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Regulation of the rate of synthesis of nitric oxide by Mg^{2+} and hypoxia. Studies in rat heart mitochondria

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Summary. In isolated rat heart mitochondria, L-arginine is oxidized by a nitric oxide synthase (mtNOS) achieving maximal rates at 1 mM L-arginine. The NOS inhibitor N^G-nitro-L-arginine methyl ester (NAME) inhibits the increase in NO production. Extramitochondrial free magnesium inhibited NOS production by 59% at 3.2 mM. The mitochondrial free Mg^{2+} concentration increased to different extents in the presence of L-arginine (29%), the NO donor (S-nitroso-N-acetylpenicillamine) (105%) or the NOS inhibitors L-NAME (48%) or N^G-nitro-L-arginine methyl ester, N^G-monomethyl-L-arginine (L-NMMA) (53%). Under hypoxic conditions, mtNOS activity was inhibited by Mg^{2+} by up to 50% after 30 min of incubation. Reoxygenation restored the activity of the mtNOS to pre-hypoxia levels. The results suggest that in heart mitochondria there is an interaction between Mg^{2+} levels and mtNOS activity which in turn is modified by hypoxia and reoxygenation.

Keywords: Amino acids – Nitric oxide – Free magnesium – Hypoxia-reoxygenation – L-Arginine – Heart mitochondria

Introduction

In modern society, heart disease is a major health problem, leading to increased mortality in people over 65 years old and in those living under stress (Swynghedauw et al., 1995). In experimental heart ischemia, a tissular edema is produced with disorders in cardiac contraction. At 20 min after the onset of the ischemic episode, the cellular concentration of glycogen granules diminishes and mitochondrial distortion appears. After 60 min of ischemia, myocardial fiber edema increases as well as internal mitochondrial cristae occurs. If exposure to ischemia continues, mitochondria become fragmented and cells die (Dutka et al., 1996). During cell death, proteases become activated in the

intermembrane mitochondrial space and are released into the cytoplasm (Scarlett and Murphy, 1997).

Nitric oxide (NO) is a free radical generated in biological systems by nitric oxide synthases (NOS) (Moncada et al., 1988). Physiologically, NO participates in neurotransmission, vasodilatation, and in the immune response (Ignarro, 1989; Bredt and Sneyder, 1990). During ischemia, NO seems to damage mitochondrial Fe-S proteins, with an irreversibly effect on respiration (Borutaité and Brown, 1996). However, it has been shown that NO plays a role in the modulation of O_2 consumption in rat liver mitochondria (Giulivi et al., 1998). Immunochemical studies have revealed the presence of a NOS in mitochondria (mtNOS) from different tissues (Bates et al., 1995); in rat liver mitochondria, mtNOS was found to be associated with the inner membrane (Ghafourifar and Richter, 1997; Giulivi et al., 1998).

Magnesium is an important intracellular cation involved in several functions, such as a cofactor of the Na^+/Ca^{2+} ATPase and regulating cotransport of Na^+, K^+ and Cl^- (Flatman, 1991). The addition of cyclic AMP to mitochondria induces a net efflux of matrix Mg^{2+} (Romani et al., 1991). However, other investigators have reported changes in the content of cell Mg^{2+} and Ca^{2+} in Mg^{2+}-containing media (Okada et al., 1992; Wolf et al., 1994; Zhang and Melvin, 1994). Recent data described that intramitochondrial matrix free Mg^{2+} ($[Mg^{2+}]_m$) modulates the rate of citrulline synthesis through a direct interaction with carbamyl-phosphate synthase I (ammonia) (Rodríguez-Zavala et al., 1997); under ischemia, Mg^{2+} inhibits calcium transport and maintains the ionic balance between sarcolemma membranes (Shechter et al., 1995; Leor and Kloner, 1995). It has been described that cytosolic free Mg^{2+} inhibits the activity of the cytoplasmic NOS in porcine aortic endothelial cells due to a change in the intracellular magnesium concentration, possibly by a release of the cation from mitochondria (Howard et al., 1995).

The relationship between intramitochondrial free Mg^{2+} and NO synthesis has not been yet established in rat heart mitochondria, or during hypoxia-reoxygenation. There are, however, extensive studies on the effects of cytoplasmic nitric oxide synthesis and its effects on the respiratory chain (Borutaité and Brown, 1996; Giulivi et al., 1998). Thus, we hypothesized that in hypoxic conditions the levels of the Mg-ATP complex decreases and release of Mg^{2+} occurs, and this should modify mtNOS activity. We have detected mtNOS activity in mitochondria from heart, brain and kidney in addition to the liver (Manzo-Ávalos and Saavedra-Molina, 1998). In this paper the effects of different concentrations of extramitochondrial free Mg^{2+} on the mtNOS activity of isolated heart mitochondria were explored during a cycle of hypoxia-reoxygenation.

Materials and methods

Materials

All reagents were of pure analytical grade from Sigma Chemical (St. Louis, MO. USA). Mag-Fura-2/AM from Molecular Probes (Eugene, OR. USA).

Isolation of heart mitochondria

Rat heart mitochondria were isolated according to the modified method of Moreno-Sánchez and Hansford (1988). Briefly, male Wistar rats weighing between 200–250 g were used and after decapitation the heart was extracted and fragmented into small pieces, resuspended in SHE medium (250 mM sucrose, 10 mM HEPES (N-[2-hidroxyethyl]piperazine-N′-[2-ethane sulfonic acid), pH 7.4, 1 mM EGTA (ethylen glycol bis(β-aminoethyl ether) N, N, N′, N′-tetraacetic acid) and incubated 9 min in the presence of the protease Nagarse, then the suspension was centrifuged at 9,000 rpm/5 min at 4°C to remove the protease. A gentle homogenization was followed by differential centrifugation and the final supernatant was resuspended in SHE medium added with 0.2% bovine serum albumin and 1 mM ADP (adenosine 5′-diphosphate, sodium). The presence of substrates and cofactors to the incubated rat heart mitochondrial suspension required for the NOS activity, the addition of L-arginine (L-arg), $CaCl_2$, NADPH, BH_4 (5, 6, 7, 8-tetrahydrobiopterin) and the availability of O_2, which were situations that needs integral functionality of mitochondria. In all the experiments mitochondria were used with a respiratory control ratio of 9.32 ± 0.82 (n = 12).

Nitric oxide determination

Nitric oxide was determined as described by Green et al. (1982) after each experimental protocol in permeabilized (0.005% Triton X-100) mitochondria without further additions using the Griess reagent. Substrate (L-arg) and the inhibitors N^G-nitro-L-arginine methyl ester (L-NAME), N^G-monomethyl-L-arginine (L-NMMA) and the nitric oxide donor S-nitroso-N-acetylpenicillamine (SNAP) were incubated separately during 1 min at 25°C. NO was quantified in a Beckman DU-640 spectrophotometer at 546 nm using a calibration curve.

Determination of mitochondrial matrix free Mg^{2+} concentrations

The indicated external free Mg^{2+} concentrations were stabilized by the addition of appropriate Mg^{2+}/EGTA buffers (Shoenmakers et al., 1992). Intramitochondrial free Mg^{2+} ($[Mg^{2+}]_m$) was determined incubating heart mitochondria (30 mg/ml) in SHE medium (added with 1 mM ADP, 1 mM $MgCl_2$, 0.2% bovine serum albumin) in the presence of the fluorescent magnesium indicator Mag-Fura-2/AM (5μM), as described by Rodríguez-Zavala et al. (1997). Mag-Fura-2 acid responded to different concentrations of Mg^{2+} to obtain a Kd which was in the same range as that reported by Jung et al. (1996). Fluorescence was recorded in a Shimadzu RF-5000U spectrofluorometer.

Hypoxia-reoxygenation studies

Heart mitochondria were subjected to hypoxia (N_2/CO_2, 95%/5%) in 120 mM KCl, 20 mM MOPS (4-Morpholine-propanesulfonic acid) (pH 7.4), 0.5 mM EGTA medium plus 5 mM glutamate-malate, 10 mM NaCl, 5 mM NaH_2PO_4. Reoxygenation was obtained saturating the incubation medium with 95% O_2/5% CO_2 without any pH changes during the experiment.

Results

The apparent toxicity of NO and the reports of a mtNOS in some tissues (Bates et al., 1995; Ghafourifar and Richter, 1997; Giulivi et al., 1998; Manzo-

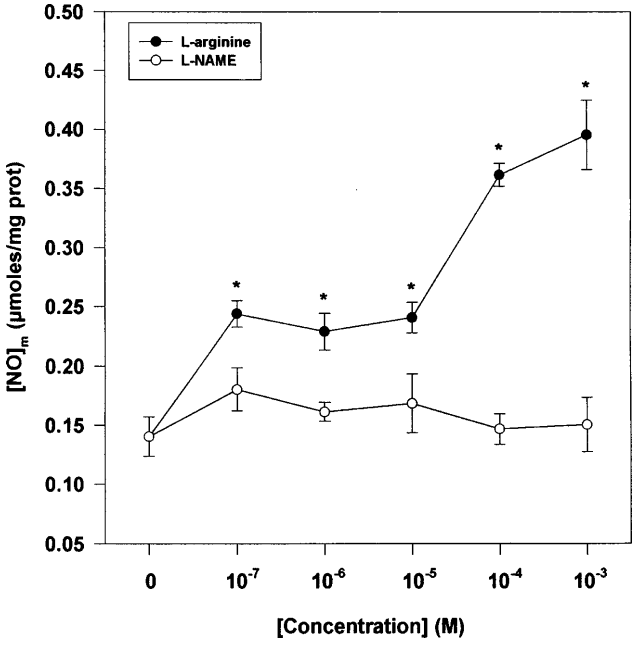

Fig. 1. Effect of L-arginine and L-NAME on NO synthesis in permeabilized mitochondria. Rat heart mitochondria (1 mg/ml) were incubated in 120 mM KCl, 20 mM MOPS (pH 7.4), 0.5 mM EGTA medium plus 5 mM glutamate-malate, 10 mM NaCl and 5 mM NaH_2PO_4. Mitochondria were permeabilized with Triton X-100 (0.005%). Nitric oxide was determined in the presence of the Griess reagent (Green et al., 1982). Data represent the mean ± SEM of at least five experiments in duplicate. Other details as in Materials and methods section. *($P < 0.05$)

Ávalos and Saavedra-Molina, 1998) led us to determine the effect of L-arg and the competitive inhibitor L-NAME on NOS activity in rat heart mitochondria. A high basal mtNOS activity of 0.13 ± 0.01 μM NO (n = 5) was detected, which indicates the presence of endogenous L-arg (Fig. 1). In addition, as the substrate L-arginine was added, NO biosynthesis in permeabilized rat heart mitochondria increased (Fig. 1). From 10^{-7} to 10^{-6} M L-arginine, the mtNOS activity increased to 1.74 times the basal rate. Then, at 10^{-4} and 10^{-5} a larger increase, to 2.8 times the basal rate was observed. The maximum increase in NO synthesis was obtained in the presence of 1 mM L-arginine (Fig. 1). In contrast, when the NOS inhibitor L-NAME was included in the assay mixture, L-arginine did not lead to any increase in NOS activity. Our results are in agreement with those observed in liver mitochondria (Ghafourifar and Richter, 1997; Giulivi et al., 1998) and with those observed in the cat mesenteric artery and intestinal segments (Lizasoain et al., 1996).

In order to determine whether Mg^{2+} modulated the mtNOS activity, different concentrations of extramitochondrial free Mg^{2+} ($[Mg^{2+}]_e$) were added in order to modify intramitochondrial free Mg^{2+} (Rodríguez-Zavala et al., 1997). When permeabilized heart mitochondria were incubated in the presence of L-arginine and increasing concentrations of external free Mg^{2+}, rang-

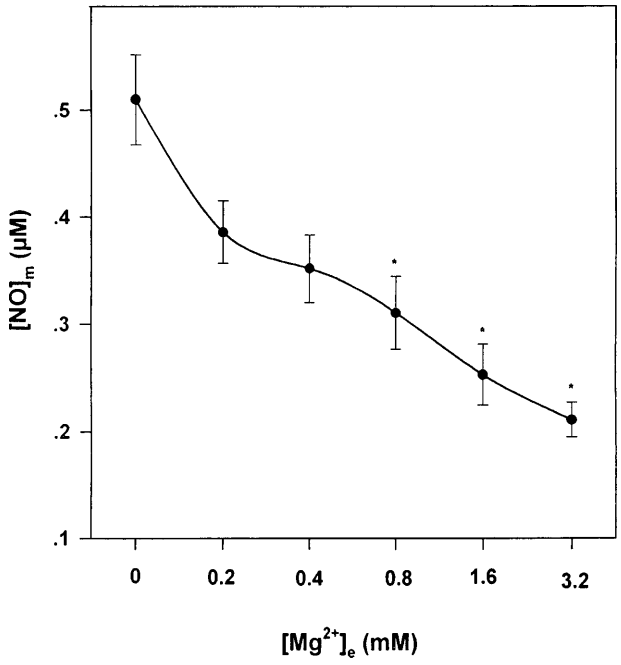

Fig. 2. Effect of extramitochondrial free Mg^{2+} on heart mitochondria. Rat heart mitochondria (1 mg/ml) were incubated in 120 mM KCl, 20 mM MOPS (pH 7.4), 0.5 mM EGTA medium as in figure 1. Other details as in Materials and methods section. Each point represent the mean ± SEM of at least five experiments in duplicate. *($P < 0.05$)

ing from 0.2 to 3.2 mM (Fig. 2), mtNOS activity was inhibited from 24% to 59% as Mg^{2+} increased. Once it was established that Mg^{2+} inhibits the synthesis of NO, it was decided to examine whether there is an effect of the substrate L-arginine or of the other NOS effectors on Mg^{2+} concentration. L-arginine, the inhibitors L-NAME and L-NMMA and the NO donor, SNAP were tested (Table 1). In the presence of the Mg^{2+} fluorescent indicator, Mag-Fura-2, the values of free mitochondrial Mg^{2+} increased in the presence of L-arginine by 29%, from 0.357 ± 0.01 mM to 0.461 ± 0.02 mM ($p < 0.05$). The addition of the inhibitor L-NAME produced a higher increase than L-arginine on $[Mg^{2+}]_m$, to about 48% (Table 1). A higher increase was obtained when L-NMMA was added (53%). The NO donor SNAP exerted the highest increase in $[Mg^{2+}]_m$ (105%). These results demonstrate that fluctuations in $[Mg^{2+}]_m$ exerted by NO-related compounds modulate NO synthesis in heart mitochondria.

Hypoxia-reoxygenation is an important event during the evolution of an ischemic heart lesion. It is recognized that cells surviving the anoxic phase may die during reperfusion, probably as a result of free radical accumulation (Caraceni et al., 1994) and of the inhibition or activation of some metabolic pathways such as glycogenolisis or ureagenesis (Villalobos-Molina et al., 1998). Thus, we evaluated NO synthesis in mitochondria subjected to a hypoxia-reoxygenation cycle. During hypoxia, a significative decrease in the

Table 1. Effect of NO-related compounds on intramitochondrial free Mg^{2+}. Mag-Fura-2-loaded-mitochondria (0.5 mg/ml) were incubated as described in Materials and methods. Fluorescence excitation (395 nm) and emission (495 nm) spectra were recorded under Materials and methods section; L-arginine (L-arg), L-NAME, L-NMMA were added 1 mM; SNAP 0.41 mM. Values presented are means ± SEM with the number of experiments in duplicate indicated in parentheses

$[Mg^{2+}]_m$				
Control	L-arg	L-NAME	L-NMMA	SNAP
0.357 ± 0.01 (7)	0.461 ± 0.02 (4)	0.528 ± 0.03 (4)	0.548 ± 0.06 (6)	0.735 ± 0.04 (6)

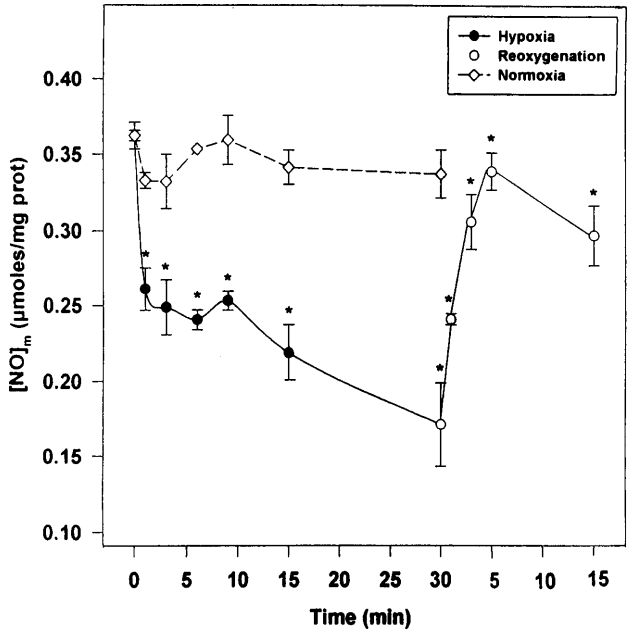

Fig. 3. Effect of hypoxia-reoxygenation on NO synthesis in heart mitochondria. Heart mitochondria (1 mg/ml) were incubated in 120 mM KCl, 20 mM MOPS (pH 7.4), 0.5 mM EGTA medium plus 5 mM glutamate-malate, 10 mM NaCl, 5 mM NaH_2PO_4. Hypoxia was obtained saturating the incubation medium with 95% N_2/5% CO_2 and reoxygenation with 95% O_2/5% CO_2 without any pH changes during the experiment. Nitric oxide was determined under Methods. Data represent the mean ± SEM of three separate experiments in duplicate. *($P < 0.05$)

concentration of mitochondrial NO was observed (Fig. 3). From 0.36 ± 0.02 μM in the control to 0.24 ± 0.01 μM after 5 min of hypoxia, to 0.22 ± 0.01 μM after 15 min and to 0.17 ± 0.02 μM at 30 min after the onset of hypoxia. Then, when oxygenation was reinstalled, $[NO]_m$ was recovered reaching a nearly normal value of 0.34 ± 0.01 μM after 5 min of reoxygenation (Fig. 3). Thus our data demonstrate that NO production is rapidly responsive to oxygen concentration (Fig. 3).

Discussion

The present results corroborate compelling evidence of a nitric oxide synthase activity in heart mitochondria, as we have previously reported (Ríos et al., 1998) which is comparable to the activity reported in liver mitochondria (Ghafourifar and Richter, 1997; Giulivi, 1998). A calcium-dependence in mtNOS activity in liver mitochondria was demonstrated by Ghafourifar and Richter (1997), which was comparable to the response observed in heart mitochondria, i.e. from 0.51 ± 0.3 (in the absence of Ca^{2+}) to 2.74 ± 0.1 nmoles/mg prot/min, (in the presence of Ca^{2+}). In our hands, mtNOS activity was inhibited by L-NAME, a L-arginine analogue (Fig. 1). NO production was better detected in intact mitochondria than in permeabilized mitochondria and for that reason all the experiments were conducted in intact mitochondria; however, when the $[Mg^{2+}]_e$ was controlled, this condition was not a problem, and NO synthesis was detected successfully (Fig. 2). Mg^{2+} ions have been reported to have a dual regulation on NOS activity, suggesting a direct relationship between $[Mg^{2+}]$ and the activity of the endothelial-derived relaxing factor (EDRF), while others suggest an opposite reaction (Howard et al., 1995).

Here, hypoxia inhibited mtNOS activity in heart mitochondria as previously was reported for the cytoplasmic NOS in other models such as in perfused post-ischemic rat heart (Maulik et al., 1995), rat kidney cells (Abu-Soud et al., 1996) and for mtNOS in liver isolated mitochondria (Bates et al., 1996). This is in contrast to results in coronary artery (Griffith and Stuehr, 1995) and rabbit heart myocytes (Depré, 1997). It has been reported that L-arginine causes a decrease in mitochondrial respiration in rat liver (Bates et al., 1996), while blocking cytoplasmic NO synthesis reduces myocardic oxygen consumption (Sherman et al., 1997). This inhibition of respiration is due to degradation of the Fe-S centres of the respiratory complexes (Zhang et al., 1996), which is in accord with the report of Lizasoain et al. (1996), in which a NO donor, 3-morpholinosydnonimine, (SIN-1) inhibited respiration in brain submitochondrial particles. Under our conditions, in the presence of the respiratory substrate glutamate-malate heart mitochondria exhibited a decrease in NO synthesis without significant differences in respiratory control in the presence of L-arginine or L-NAME (data not shown). In liver mitochondria, intramitochondrial NO is implicated in ATP synthesis and mitochondrial respiration (Ghafourifar and Richter, 1997) through modulation of cytochrome oxidase activity (Giulivi, 1998). In heart mitochondria NO synthesis was also affected by hypoxia-reoxygenation. Further experiments are required to elucidate the precise role of intramitochondrial NO in different metabolic pathways in mammals.

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References

Abu-Soud HM, Rousseau DL, Stuehr DJ (1996) Nitric oxide binding to the heme of neuronal nitric-oxide synthase links its activity to changes in oxygen tension. J Biol Chem 271: 32515–32518

Bates TE, Loesch A, Burnstock G, Clark JB (1995) Inmunocytochemical evidence for a mitochondrially located nitric oxide synthase in brain and liver. Biochem Biophys Res Commun 213: 896–900

Borutaité V, Brown GC (1996) Rapid reduction of nitric oxide by mitochondria, and reversible inhibition of mitochondrial respiration by nitric oxide. Biochem J 315: 295–299

Bredt DS, Snyder SH (1990) Isolation of nitric oxide synthase, a calmodulin-requiring enzyme. Proc Natl Acad Sci USA 87: 682–685

Caraceni P, Rosenblum E, Van Thiel DH, Borle AB (1994) Reoxygenation injury in isolated rat hepatocytes: relation to oxygen free radicals and lipid peroxidation. Am J Phisyol 266: G799–G806

Depré C, Fieérain L, Hue L (1997) Activation of nitric oxide synthase by ischaemia in the perfused heart. Cardiovasc Res 33: 82–87

Dutka DP, Olivotto I, Ward S, Oakley CM (1996) Effects of aging on neuroendocrine activation in subjects and patients in the presence and absence of heart failure with left ventricular systolic dysfunction. Am J Physiol 77: 1197–1201

Flatman PW (1991) Mechanisms of magnesium transport. Annu Rev Physiol 53: 259–271

Ghafourifar P, Richter C (1997) Nitric oxide synthase activity in mitochondria. FEBS Lett 418: 291–296

Giulivi C (1998) Functional implications of nitric oxide produced by mitochondria in mitochondrial metabolism. Biochem J 332: 673–679

Giulivi C, Poderoso J, Boveris A (1998) Production of nitric oxide by mitochondria. J Biol Chem 273: 11038–11043

Green LC, Wagner DA, Glogowski J, Skipper PI, Wishnok JS, Tannenbaum SR (1982) Analysis of nitrate, and [^{15}N]nitrate in biological fluids. Anal Biochem 126: 131–138

Griffith OW, Stuehr DJ (1995) Nitric oxide synthases: properties and catalytic mechanism. Annu Rev Physiol 57: 707–736

Howard AB, Alexander RW, Taylor WR (1995) Effects of magnesium on nitric oxide synthase activity in endothelial cells. Am J Physiol 269: C612–C618

Ignarro LJ (1989) Endothelium-derived nitric oxide: actions and properties. FASEB J 3: 31–36

Jung DW, Chapman CJ, Baysal K, Pfeiffer DR, Brierley GP (1996) On the use of fluorescent probes to estimate free Mg^{2+} in the matrix of heart mitochondria. Arch Biochem Byophys 232: 19–29

Leor J, Kloner RA (1995) An experimental model examining the role of magnesium in the therapy of acute myocardial infarction. Am J Cardiol 75: 1292–1293

Lizasoain I, Moro MA, Knowles RG, Darley-Usmar V, Moncada S (1996) Nitric oxide and peroxynitrite exert distinct effects on mitochondrial respiration which are differentially blocked by glutathione or glucose. Biochem J 314: 877–880

Manzo-Ávalos S, Saavedra-Molina A (1998) Mg^{2+} and nitric oxide in heart mitochondria. FASEB J 12: A1480

Maulik N, Engelman DT, Watanabe M, Engelman RM, Maulik G, Cordis GA, Das DK (1995) Nitric oxide signaling in ischemic heart. Cardiovasc Res 30: 593–601

Moncada S, Radomski MW, Palmer RM (1988) Endothelium-derived relaxing factor. Identification as nitric oxide and role in the control of vascular tone and platelet function. Biochem Pharmacol 37: 2495–2501

Moreno-Sánchez R, Hansford GR (1988) Dependence of cardiac mitochondrial pyruvate dehydrogenase activity on intramitochondrial free Ca^{2+} concentration. Biochem J 256: 403–412

Okada K, Ishikawa S, Saito T (1992) Cellular mechanisms of vasopressin and endothelin to mobilize $[Mg^{2+}]_i$ in vascular smooth muscle cell. Am J Physiol 32: C873–C878

Ríos M, Clemente-Guerrero M, Manzo-Ávalos S, Moreno-Sánchez R, Saavedra-Molina A (1998) Citrulline and nitric oxide synthesis in mitochondria of several organs FASEB J 12: A1480

Rodríguez-Zavala JS, Saavedra-Molina A, Moreno-Sánchez R (1997) Effect of intramitochondrial Mg^{2+} on citrulline synthesis in rat liver mitochondria. Biochem Mol Biol Int 41: 179–187

Romani A, Dowell E, Scarpa A (1991) Cyclic AMP-induced Mg^{2+} release from rat liver hepatocytes, permeabilized hepatocytes, and isolated mitochondria. J Biol Chem 266: 24376–24384

Scarlett JL, Murphy MP (1997) Release of apoptogenic proteins from the mitochondrial permeability transition. FEBS Lett 418: 282–286

Shechter M, Hod H, Chouraqui P, Kaplinsky E, Rabinowits B (1995) Magnesium therapy in acute myocardial infarction when patients are not candidates for thrombolytic therapy. Am J Physiol 75: 321–323

Sherman AJ, Davies III CA, Clocke FJ, Harris KR, Srinivasan G, Yaacoub SS, Quinn DA, Ahlin KA, Jang JJ (1997) Blockade of nitric oxide synthesis reduces myocardial oxygen consumption in vivo. Circulation 95: 1328–1334

Shoenmakers TJM, Visser GJ, Flik G, Theuvenet PR (1992) Chelator: an improved method for computing metal ion concentrations in physiological solutions. Biotech 12: 870–879

Swynghedauw B, Besse S, Assayag P, Carré F, Chevalier B, Charlemagne D, Delcayre C, Hardouin S, Heymes C, Moalic JM (1995) Molecular and cellular biology of the senescent hypertrophied and failing heart. Am J Physiol 76: 2D–7D

Villalobos-Molina R, Saavedra-Molina A, Devlin TM (1998) Effect of hypoxia and reoxygenation on metabolic pathways in rat hepatocytes. Arch Med Res 29: 219–223

Wolf FI, Di Francesco A, Covacci V, Cittadini A (1994) cAMP activates magnesium efflux via the Na^+/Mg^{2+} antiporter in Ascites cells. J Biol Chem 202: 1209–1214

Zhang GH, Melvin JE (1994) Intracellular Mg^{2+} movement during muscarinic stimulation. Mg^{2+} uptake by the intracellular Ca^{2+} store in rat sublingual mucous acini. J Biol Chem 269: 10352–10356

Zhang X, Xie YW, Nasjletti A, Xu X, Wolin MS, Hintze TH (1996) ACE inhibitors promote nitric oxide accumulation to modulate myocardial oxygen consumption. Circulation 95: 176–182

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