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ROLE OF NITRIC OXIDE IN THE INDUCTION OF MEMBRANE PERMEABILITY TRANSITION IN RAT LIVER MITOCHONDRIA

Garcia, R.¹; Leite, A.C.R.²; Utino F.L.¹; Castilho, R.F.¹; Oliveira, H.C.F.²; Vercesi, A.E.¹.
Departamentos de ¹Patologia Clínica e ²Fisiologia e Biofísica, UNICAMP, Campinas, SP, Brasil.

BACKGROUND

Nitric oxide (NO^{*}) generated in mitochondria seems to regulate energy metabolism, O₂ consumption, and reactive oxygen species (ROS) formation by this organelle. The synthesis of NO^{*} from L-arginine and O₂ is catalyzed, in mitochondria, by a calcium-dependent nitric oxide synthase (mtNOS). Therefore, the activity of this mtNOS is linked to the critical function of mitochondria in maintaining matrix calcium homeostasis. In this regard, mitochondrial permeability transition (MPT), a non-selective permeabilization of the inner mitochondrial membrane is triggered by the accumulation of excessive quantities of Ca²⁺ in mitochondria. High concentrations of NO^{*} and its derivatives can promote MPT, whereas low rates of NO^{*} formation can inhibit Ca²⁺ accumulation and thus prevent MPT. Previous results from our group have shown that NOS inhibitors induce Ca²⁺-dependent MPT in rat liver mitochondria.

RESULTS

In the present study we show that low amounts of SNAP (a NO^{*} donor) can avoid MPT triggered by Ca²⁺ and protect against MPT stimulated by L-NAME (a NOS inhibitor). It was also observed that SNAP, at low quantities, not only delays Ca²⁺ release from control mitochondria, but also from those treated with L-NAME. A direct effect of L-NAME on MPT was ruled out by the lack of effect of its isomer D-NAME.

MATERIALS AND METHODS

Rat Liver Mitochondria from female Wistar rats were isolated by conventional differential centrifugation according to Kaplan and Pedersen, JBC 212: 279. (1983).

Standard Incubation Procedure - The experiments were carried out at 28 °C in a reaction medium containing 125 mM sucrose, 65 mM KCl, 10 mM Hepes buffer, pH 7.2, and a complex I substrate mixture made of 3.4 mM malate, 1.7 mM pyruvate, 1.7 mM ketoglutarate, 1.7 mM glutamate. Other additions are indicated in the figure legends. The results shown are representative of a series of at least 6 experiments, done in duplicates.

Mitochondrial swelling - Mitochondrial swelling was determined as the decrease in the turbidity of the mitochondrial suspension measured at 520 nm in a Hitachi U-3000 spectrophotometer.

Mitochondrial membrane potencial disruption - Safranin O fluorescence (Akerman and Wikstrom, FEBS Lett. 68: 1976) at Ex = 495 nm and Em = 586 nm was used to estimate mitochondrial membrane potentials by following its fluorescence on a Hitachi F4500 spectrofluorometer with temperature control (28°C) and gentle stirring, under the conditions described in the figure legend.

Mitochondrial calcium uptake - Calcium uptake by isolated liver mitochondria was monitored following the fluorescence of 0.1 μM calcium green-5N hexapotassium salt (Molecular Probes) in a Hitachi F4500 spectrofluorometer operating in excitation and emission wavelengths of 506 and 531 nm, respectively, and slit widths of 5.0 nm (Murphy et al., 1996). Fluorescence was continuously monitored at 28 °C.

Mitochondrial nitric oxide production - DAF-FM (5 μmol/l) was used to monitor RNS release rates in rat liver mitochondrial suspension. RNS production was measured using a temperature-controlled Hitachi F4500 spectrofluorometer at excitation and emission wavelengths of 495 and 515 nm, respectively. Superoxide dismutase (1 μM) and catalase (1 μM) were added to prevent interference by O₂ and H₂O₂.

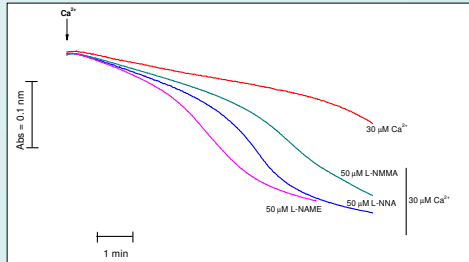


Figure 1: Mitochondrial swelling induced by NOS inhibitors *in vitro*. RLM (0.5 mg/ml) were incubated in the standard medium containing 50 μM L-NAME or L-NMMA or L-NNA. The results are representative of four independent experiments performed in duplicates. The absorbances at 10 min were: 1.116 ± 0.156 (L-NMMA), 1.103 ± 0.169 (L-NNA), and 1.158 ± 0.133 (L-NAME) vs. 1.246 ± 0.057 (30 μM Ca²⁺), p < 0.05. The results are representative of four independent experiments performed in duplicates.

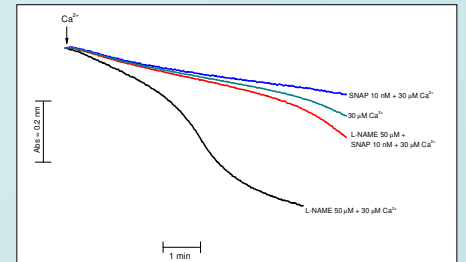


Figure 4: Effect of SNAP and L-NAME on the mitochondrial swelling. RLM (0.5 mg/ml) were incubated in the standard medium containing 30 μM Ca²⁺. The absorbances in 11 minutes were: 1.469 ± 0.038 (30 μM Ca²⁺ + 50 μM L-NAME) 1.469 ± 0.038 (30 μM Ca²⁺ + 10 nM SNAP + 50 μM L-NAME) vs. 1.454 ± 0.051 (30 μM Ca²⁺), p < 0.05. The results are representative of four independent experiments performed in duplicates.

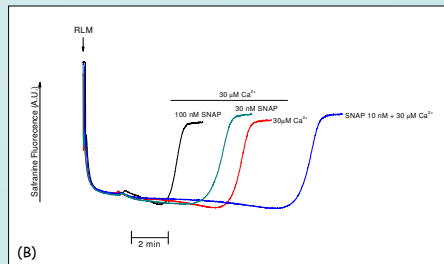
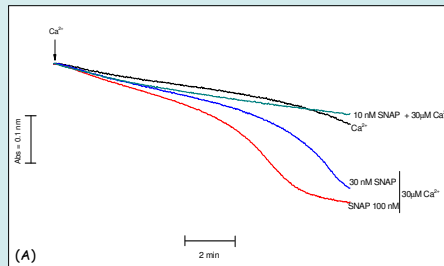


Figure 2: Mitochondrial membrane potential disruption and swelling induced by the NO donor SNAP. RLM (0.5 mg/ml) were incubated in the standard medium containing 30 μM Ca²⁺. Panel A: the absorbances in 10 minutes were: 0.97 ± 0.09 (100 nM SNAP + 30 μM Ca²⁺), 1.01 ± 0.06 (30 nM SNAP + 30 μM Ca²⁺), 1.04 ± 0.03 (10 nM SNAP + 30 μM Ca²⁺) vs. 1.03 ± 0.04 (30 μM Ca²⁺), p < 0.05. Panel B: Δψ (mV) at 15 min was 89.22 ± 25.7 (100 nM SNAP + 30 μM Ca²⁺), 84.15 ± 23.4 (30 nM SNAP + 30 μM Ca²⁺), 79.71 ± 22.2 (10 nM SNAP + 30 μM Ca²⁺), vs. 81.52 ± 21.6 (30 μM Ca²⁺), p < 0.05. The results are representative of four independent experiments performed in duplicates.

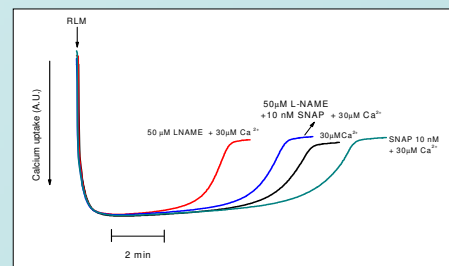


Figure 3: SNAP protects against L-NAME-induced mitochondrial swelling and calcium release. RLM (0.5 mg/ml) were incubated in standard medium containing Ca²⁺. Green to measure extramitochondrial Ca²⁺. The fluorescence at 10 min were: 853.95 ± 393.8 (10 nM SNAP + 30 μM Ca²⁺), 594.66 ± 291.6 (50 μM L-NAME + 10 nM SNAP + 30 μM Ca²⁺), 609.14 ± 315.6 (50 μM L-NAME + 30 μM Ca²⁺) vs. 575.04 ± 292.2 (10 μM Ca²⁺) p < 0.05. The results are representative of four independent experiments performed in duplicate.

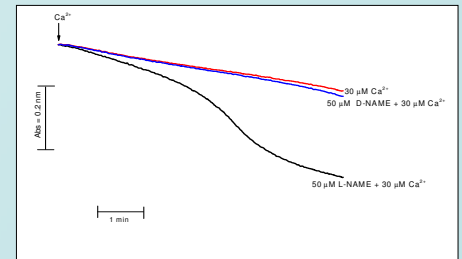


Figure 5: D-NAME (the L-NAME isomer) does not induce mitochondrial swelling. RLM (0.5 mg/ml) were incubated in the standard medium containing 30 μM Ca²⁺. The absorbances in 8 minutes were: 1.098 ± 0.134 (50 μM L-NAME + 30 μM Ca²⁺), 1.175 ± 0.105 (50 μM D-NAME + 30 μM Ca²⁺), vs. 1.163 ± 0.114 (30 μM Ca²⁺), p < 0.05. The results are representative of four independent experiments performed in duplicates.

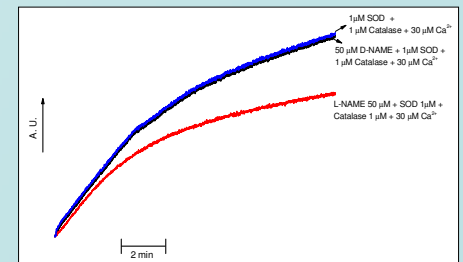


Figure 6: Inhibition of mitochondrial NO generation by L-NAME. NO^{*} production by purified mitochondria was measured using DAF-FM (a fluorescent probe), in the standard medium and in the presence of 1 μM SOD and 1 μM Catalase. The fluorescence at 15 minutes were: 12.57 ± 2.91 (10 μM Ca²⁺ + 50 μM L-NAME), 15.18 ± 2.16 (10 μM Ca²⁺ + 50 μM D-NAME), vs. 14.97 ± 4.15 (10 μM Ca²⁺) p < 0.05. The results are representative of 7 independent experiments performed in duplicates.

CONCLUSION

In the light of these results, we conclude that the release of physiological amounts of NO^{*} by NO^{*} donors can partially inhibit MPT brought about by the inhibition of the mtNOS activity.

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