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Inhibition of creatine kinase by *S*-nitrosoglutathione

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Abstract The sarcoplasmic reticulum-bound creatine kinase from rabbit skeletal muscle was inhibited by the nitric oxide donor *S*-nitrosoglutathione (GSNO). This led to a decrease in Ca^{2+} uptake in sarcoplasmic reticulum vesicles when the transport was driven by ATP generated from phosphocreatine and ADP. In contrast, the Ca^{2+} transport measured using 2 mM ATP as substrate was unaffected by GSNO up to 200 μM. GSNO (5—20 μM) inhibited the activity of both soluble and membrane-bound creatine kinase. Oxyhemoglobin (15–40 μM) protected creatine kinase against inactivation by GSNO. The inhibition by 10 μM GSNO was reversed by the addition of dithiothreitol (2 mM). The results indicate that nitric oxide (NO, including NO^+, NO and NO^-) inactivates creatine kinase in vitro by promoting nitrosylation of critical sulphydryl groups of the enzyme.

Key words: Nitric oxide; Creatine kinase; Ca^{2+}-ATPase; Sarcoplasmic reticulum; Skeletal muscle; *S*-nitrosylation

1. Introduction

Nitric oxide is an intercellular messenger molecule that is synthesized from L-arginine in several tissues by a reaction catalyzed by NO synthases [1]. Different enzymes involved in energy transduction are inhibited by NO. These include glyceraldehyde-3-phosphate dehydrogenase, aconitase and complex I and II of the respiratory chain [1]. Skeletal muscle expresses high levels of neuronal type NO synthase [2]. Recently, it has been proposed that NO could affect skeletal muscle via two different mechanisms. One is related to activation of guanylyl cyclase which decreases the force generation by skeletal muscle fibers [3]. The other involves a possible *S*-nitrosylation of thiol groups in the sarcoplasmic reticulum such as those from the Ca^{2+} pump [3]. In the present report, we investigate whether NO donors affect the Ca^{2+} transport of sarcoplasmic reticulum.

2. Materials and methods

2.1. Vesicle preparation

Sarcoplasmic reticulum vesicles were prepared from rabbit fast skeletal muscle as described [4].

2.2. Ca^{2+} uptake

Ca^{2+} uptake was measured by filtration through Millipore filters (0.45 μm). The filter was washed six times with 5 ml of 3 mM $La(NO_3)_3$ and the remaining radioactivity was determined in a scintillation counter.

2.3. Creatine kinase activity

MM-creatine kinase (Sigma) or sarcoplasmic reticulum vesicles were incubated in the presence of various concentrations of GSNO, 50 mM MOPS-Tris (pH 7.0), 5 mM $MgCl_2$, 100 mM KCl and diluted 10-fold in a medium containing 50 mM MOPS-Tris (pH 7.0), 5 mM $MgCl_2$, 100 mM KCl, 10 mM phosphocreatine, 1 mM ADP, 1 mM NAD^+, 20 mM glucose, 2 U/ml hexokinase (Sigma), 2 U/ml glucose-6-phosphate dehydrogenase (Sigma) and 10 μM AP_5A. The forward reaction of creatine kinase was measured by NADH formation monitored by changes in absorbance at 340 nm. The NO donors in the concentration range used in this study had no effect on hexokinase or glucose-6-phosphate dehydrogenase activity measured by direct addition of ATP to the cuvette.

2.4. GAPDH activity

Purified GAPDH (1 U/ml, Boehringer Mannheim) was incubated in either the absence or presence of various GSNO concentrations in a medium with the same composition as that described for creatine kinase. After 2 min the enzyme was diluted 5-fold in a medium containing 100 mM HEPES-Tris (pH 7.7), 1 mM ATP, 0.9 mM EDTA, 2 mM $MgSO_4$, 6 mM 3-phosphoglyceric acid, 0.2 mM NADH and 10 U/ml 3-phosphoglycerate kinase (Boehringer Mannheim). NADH consumption was monitored at 340 nm.

2.5. Reagents

SNAP and GSNO were gifts from Dr. J. Assreuy (Department of Pharmacology, Federal University of Rio de Janeiro). Oxy- and methemoglobin were prepared according to Martin et al. [5].

3. Results

The Ca^{2+} pump is a membrane-bound protein that pumps

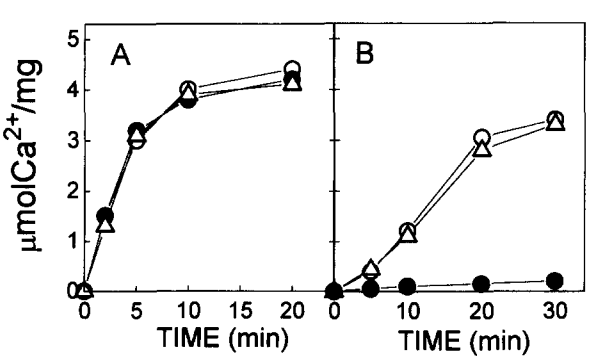

Fig. 1. Effect of GSNO on Ca^{2+} uptake in sarcoplasmic reticulum vesicles. Vesicles were incubated for 20 min in order to ensure adequate release of NO in a medium containing 50 mM MOPS-Tris (pH 7.0), 100 mM KCl, 20 mM P_i, 0.02–0.05 mg vesicle protein/ml, 10 μM AP_5A, in the absence (○) or presence of 200 μM GSNO (●), or 1 mM dcGMP (△). Ca^{2+} uptake was started by the addition of either 2 mM ATP (A) or 10 mM phosphocreatine plus 1 mM ADP (B). The values are representative of 3–4 experiments with two different vesicle preparations.

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Abbreviations: dcGMP, $N^2,O^{3'}$-dibutyrylguanosine 3',5'-cyclic monophosphoric acid; DTT, dithiothreitol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSNO, *S*-nitrosoglutathione; Mops, 4-morpholinopropanesulfonic acid; NO, nitric oxide (including NO^+, NO and NO^-); SNAP, *S*-nitroso *N*-acetylpenicillamine.

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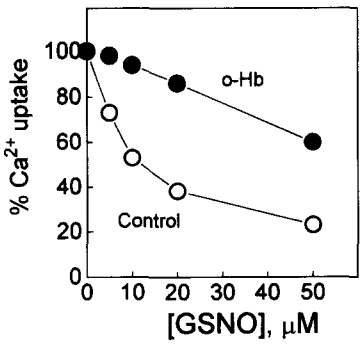

Fig. 2. Protection by oxyhemoglobin against inhibition of Ca^{2+} uptake by GSNO. Ca^{2+} uptake was measured as described in the legend to Fig. 1, using 1 mM ADP and 10 mM phosphocreatine to load the vesicles in the presence of various GSNO concentrations, in either the absence (○) or presence of 40 μM oxyhemoglobin (●). The reaction was stopped after 10 min as described in Section 2. 100% Ca^{2+} uptake corresponded to 1.5 μmol Ca^{2+}/mg vesicle protein. The values are representative of three experiments with two different vesicle preparations.

Ca^{2+} from the cytosol to the lumen of sarcoplasmic reticulum using the energy derived from ATP hydrolysis [6]. GSNO, up to 200 μM had no effect on Ca^{2+} uptake in sarcoplasmic reticulum vesicles when a high ATP concentration (2 mM) was used as substrate (Fig. 1A). It has been shown that sarcoplasmic reticulum vesicles retain a membrane-bound creatine kinase that supports local ATP regeneration [7,8]. Thus, in the presence of phosphocreatine plus ADP, the Ca^{2+}-ATPase pumps Ca^{2+} using the ATP generated at the surface of the membrane by the creatine kinase (Fig. 1B). We found that GSNO inhibited Ca^{2+} uptake when ADP and phosphocreatine were present in the medium (Fig. 1B). Half-maximal inhibition was achieved at 10 μM GSNO (Fig. 2). The Ca^{2+} uptake measured using the ATP synthesized by other ATP regenerating systems, such as pyruvate kinase and adenylate kinase, was not affected by GSNO up to a concentration of 200 μM (data not shown). Thus, in the experiments in Figs. 1 and 2, AP_5A (10 μM) was added to the assay medium in order to prevent the synthesis of ATP from ADP catalyzed by adenylate kinase activity. The effect of GSNO in sarcoplasmic reticulum vesicles was not due to peroxynitrite formation, since the addition of superoxide dismutase (50 U/ml) did not abolish the effect of GSNO (data not shown). In addition, dcGMP (1 mM) had no effect (Fig. 1), indicating that the effects observed in the present study are not mediated by activation of guanylyl cyclase and production of cGMP. Oxyhemoglobin, which binds NO with high affinity, prevented inhibition when added to the medium prior to GSNO (Fig. 2). These results indicate that the effects observed are mediated by NO itself, and not by a non-specific effect of the NO donors.

GSNO inhibits the activity of both the purified (Fig. 3A) and the sarcoplasmic reticulum-bound creatine kinase (data not shown). Even though the incubation time used was only 2 min, 10 μM GSNO promoted almost total inhibition of purified creatine kinase activity. Under similar conditions, half-maximal inhibition of GAPDH activity was attained at 40 μM GSNO (data not shown). As observed for Ca^{2+} uptake, oxyhemoglobin protected the enzyme against inactivation by GSNO (Fig. 3A). The half-life of GSNO is in the range of several hour to days and the product of NO release is the thiol disulphide GSSG [9]. In control experiments it was observed that the addition of 10 μM of a 1-week-old solution of GSNO had no effect on creatine kinase activity (data not shown). Another NO donor, SNAP (100–500 μM) also inhibited creatine kinase activity and Ca^{2+} uptake (data not shown). DTT (2 mM) was able to reverse the inhibition of both purified creatine kinase (Fig. 3B) and Ca^{2+} uptake (data not shown). Under the same conditions, but without NO donors, DTT had no effect on creatine kinase activity (not shown). These results indicate that the mechanism of NO inhibition is mediated by nitrosylation of critical SH groups of creatine kinase.

4. Discussion

GSNO used in the present study has been found endogenously in neutrophils and human airways at micromolar concentrations [10,11]. Several effects of NO are not mediated by guanylyl cyclase activation. NO (in the NO^+ and NO^- forms) has high affinity for sulphydryl groups and promote *S*-nitrosylation of numerous proteins [9]. On this basis, it has been postulated that NO may affect Ca^{2+} fluxes in the sarcoplasmic reticulum [3]. We now show that the sarcoplasmic reticulum Ca^{2+}-ATPase is not directly inhibited by NO (Fig. 1). Ca^{2+} transport, however, may be indirectly affected by the inhibition of local ATP regeneration by creatine kinase.

Creatine kinase plays a crucial role in the process of energy transduction and occurs both in the soluble cytoplasmic compartment and bound to subcellular organelles such as mitochondria, myofilaments and endoplasmic reticulum [7,8,12–14]. Creatine kinase possesses 8 sulphydryl groups, and several sulphydryl reagents inhibit the enzyme activity [15]. However, not all sulphydryl reagents are able to inactivate the enzyme, suggesting that the cysteines may not be essential for catalysis [15]. In order to explain this apparent discrepancy, Hou and Vollmer [16] proposed that creatine kinase is able to regenerate free thiol at the active site. The reversal of inhibition by NO after the addition of DTT suggests that the inhibition involves *S*-nitrosylation of critical sulphydryl groups of the enzyme. Similar to that observed with GAPDH, creatine kinase was inhibited by GSNO concentrations which are likely

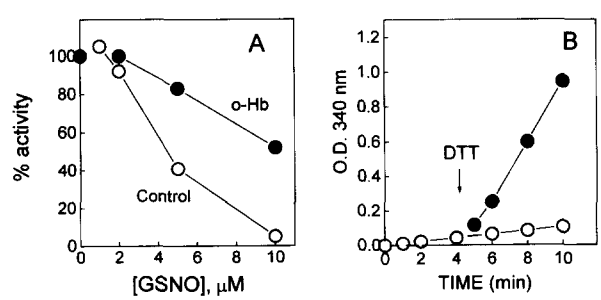

Fig. 3. Inhibition of purified creatine kinase by GSNO. Protection by oxyhemoglobin (A) and by DTT (B). Creatine kinase activity was measured as described in Section 2. (A) Purified creatine kinase (1 U/ml) was incubated for 2 min in the presence of various concentrations of GSNO, in either the absence (○) or presence of 15 μM oxyhemoglobin (●). (B) After inhibition of creatine kinase was established by preincubation for 2 min in the presence of 500 μM GSNO, DTT was added to the cuvette to a final concentration of 1 mM (●) as indicated by the arrow. The values are representative of 4 experiments.

to be found in vivo [10,11]. This implies that creatine kinase is a potential target for NO.

It has been shown that NO inhibits energy-generating pathways and decreases ATP levels in synaptosomes [17]. It is noteworthy that mistargeting of NO synthase from the sarcolemma to the cytoskeleton may be involved in pathological states such as muscle dystrophy [18]. In such conditions, NO may exert inhibition of key enzymes at unwanted sites. This finding and those shown in the present report led us to hypothesize that the inhibition of creatine kinase by NO might be involved in mechanisms of cell injury.

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