Nitric oxide inhibits cardiac energy production via inhibition of mitochondrial creatine kinase

Allen Kaasik[a,b,*], Ave Minajeva[a,c], Elvira De Sousa[a], Renée Ventura-Clapier[a], Vladimir Veksler[a]

[a] *Laboratoire de Cardiologie Cellulaire et Moléculaire INSERM U-446, Université de Paris-Sud, Faculté de Pharmacie, 5, rue Jean-Baptiste Clément, Tour D4, 5éme étage, Châtenay-Malabry Cedex, France*
[b] *Department of Pharmacology, University of Tartu, 18 Ülikooli, EE-2400 Tartu, Estonia*
[c] *Department of Pathoanatomy, University of Tartu, Tartu, Estonia*

Received 13 November 1998; received in revised form 30 December 1998

Abstract Nitric oxide biosynthesis in cardiac muscle leads to a decreased oxygen consumption and lower ATP synthesis. It is suggested that this effect of nitric oxide is mainly due to the inhibition of the mitochondrial respiratory chain enzyme, cytochrome *c* oxidase. However, this work demonstrates that nitric oxide is able to inhibit soluble mitochondrial creatine kinase (CK), mitochondrial CK bound in purified mitochondria, CK in situ in skinned fibres as well as the functional activity of mitochondrial CK in situ in skinned fibres. Since mitochondrial isoenzyme is functionally coupled to oxidative phosphorylation, its inhibition also leads to decreased sensitivity of mitochondrial respiration to ADP and thus decreases ATP synthesis and oxygen consumption under physiological ADP concentrations.

Key words: Nitric oxide; Creatine kinase; Mitochondrion

1. Introduction

Recent studies have suggested that oxygen consumption in cardiac muscle is negatively regulated by nitric oxide (NO). Decreases in myocardial oxygen consumption rate have been observed in isolated rat hearts perfused with NO solutions [1]. Furthermore, inhibition of NO formation has been shown to increase the myocardial oxygen consumption [2]. Two major mechanisms have been thought to be responsible for this effect: NO reduces the activity of several mitochondrial enzymes irreversibly via nitrosylation of the iron-sulphur centres and reversibly by interacting with the haem group of cytochrome *c* oxidase (for review, see [3]). However, the levels of NO that have been used to nitrosylate the iron-sulphur centres are very high relative to the levels of NO likely to occur in vivo. At the same time, the inhibition of cytochrome *c* oxidase occurs at relatively lower concentrations, suggesting that its inhibition may be the main mechanism by which NO decreases the oxygen consumption and thus decreases the ATP synthesis [3,4]. Nevertheless, there are two recent reports [5,6] showing that NO is able to inhibit MM-type creatine kinase at very low concentrations. Creatine kinases (CK) are key enzymes of energy metabolism, responsible for phosphoryl transfer between ATP and phosphocreatine in mitochondria (mitochondrial isoform) and in extramitochondrial spaces (MM, MB, and BB isoforms). Mitochondrial isoenzyme (mi-CK) is functionally coupled to oxidative phosphorylation through its close proximity to the ATP-ADP translocator on the inner mitochondrial membrane (for review, see [7]). Because of this close co-localisation, nascent ADP does not readily diffuse out of the mitochondrial intermembrane space, but rather is transported back into the mitochondrial matrix via the adenine nucleotide translocase [8]. This increases the ADP concentration that can be used for ATP synthesis that in turn leads to an increased rate of oxygen consumption. Thus, if NO inhibits also mi-CK, this inhibition may therefore lead to a significant decrease in oxygen consumption and ATP production by changes in kinetics of ADP regulation. To test this hypothesis, an attempt was made to determine whether the catalytic and functional activities of mi-CK are inhibited in situ by a NO donor.

2. Materials and methods

2.1. Preparation of mitochondria and skinned fibres

Adult Wistar rats were anaesthetised with pentobarbital, the hearts were removed and minced in ice-cold Krebs solution. Pieces of the tissue were incubated for 15 min with 0.125 mg/ml trypsin and then for a few minutes with trypsin inhibitor (0.3 mg/ml). The pieces were collected and homogenised with a glass-teflon homogeniser in medium containing 300 mM sucrose, 10 mM HEPES, 0.2 mM EDTA and 1 mg/ml BSA (pH 7.2). The resulting homogenate was centrifuged ($600\times g$ for 10 min at 4°C), the supernatant was collected and centrifuged again ($8000\times g$ for 15 min). The resulting pellet was washed twice and kept at 4°C until use. Part of the mitochondria was solubilised by adding 1% Triton X-100.

For skinned fibre preparation, thin fibre bundles (<250 μm in diameter) were excised from the subendocardial surface of the left ventricle. The bundles were incubated with shaking for 30 min in a solution containing 7.23 mM K_2EGTA, 2.77 mM CaK_2EGTA (free [Ca^{2+}] 0.1 μM), 6.56 mM $MgCl_2$ (free [Mg^{2+}] 1 mM), 5.7 mM Na_2ATP, 15 mM phosphocreatine, 20 mM taurine, 0.5 mM DTT, 20 mM imidazole, 50 mM potassium methane sulphonate (pH 7.1) and 50 μg/ml saponin to selectively destroy the sarcolemma. The fibres were then washed and kept in the same solution without saponin. All these procedures were carried out at 4°C.

2.2. Catalytic activity of CK

CK activity was assayed using the coupled-enzyme system of glucose 6-phosphate dehydrogenase (G-6-PDH) and hexokinase by measuring NADPH production at 340 nm in the solution for respiration (R, see below) containing, in addition, ADP 1 mM, AMP 10 mM, glycose 20 mM, magnesium acetate 3 mM, NADP 5 mM, phosphocreatine 10 mM, sodium azide 5 mM, hexokinase 2 U/ml, G-6-PDH 2 U/ml at a pH 7.1 and 22°C. Reaction was initiated by fibre addition. Purified MM-CK was obtained from Sigma Chemical Co.

2.3. Functional activity of mi-CK

The saponin-permeabilised bundles were transferred into solution R containing 7.23 mM K_2EGTA, 2.77 mM CaK_2EGTA (free [Ca^{2+}] 0.1 μM), 1.38 mM $MgCl_2$ (free [Mg^{2+}] 1 mM), 3 mM KH_2PO_4, 20 mM taurine, 90 mM potassium methane sulphonate, 10 mM sodium meth-

*Corresponding author. Fax: (372) (7) 375426.
E-mail: allen@ut.ee

PII: S0014-5793(99)00033-2

ane sulphonate, 20 mM imidazole (pH 7.1), 5 mM glutamic acid and 2 mM malic acid for 10 min to wash out adenine nucleotides and PCr. Respiratory rates were determined by a Clark electrode in an oxygraphic cell in 3 ml of the same solution at 22°C with continuous stirring. After measurement, the bundles were removed and dried. Respiration rates were expressed as nmol O_2/min/mg dry weight. The solubility of oxygen was taken to be 230 nmol O_2/ml.

To determine the dependencies of respiration on external [ADP], the fibres were exposed to increasing [ADP] in the presence (20 mM) or in the absence of creatine. The ADP-stimulated respiration above basal oxygen consumption was plotted in order to determine the apparent K_m for ADP.

2.4. Statistical analysis

The data are expressed as the mean ± S.E.M. Mean values were compared using one-way ANOVA. A difference was considered statistically different when the P value was less than 0.05. Non-linear fits to Michaelis-Menten kinetics were computed by a non-linear least-squares routine.

3. Results

S-Nitrosoglutathione (SNOG) was used as an exogenous NO-releasing agent because of its well characterised effect of inhibiting MM-type CK [6]. First, the effect of SNOG on mi-CK activity in Triton-solubilised and non-solubilised mitochondrial preparations was compared. Fig. 1 demonstrates that SNOG concentrations in the range of 10^{-5}–10^{-3} M dose-dependently inhibited both soluble and bound mi-CK activity. In both cases, half-maximal inhibition was achieved at 30–35 μM SNOG. Thus, SNOG inhibited the activity of both solubilised and bound mi-CK almost totally and the sensitivities of the preparations to SNOG were similar.

Our next step was to determine the characteristics of mi-CK inhibition by SNOG in skinned cardiac fibres which maintain the architecture and spatial relationships between the intracellular compartments. As shown in Fig. 1, SNOG inhibited the CK activity in saponin-skinned fibres, but at significantly higher doses (IC_{50} = 90 μM) than in mitochondrial or solubilised mitochondrial preparations. To check if this higher resistance of CK to SNOG is due to lower sensitivity of the MM isoform in the fibres, we studied the effect of SNOG on commercially available MM-CK. We found that this isoenzyme in solution had a sensitivity to SNOG inhibition similar to that of solubilised mi-CK (IC_{50} = 20 μM) (results not shown).

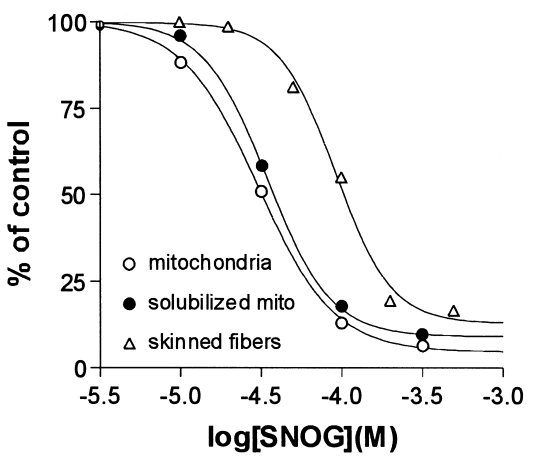

Fig. 1. Inhibition of creatine kinase in different preparations by SNOG. Preparations were incubated for 10 min in the presence of various concentrations of SNOG. Each point represents a single separate experiment. Values are represented as a percentage of the value of the control incubated without SNOG.

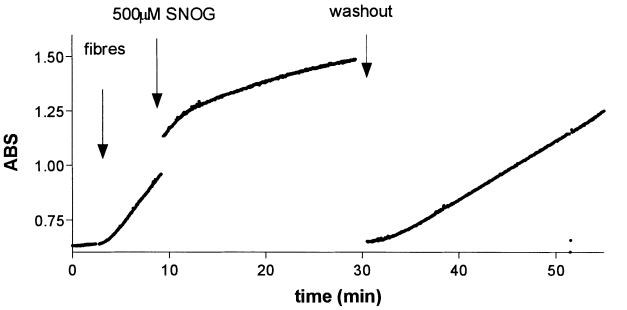

Fig. 2. Actual recording of changes in creatine kinase reaction over time. There was a significant decrease in the slope after the addition of SNOG. The removal of SNOG led to only partial recovery of CK activity.

Since CK reaction catalysed by the mitochondrial isoform increases the availability of ADP for oxidative phosphorylation, the sensitivity of mitochondrial respiration to ADP can be used as an index of mi-CK functional activity. Therefore we tested how SNOG alters the sensitivity of mitochondrial respiration for ADP in the presence or absence of the CK substrate, creatine. In control experiments without SNOG, the saponin-permeabilised fibres in the absence of ADP demonstrated a relatively weak respiration (6.6 ± 0.7 μmol O_2/min/mg dry weight; n = 6) that was considerably stimulated by 2 mM ADP (28.5 ± 2.9 μmol O_2/min/mg dry weight). This gave an acceptor control ratio value as high as 4.53 ± 0.37. SNOG inhibited mitochondrial respiration even in the absence of creatine (IC_{50} = 280 μM) which was obviously due to the well-known inhibition of cytochrome *c* oxidase. To eliminate this effect we preincubated the fibres with 500 μM SNOG and then measured the respiration parameters in the solution containing no SNOG or reducing agents. Since cytochrome *c* oxidase inhibition by NO is completely reversible but nitrosylation of CK is more or less irreversible, we expected that removal of SNOG reverses the effect of SNOG on cytochrome *c* oxidase but not on mi-CK. Indeed, our experiments showed that such an incubation changed neither maximal ADP-stimulated respiration (29.1 ± 2.2 μmol O_2/min/mg dry weight; n = 5) nor the acceptor control ratio. Nevertheless,

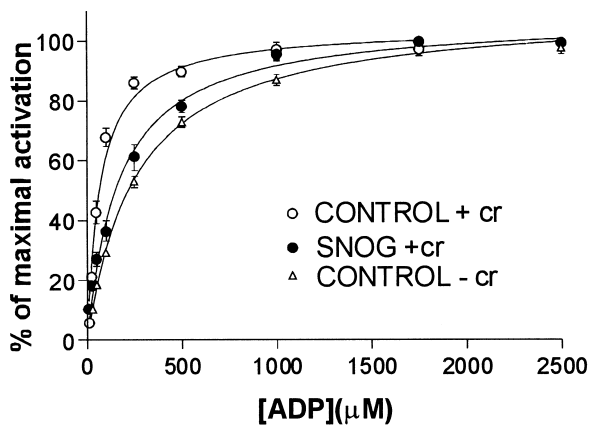

Fig. 3. Effect of ADP on O_2 consumption by skinned fibres preincubated without and with 500 μM SNOG. Values are presented as mean ± S.E.M., n = 3–5.

65% of previously inhibited CK-specific activity still remained inhibited after removing SNOG from the reaction media (Fig. 2). However, CK activity recovered when 1 mM DTT was added to the reaction media.

The respiration experiments performed using the same protocol demonstrate that addition of creatine into the respiration media decreased the K_m for ADP in control fibres from 246 ± 6 μM to 70 ± 7 μM ($n = 3$) (Fig. 3). These creatine-dependent decrease in K_m reflects the functional activity of mi-CK. At the same time, in the SNOG-preincubated group, creatine decreased K_m from 238 ± 9 μM only to 177 ± 22 μM ($n = 3$–5; $P = 0.01$ compared with control). Thus, preincubation with SNOG significantly depresses the functional activity of mi-CK.

4. Discussion

NO has been implicated in many different physiological responses and pathological conditions. It has been reported that in ischaemic central nervous system the concentration of NO can rise to higher than 10 μM [9]. These concentrations are probably high enough to inhibit mitochondrial enzymes involved in aerobic energy production. Here we demonstrate that a nitric oxide donor also inhibits mi-CK specific activity both in vitro and in situ in skinned fibres as well as the functional activity of mi-CK. Creatine kinase possesses eight sulphydryl groups and it has been suggested that the inhibition by NO involves *S*-nitrosylation of critical sulphydryl groups of this enzyme [10]. Konorev et al. [11] and Stachowiak et al. [12] have recently shown that also peroxynitrite irreversibly inhibited CK, including mi-CK. However, in our fibres most of the inhibited CK activity recovered after removal of SNOG in the presence but not in the absence of 1 mM DTT. This strongly suggests that NO but not peroxynitrite was responsible for the inhibition of CK in our preparations.

The results obtained show that CK bound to the intracellular structures in fibres is less sensitive to NO than the solubilised enzyme or mi-CK in isolated mitochondria. Since the saponin-permeabilised fibres also contain MM-CK bound to myofibrils, sarcoplasmic reticulum, resting parts of the sarcolemma, one may suggest that all these enzymes are more resistant to NO than mi-CK. Such a higher stability of mi-CK in fibres could be a result of a relatively slow diffusion of the NO donor to the enzymes. It is not surprising taking into consideration the much lower sensitivity to ADP found in permeabilised isolated cardiomyocytes than in isolated mitochondria [13]. Though NO is believed to be freely diffusible [14], a rapid decomposition of this substance may blunt its effects if the source of NO is not located in the same subcellular compartment. This emphasises the important role of the intracellular localisation of NO synthases, particularly those situated in the mitochondrial compartment [15,16]. The lower sensitivity of fibre CK to NO may also be due to the presence of some protective factor in situ that could be lost during the procedure of mitochondrial isolation.

We have shown that mi-CK inhibition by the NO donor induces a considerable decrease in mitochondrial ADP sensitivity. In contrast to the NO effect of maximal mitochondrial respiration capacity, this inhibition is not readily reversible. This suggests a chemical modification of the enzyme probably due to nitrosylation of essential group(s). Inhibition of mi-CK significantly changes the regulation of the mitochondrial respiration. This inhibition, in turn, leads to decreased sensitivity of mitochondrial respiration to ADP. In living cells where the ADP is present at submaximal concentrations this decreased sensitivity to ADP leads to a substantial decrease in energy production and respiration rate. Interestingly, the inhibition of mi-CK activity in our experimental conditions occurred at lower concentrations of the NO donor than the effect on mitochondrial respiration via cytochrome *c* oxidase inhibition. This allows us to suggest that the mi-CK inhibition combined with the effect of NO on the respiratory chain may play a very important role in the mechanisms of inhibition of the mitochondrial energy production by NO.

However, the relevance of mi-CK inhibition by NO in vivo remains still open. The cells containing glutathione (reducer of nitrosylated SH residues) and less oxygen than was present in our experiments may respond differently to NO. However, after ischemic injury resulting to depletion of the cellular pools of glutathione and/or activation the synthesis of NO, the inhibition of CK may be as relevant as the inhibition of cytochrome *c* oxidase in regulation of energy production.

Acknowledgements: A. Kaasik was supported by a FEBS Short Term fellowship. We thank Mr Philippe Mateo for his advice and help.

References

[1] Poderoso, J.J., Peralta, J.G., Lisdero, C.L., Carreras, M.C., Radisic, M., Schopfer, F., Cadenas, E. and Boveris, A. (1998) Am. J. Physiol. 274, C112–C119.
[2] Bernstein, R.D., Ochoa, F.Y., Xu, X., Forfia, P., Shen, W., Thompson, C.I. and Hintze, T.H. (1996) Circ. Res. 79, 840–848.
[3] Wolin, M.S., Hintze, T.H., Shen, W., Mohazzab, K.M. and Xie, Y.-W. (1997) Biochem. Soc. Trans. 25, 934–939.
[4] Nathan, C. (1992) FASEB J. 6, 3051–3064.
[5] Gross, W.L., Bak, M.I., Ingwall, J.S., Arstall, M.A., Smith, T.W., Balligand, J.L. and Kelly, R.A. (1996) Proc. Natl. Acad. Sci. USA 93, 5604–5609.
[6] Wolosker, H., Panizzutti, R. and Engelender, S. (1996) FEBS Lett. 392, 274–276.
[7] Wyss, M., Smeitink, J., Wevers, R.A. and Wallimann, T. (1992) Biochim. Biophys. Acta 1102, 119–166.
[8] Saks, V.A., Khuchua, Z.A., Vasilyeva, E.V., Belikova, Y.O. and Kuznetsov, A.V. (1994) Mol. Cell. Biochem. 133/134, 155–192.
[9] Tominaga, T., Sato, S., Ohnishi, T. and Ohnishi, S.T. (1994) J. Cereb. Blood Flow Metab. 14, 715–722.
[10] Hou, L.W. and Vollmer, S. (1994) Biochim. Biophys. Acta 1205, 83–88.
[11] Konorev, E.A., Hogg, N. and Kalyanaraman, B. (1998) FEBS Lett. 427, 171–174.
[12] Stachowiak, O., Dolder, M., Wallimann, T. and Richter, T. (1998) J. Biol. Chem. 273, 16694–16694.
[13] Saks, V.A., Belikova, Y.O. and Kuznetsov, A.V. (1991) Biochim. Biophys. Acta 1074, 302–311.
[14] Knowles, R.G. and Moncada, S. (1992) Trends Biochem. Sci. 17, 399–402.
[15] Kobzik, L., Stringer, B., Balligand, J.-L., Reid, M.B. and Stamler, J.S. (1995) Biochem. Biophys. Res. Commun. 211, 375–391.
[16] Giulivi, C., Poderoso, J.J. and Boveris, A. (1998) J. Biol. Chem. 273, 11038–11043.