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Synergistical effect of NO and Ca^{2+} on mitochondrial function

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Nitric oxide (NO) and Ca^{2+} concentration in tissue is reported to increase during various myocardial pathologies, including ischemia. In this study, we investigated the effect of 4 μM NO on isolated rat heart mitochondria in the presence of 5 μM of free Ca^{2+}, conditions resembling the ischemic situation. We found NO together with Ca^{2+} to inhibit the NAD-substrate oxidation pathway due to inactivation of Complex I. Inhibition of Complex I was prevented by superoxidedismutase and urate, and reversed by light and thiols, suggesting the inhibition that might occur due to nitrosation of some critical thiols by peroxynitrite.

Key words: mitochondria, nitric oxide, respiration, nitrosation, Complex I, peroxynitrite

INTRODUCTION

Nitric oxide (NO), a small inorganic free radical molecule, has a unique variety of functions in normal- as well as in pathological physiology. It acts as an intra- and transcellular signal [1], as a cytotoxic host defence factor [2], as a source of reactive nitrogen species [1]. High NO is a result of immune response, so it is common to many diseases that cause inflammation [3]. Ischemia is also reported to increase NO from nanomolar level up to several micromols [4]. NO is known to regulate mitochondrial activity by reversibly inhibiting cytochrome c oxidase at the oxygen binding site [5, 6], however, little is known about the other effects of NO on mitochondria, their mechanism and reversibility. We tested how NO affects the respiration of heart mitochondria and Complex I activity at normal and elevated concentrations of Ca^{2+}, conditions characteristic of myocardial ischemia.

MATERIALS AND METHODS

Rat heart mitochondria were isolated using standard procedures of homogenization and differential centrifugation as in [7]. Mitochondria were incubated at pH 7.2 in a closed, stirred and termostated (37 °C) 1 ml chamber with a Clark-type oxygen electrode (Rank Brothers) and Clark-type nitric oxide-selective electrode (WPI) at 0.5 mg protein/ml. The incubation medium contained 110 mM KCl, 2.24 mM $MgCl_2$, 10 mM Tris-HCl, 5 mM nitrilotriacetate, 10 mM mannitol, 5 mM KH_2PO_4, 1 mM pyruvate + malate or 10 mM succinate + 1 mM rotenone, 4 IU/ml creatine kinase, 1 mM ATP. After a 3-min incubation of mitochondria respiring in state 2 (substrate-stimulated respiration) in the medium supplemented with 4 μM NO, or 5 μM free Ca^{2+}, or NO together with Ca^{2+}, or NO, Ca^{2+}, and superoxidedismutase, or NO, Ca^{2+}, and urate, NO was scavanged by 660 μg/ml hemoglobin, and then mitochondrial state 3 (ADP-stimulated) respiration was measured with exogenously added 60 μM cytochrome c or without it. Free Ca^{2+} and Mg^{2+} were stabilized with a Ca^{2+}–nitrilotriacetic acid buffer and their concentrations were calculated from the binding constants of the medium components according to the program published in [8].

A NO electrode was calibrated by injecting NO-saturated water into a stirred incubation chamber to give a known NO concentration. NO-saturated water was obtained by dissolving NO gas in N_2-purged water. For experiments, the aliquots of NO solution were repeatedly added into the incubation chamber in order to maintain the final NO concentration at about 4 μM.

For determination of Complex I activity the mitochondria were taken out of the oxygen electrode chamber after the mitochondrial respiration rate had been measured and centrifuged in an Eppendorf centrifuge (9980 × g, 4 min). For investigation of the nitrosative effect of NO + Ca^{2+}, mitochondrial pel-

lets were resuspended in the medium used for respiratory measurements. Suspensions were either kept on ice and illuminated with a Fiber Optic Illuminator (WPI, 150 W halogen bulb) or supplemented with 4 mM dithiotreitol or 5 mM gluthatione ethyl ester and incubated in a stirred termostated chamber (37 °C) for 10 min. Then the mitochondria were again centrifuged (9980 × g, 4 min) and pellets were resuspended in 10 mM KH_2PO_4 (pH 8) and freeze-thawed at least 3 times. Activity of Complex I was detected spectrofotometrically from the decrease of NADH concentration at λ = = 340 nm, ε = 6.81/mM/cm in the presence of added coenzyme Q_1.

Data are presented as means ± S. E. of at least 4 separate experiments. Statistical comparison between experimental groups was performed using Student's t test with a statistical significance assumed at $p < 0.05$.

Table. NO together with Ca^{2+} irreversibly inhibits mitochondrial respiration due to inactivation of Complex I
SUC – succinate, P+M – pyruvate+malate, cyt *c* – cytochrome *c*, SOD – superoxidedismutase, DTT – dithiotreitol, GSH – gluthatione ethyl ester.
* – statistically significant difference as compared to Ca^{2+}-treated group for mitochondrial respiration rate and to control group – for Complex I activity; # – statistically significant difference as compared to NO+Ca^{2+}-treated group.

Parameter / Effector	Mitochondrial respiration rate, ngatom O / min mg			Complex I activity, nmol/min mg
	SUC	P+M	P+M+cyt *c*	
Control	395 ± 30	461 ± 24	472 ± 43	229 ± 16
NO	412 ± 25	474 ± 37	485 ± 16	213 ± 19
Ca^{2+}	379 ± 54	281 ± 26	423 ± 53	218 ± 3.5
NO+Ca^{2+}	387 ± 45	128 ± 19*	269 ± 33*	129 ± 14*
NO+Ca^{2+} +SOD		180 ± 15#	406 ± 60#	226 ± 28#
NO+Ca^{2+} +urate		224 ± 12#	386 ± 25#	247 ± 14#
NO+Ca^{2+}, DTT				228 ± 9#
NO+Ca^{2+}, GSH				218 ± 7#
NO+Ca^{2+}, light				233 ± 6#

RESULTS AND DISCUSSION

NO alone up to the concentration of 4 μM had no irreversible effect on either pyruvate+malate or succinate oxidation by mitochondria (Table). 4 μM of NO in the presence of increased Ca^{2+} (5 μM free ions) caused a significant irreversible inhibition of mitochondrial respiration with substrate pyruvate+malate (Table 1). This inhibition was by 33 per cent stronger than that induced by Ca^{2+} alone. Succinate oxidation rate remained unchanged under the effect of NO+Ca^{2+} (Table), indicating that some NAD- but not FAD-dependent substrate oxidation enzymes are damaged by these compounds.

NO may react with superoxide radicals producing peroxynitrite ($ONOO^-$) [9] that may nitrosate mitochondrial enzymes [10]. Superoxidedismutase (SOD) that converts superoxide to hydrogen peroxide and water or $ONOO^-$ scavenger urate partially prevented NO+Ca^{2+}-induced inhibition of pyruvate+malate oxidation (Table). In the presence of exogenously added cytochrome *c*, SOD completely prevented the NO+Ca^{2+} effect (Table). Thus, it might be that Ca^{2+} facilitates NO convertion to $ONOO^-$, which may damage mitochondrial oxidative phosphorylation system enzymes.

NO in the presence of increased Ca^{2+} inhibited the NAD-dependent substrate oxidation pathway. It is known from the literature [11] that Complex I is the most sensitive from the respiratory chain enzyme complexes. Thus, we tested how NO acts on Complex I in the presence of increased Ca^{2+}. We found that Complex I in NO+Ca^{2+}-treated mitochondria was by 44% less active than in control mitochondria and this inactivation was completely prevented by SOD and urate (Table). Neither Ca^{2+} nor NO alone affected Complex I activity. Furthermore, inhibition of Complex I by NO+Ca^{2+} was completely reversed by thiols and light (Table), agents known to destroy nitroso-compounds, indicating that NO and Ca^{2+} acting together may induce nitrosation of some critical sulphydryl groups within the complex. We have previously shown in [12] that $ONOO^-$ also inhibits Complex I and, coming back to the present study, this inhibition is comparable to the effect of NO+Ca^{2+} (Table). Summarizing these data, NO and Ca^{2+} synergistical effect on mitochondrial respiration is caused by inhibition of Complex I, possibly due to an increased formation of $ONOO^-$ in mitochondria and subsequent nitrosation of critical –SH groups within the complex.

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SINERGETINIS NO IR CA^{2+} POVEIKIS MITOCHONDRIJØ FUNKCIJOMS

S a n t r a u k a

Azoto monoksido (NO) ir Ca^{2+} koncentracija audinyje padidëja ávairiø miokardo patologijø, ypaè ischemijos, metu. Ðiame darbe tyrëme, kaip iðskirtas þiurkës ðirdies mitochondrijas veikia 4 μM NO kartu su 5 μM Ca^{2+}, – panaðios sàlygos susidaro ischemijos metu. Nustatëme, kad NO kartu su Ca^{2+} inhibuoja NAD-substratø oksidacijà dël I komplekso inaktyvacijos. Nuo I komplekso inhibicijos apsaugojo superoksidismutazë bei uratas, o ðià inhibicijà paðalino ðviesa bei redukuoti tioliai. Tai leidþia manyti, kad ði inhibicija atsirado dël funkcijai svarbiø tiolio grupiø nitrozinimo peroksinitritu.