing 0.15 M saline (PBS) under air atmospheric conditions at 37° for 2 days. The released NO from these donors was determined by the method of Saville [14]. The decomposed products of NOC7 did not generate NO under the present experimental conditions.

Preparation of Erythrocytes

Human erythrocytes were obtained from fresh blood samples from healthy volunteers. The cells were washed three times in 2 vol. of PBS 6 mM KCl and 1 mM $MgCl_2$ (KRP). Cells were suspended in KRP containing 1 mM $CaCl_2$ and used for the experiments.

Measurement of Glucose, Lactate, and ATP

Glucose concentrations in the medium were measured by the glucose assay kit using glucose oxidase. Lactate concentrations in the medium were determined by the method of Goodall and Byers [16]. Samples were incubated with lactate dehydrogenase, and enzymic reduction of NAD to NADH was monitored as the change in the absorbance at 340 nm. Alanine aminotransferase was added to remove pyruvate, thereby preventing the reverse reaction and product inhibition of the enzyme. Cellular levels of ATP were measured with a bioluminescence assay kit using luciferase.

Statistical Analysis

Data are expressed as means ± standard error (SEM) and were analyzed by one-way ANOVA. A P value <0.01 was considered statistically significant.

RESULTS

Effects of NO Donors on Cellular ATP Levels

To test whether NO affects the cellular level of ATP, erythrocytes were incubated at 37° in KRP containing 1 mM $CaCl_2$ in the presence or absence of NO donors. Figure 1 shows the effect of varying doses of NOC7 on cellular ATP levels. In the absence of NOC7, cellular ATP levels decreased fairly slowly. Under the standard conditions, the cellular level of ATP decreased from 1.1 ± 0.1 mM to 0.86 ± 0.04 mM during the incubation. NOC7 dose dependently enhanced the rate of decrease in the cellular ATP level. Figure 2 shows the effect of NO and related compounds on cellular levels of ATP 60 min after incubation. Although NO and its oxidized metabolites (NO_2^-, NO_3^-) did not affect cellular levels of ATP, both NOC7 and CysNO significantly decreased ATP levels. Although both NOC7 and CysNO stoichiometrically release NO, the decrease in the cellular ATP level was more marked with the former than with the latter. Such a difference might reflect, at least in part, the difference in the rate of NO release from the two compounds [17–23]. Because SIN-1 generates equimolar amounts of NO and superoxide (O_2^-) [24], thereby forming peroxynitrite [25], the effect of this donor was also tested in the presence of SOD. However, the presence of both SIN-1 and SOD did not affect the cellular levels of ATP. Thus, under the present experimental conditions, NO itself did not affect ATP levels in erythrocytes. We also tested the effects of the decomposed products of CysNO and NOC7 on cellular ATP levels. Incubation of erythrocytes with either cysteine, cystine, or degraded products of NOC7 significantly decreased cellular ATP levels. These results indicate that NO donors and/or their decomposed products, but not NO and its oxidized metabolites, might be responsible for the decrease in cellular ATP levels.

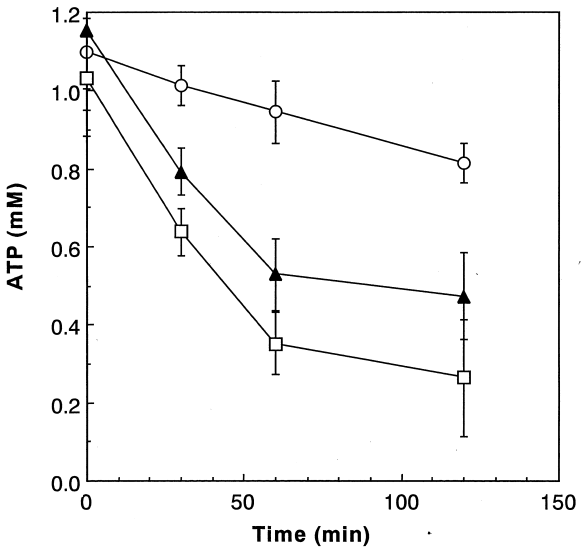

FIG. 1. Effect of NOC7 on ATP levels in erythrocytes. Erythrocytes (12% v/v) were incubated with varying concentrations of NOC7 at 37° as described in the text. At the indicated time, cellular ATP levels were determined as described in the text. Open circles, control; closed triangles, 2 mM NOC7; open squares, 5 mM NOC7. Data are expressed as means ± SEM, N = 4.

Effect of NOC7 and Glucose on Cellular ATP Levels

Among various NO donors examined, NOC7 exhibited the strongest activity in decreasing cellular ATP. Because ATP levels in erythrocytes are maintained by glycolysis, we also

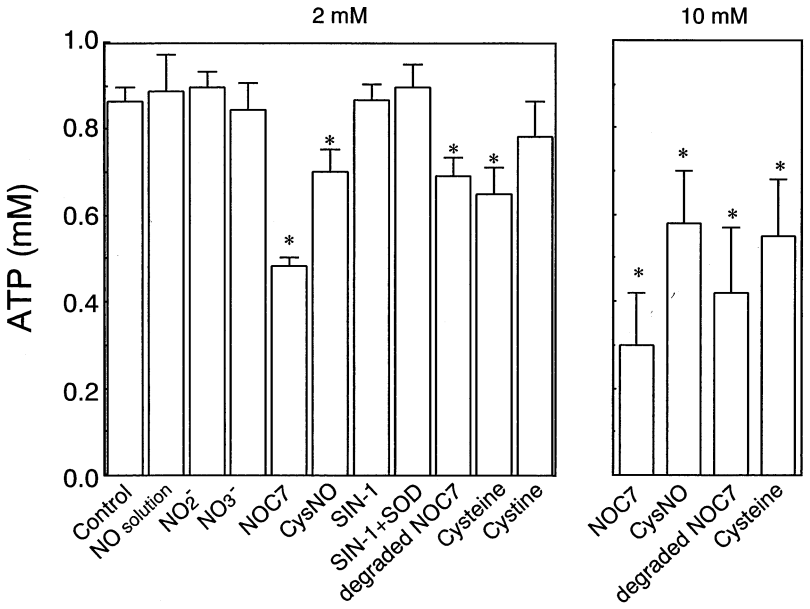

FIG. 2. Effect of NO and related compounds on ATP levels in erythrocytes. Under air atmospheric conditions, erythrocytes (12% v/v) were incubated in KRP containing 1 mM $CaCl_2$ and 2 mM (left panel) or 10 mM (right panel) of various compounds (except NO [400 μM]) at 37° for 60 min. Fifty units of SOD were also added in the experiment using SIN-1. Then, cellular ATP was determined as described in the text. Data are expressed as means ± SEM N = 4. *, $P < 0.01$ against control.

observed the effect of NOC7 and its decomposed products on cellular ATP in the presence and absence of glucose. Figure 3 shows the effect of NOC7 and its degraded products on cellular ATP levels in the presence or absence of glucose. Although NOC7 and its decomposed products dose dependently decreased cellular ATP levels in the absence of 1 mM glucose, they failed to affect ATP levels in the presence of glucose up to its concentration of 5 mM. However, a fairly high concentration of NOC7 (10 mM) markedly decreased cellular ATP levels even in the presence of glucose. Because glucose inhibited the effect of both NOC7 and its degraded products, enhanced utilization of ATP rather than inhibition of glycolysis might underlie the mechanism for the decrease in cellular ATP. To test this possibility, we examined the effect of NOC7 and its decomposed products on glycolytic activity in erythrocytes. When incubated with 1 mM glucose, the rates of its consumption and lactate formation were increased by decomposed products of NOC7 in a dose-dependent manner (Fig. 4)

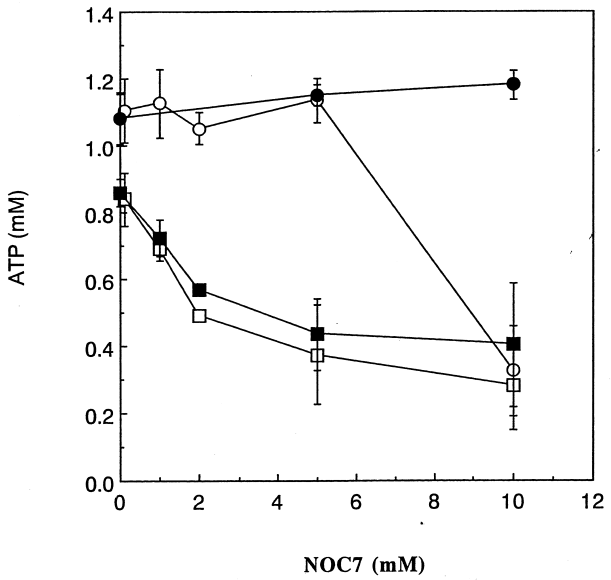

FIG. 3. Effect on NOC7 and glucose on cellular ATP levels. Erythrocytes were incubated with varying concentrations of NOC7 (open symbols) and its metabolites (closed symbols) at 37° for 60 min under air atmospheric conditions in the presence (circles) and absence (squares) of 1 mM glucose. Then, the cellular ATP level was determined. Other conditions were as described in the text. Data are expressed as means ± SEM, N = 4.

DISCUSSION

The present work demonstrates that NOC7, CysNO, and their degraded products, but not NO and its oxidized metabolites nor SIN-1, decreased ATP levels in erythrocytes. These results indicate that NOC7 and CysNO and/or their decomposed product(s), but not NO, might be principally responsible for the enhanced decrease in cellular ATP levels. The ATP level in erythrocytes is determined by the equilibrium between its utilization and regeneration by glycolysis. Because the decrease in the cellular ATP level was associated with enhanced glycolysis at NOC7 concentrations lower than 5 mM, this donor might principally increase the utilization of cellular ATP, thereby enhancing glycolysis. Both NOC7 and its decomposed product (presumably (3-*N*-methylaminopropyl)-methylamine) decreased cellular ATP levels, although the effect was more marked with the former than with the latter. Because NO had no appreciable effect on cellular ATP levels, utilization of ATP might be enhanced by both NOC7 and its degraded product. It should be noted that a high concentration (10 mM) of NOC7, but not its decomposed products, decreased cellular ATP even in the presence of glucose. Under identical conditions, glycolysis occurring in erythrocytes

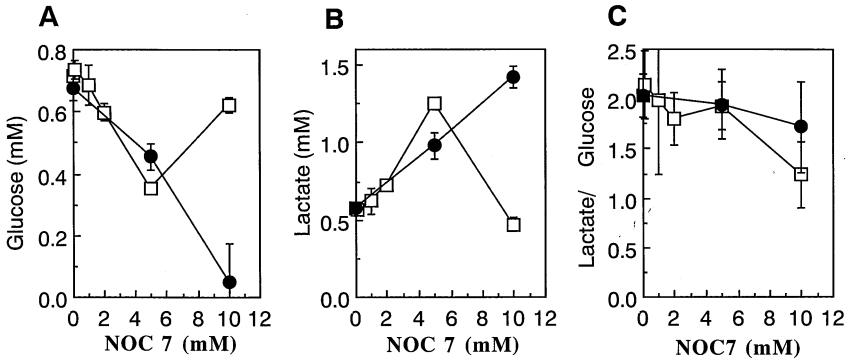

FIG. 4. Effect of NOC7 and its metabolites on glycolysis. Erythrocytes were incubated with varying concentrations of NOC7 (open squares) and its degraded products (closed circles) in the presence of 1 mM glucose. After incubation at 37° for 60 min, concentrations of lactate (A) and glucose (B) inthe medium were measured as described in the text. Panel (C) shows the ratio of lactate formed and glucose consumed. Data are expressed as means ± SEM, N = 4.

was also inhibited by NOC7, but not by its decomposed products (see Fig. 4). Thus, the decrease in cellular ATP induced by high concentrations of NOC7 might be responsible for the enhanced utilization and inhibited regeneration of ATP. Consistent with this notion is the finding that NO inhibited glyceraldehyde-3-phosphate dehydrogenase *in vitro* [26]. Although this enzyme does not function as a rate-limiting enzyme in glycolysis under physiological conditions, strong inhibition of the enzyme might decrease cellular ATP levels.

Because 1 mol of NOC7 generates 1.5 mol of NO, 10 mM of the donor might theoretically generate 15 mM of NO. The present experiments were performed with 12% erythrocytes (v/v), which contained about 0.72 μmol of hemoglobin per mL of the mixture. Thus, although NO has extremely high affinity for heme iron [27, 28], the concentration of NO released from NOC7 would be significantly higher than that of hemoglobin in the mixture. It should be noted that NO and/or its metabolites also react with free thiols [29–31]. Because erythrocytes contain about 3 mM of GSH [32] and hemoglobin has eight free cysteinyl residues on its surface [33], some fractions of NO (and its reactive intermediates) generated from NOC7 might also react with these thiols (< 6.1 μmol/mL) [34]. However, significant amounts of NO (~8 μmol/mL) would have been used for the interaction with other molecules than thiols in erythrocytes. Although glycolysis could be inhibited by fairly high concentrations of NOC7, such inhibition may not occur in the circulating erythrocytes. It should be noted that pharmacokinetic properties of NO donors significantly differ from one compound to another and, hence, local concentrations of these compounds and their metabolites might differ from each other. Because the cytoplasmic sides of cells are negatively charged, organic cations are often taken up rapidly into cells. It has been reported that both NOC7 and its decomposed product NONOate are organic cations [17] which could be actively taken up by some tissues, such as liver and kidney. Thus, these compounds might accumulate in these organs in sufficiently high concentrations to exhibit their pharmacological effects as described in the present work. Thus, the biological action of these compounds may also operate in liver, kidney, and other tissues that have active transport systems for organic cations.

Extensive accumulation of cationic molecules often perturb cellular metabolism and decrease membrane potential. Under such conditions, cells actively transport various solutes across their membranes and consume ATP to maintain cellular homeostasis. Such changes may underlie the mechanism by which cellular ATP levels were decreased by NOC7 and its decomposed products. It should be noted that CysNo decomposes to form NO (NO^+), cysteine, and their oxidized compounds. Oxidation of sulfhydryl groups on plasma membranes by these metabolites might rapidly decrease the membrane potential of various cells [35]. Decreased membrane potential of erythrocytes could be normalized predominantly by Na^+, K^+-ATPase. Thus, similar changes induced by cyst(e)ine might underlie the mechanism for the decrease in cellular ATP levels. This possibility should be studied further.

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