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Protective role of nitric oxide synthase against ischemia-reperfusion injury in guinea pig myocardial mitochondria

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Abstract

In guinea-pig myocardial mitochondria preparation, lowering the Ca^{2+} concentration or pH level in the perfusate rapidly elevated the fura-2 Ca^{2+} signal ($[Ca^{2+}]_m$). Pretreatment with 10^{-4} M L-Arg inhibited the rapid $[Ca^{2+}]_m$ influx, whereas administration of 10^{-4} M L-NAME did not, suggesting some association between nitric oxide ($NO\cdot$) synthase (NOS) activation and Ca^{2+} kinetics in mitochondria. Immunoblotting analysis showed that endothelial (e)-NOS was present in mitochondria, but not inducible (i)-NOS or brain (b)-NOS. Electron microscopy observations revealed that the e-NOS antibody-reactive site in the mitochondria was the inner cristae. The production of reactive oxygen species and $NO\cdot$ in isolated mitochondria was detected by the spin trapping technique with electron paramagnetic resonance (EPR) spectrometry. Pretreatment with 10^{-5} M *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) and 10^{-5} M 3-[2-Hydroxy-1-(1-methylethyl)-2-nitrosohydrazino]-1-propananine (NOC 5), which spontaneously generate $NO\cdot$, completely inhibited the $[Ca^{2+}]_m$ uptake. In addition, *N*-morpholino sydnonimine hydrochloride (SIN-1) (10^{-5} M), which simultaneously generates $NO\cdot$ as well as $\cdot O_2^-$ and peroxynitrite anion ($ONOO^-$), inhibited the increase in $[Ca^{2+}]_m$. $ONOO^-$ (3×10^{-4} M) itself also inhibited this increase. Pretreatment with the $\cdot O_2^-$-scavenger manganese superoxide dismutase or catalase (200 units/ml) completely inhibited the increase in $[Ca^{2+}]_m$ caused by lowering of either the Ca^{2+} concentration or the pH in the perfusate. These results suggested that the formation of reactive oxygen species promoted the $[Ca^{2+}]_m$ influx. The agents that inhibited the $[Ca^{2+}]_m$ influx improved contractility even in Langendorff preparations after ischemia. Based on these findings, we concluded that e-NOS exists in mitochondria and that $NO\cdot$ may play an important protective role in reperfusion cardiac injury after ischemia, by inhibiting the Ca^{2+} influx into mitochondria which are otherwise damaged by $\cdot O_2^-$. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Myocardial mitochondria; Ischemia-reperfusion injury; Nitric oxide ($NO\cdot$); Endothelial nitric oxide ($NO\cdot$) synthase; Reactive oxygen species; EPR (Electron paramagnetic resonance) spectrometry; (Guinea pig)

1. Introduction

Myocardial ischemia, if prolonged, results in cardiac myocyte dysfunction and eventual death. Therefore, the re-establishment of blood flow to the ischemic tissue is essential if tissue death (necrosis) is to be avoided. However, it is now clear that the process of reperfusion does not result simply in the salvage of the previously ischemic myocardium but also has deleterious effects on the tissue. These effects include a variety of phenomena such as reperfusion arrhythmias, myocardial stunning, cell lysis, Ca^{2+}-overload and neutrophil infiltration leading to inflammation (Hearse, 1977; Becker and Ambrosio, 1987; Opie, 1989).

One of the key events in these processes is the acute cell lysis which occurs at the time of the readmission of O_2 to the tissue. This specific role of oxygen deprivation and re-supply in the generation of cell damage has been established using a model in which hearts are perfused under substrate-free hypoxic conditions for periods of 20–40 min

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and subsequently reoxygenated. Under these conditions, the tissue shows a significant increase in total Ca^{2+} content at the time of reoxygenation (this feature is common to a number of models of ischemia-reperfusion (Richter and Schweizer, 1997)).

A number of studies (Hearse, 1991; Gunter et al., 1994; Hansford, 1994; Ferrari, 1996) have shown that mitochondrial function is compromised by treatments such as hypoxia-reoxygenation or ischemia-reperfusion. In addition, it is clear that the mitochondria play an important role in the elevation of intracellular Ca^{2+} which occurs as a consequence of these treatments and which is generally regarded as damaging to cells. The relationship between Ca^{2+} influx and cell damage is still to be established, although a great deal of circumstantial evidence links these two events (Mc Cord and Turrens, 1994).

In the course of an investigation of protective agents for ischemia-reperfusion cardiac injury, we observed that the function of mitochondria appeared to be closely related to unrecovered cardiac contractility in Langendorff guinea-pig heart preparations (Hotta et al., 1998). The contractility has prompted considerable debate concerning the relevance of the permeability transition pore to mitochondria function under conditions of ischemia and reperfusion, as reported by Al-Nasser and Crompton (1986). The marked elevation of mitochondrial matrix fura-2 Ca^{2+} signals ($[Ca^{2+}]_m$) elicited by perfusion with a solution with a physiological low content of Ca^{2+} or acidified perfusate was reduced by pre-treatment of the mitochondria with inhibitors of Na^+–H^+ and K^+–H^+ exchange (Hotta et al., 1998) or a nitric oxide ($NO\cdot$) donor (*S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP), *N*-morpholino sydnonimine hydrochloride (SIN-1), or 3-[2-Hydroxy-1-(1-methylethyl)-2-nitrosohydrazino]-1-propananine (NOC 5)) (Morikawa et al., 1992; Weyrich et al., 1992; Malinski et al., 1993; Prado et al., 1993; Kurose et al., 1994), which have beneficial effects against ischemia-reperfusion injury of Langendorff heart preparations. The improvement of contractility was associated with a decrease in the intracellular Ca^{2+} level and a small reduction of ATP content with an increase of Pi, even during the last stage of a long period of ischemia (Hotta et al., 1998).

The purpose of the present study was to evaluate the inhibitory effects of agents generating $NO\cdot$ in response to $NO\cdot$ synthase (NOS), on the increase in $[Ca^{2+}]_m$ caused by lowering the perfusate Ca^{2+} concentration or the pH value. Since immunoblotting and electron microscopic analyses in the present study showed the existence of endothelial NOS (e-NOS) in mitochondria in cardiac muscle, the increase in $[Ca^{2+}]_m$, possibly resulting in mitochondrial swelling and damage, was tested in relation to $NO\cdot$ and $\cdot O_2^-$ radicals. In respect to the relation between the presence of NOS and Ca kinetics in mitochondria, we also discuss the physiological role of $NO\cdot$ and the relevance of NO-supplying drugs in ischemia-reperfusion cardiac injury.

2. Materials and methods

2.1. Mitochondria

2.1.1. Intramitochondrial calcium measurements with fura-2

Mitochondria were isolated from guinea-pig hearts as previously described (Hotta et al., 1998), and suspended in 70 mM sucrose, 210 mM mannitol, containing 20 mM 3-morpholinopropanesulfonic acid (MOPS)–KOH (pH 7.4). They were incubated at 24°C in a normal medium (250 mM sucrose, 1 mM $MgCl_2$, 1 mM KH_2PO_4, 10 mM succinate, 5 mM pyruvate, 5 mM malate, 20 mM MOPS–KOH buffer, pH 7.4) and in a medium with an intracellular ionic composition (100 nM Ca^{2+}, 10 mM Na^+ and 110 mM K^+, no Ca^{2+} buffers such as EGTA) containing the Ca^{2+} fluoroprobe 10 μM fura-2AM and 0.025% Cremophor EL (Hotta et al., 1989, 1995). A 0.5-ml aliquot of mitochondria was pipetted onto a glass coverslip on the stage of an inverted microscope (CAM230, Japan Spectroscopic, Tokyo) and 30 min later was washed with dye-free solution for 10 min and then continuously perfused with 10 ml medium solution. Fura-2 Ca^{2+} signals were measured at 500 nm as the ratio of the strength of fluorescence (R340/380) excited at 340 nm (F340) and 380 nm (F380). After fura-2 loading for a period of 30 min, the amplitude of F340 from the mitochondria increased by 5–7-fold compared with that of fura-2 unloaded mitochondria. The intramitochondrial Ca^{2+} signal of mitochondria preloaded with a high Ca^{2+} concentration was markedly increased by lowering the Ca^{2+} concentration from high to a physiological low level (Ca^{2+}; 1 μM → 100 nM) or by acidifying the perfusate (pH 7.5 → 6.5). We then determined whether various agents suppressed the Ca^{2+} increase induced by these maneuvers. The final mitochondrial protein level was adjusted to 30–35 mg/ml.

2.1.2. Immunoblot analysis of the mitochondrial proteins from guinea-pig myocardial cells with anti-NOS antibodies

Mitochondria from guinea-pig myocardial cells were purified by differential centrifugation in Percoll ($\rho = 1.09$ g/ml) containing protease inhibitors (Complete™, Boehringer Mannheim, Mannheim, Germany). The mitochondria were homogenized in 10 mM Tris–HCl (pH 7.4) containing protease inhibitors and 2% sodium dodecyl sulfate (SDS). SDS–polyacrylamide gel electrophoresis (PAGE, 8% gel) was carried out according to the method of Rudolph and Krueger (1979). Mitochondrial proteins separated by SDS-PAGE (8% gel) were transferred to a polyvinylidene difluoride membrane (Immobilon™, Millipore, Bedford, MA) according to the method of Towbin et al. (1979). After blotting, the PVDF membrane was cut into three fragments for further analysis. The PVDF membrane pieces were incubated in 4% skimmed milk in phosphate-buffered saline (PBS) at room temperature for 2 h. After the washing process with PBS containing 0.1%

Tween 20, each PVDF membrane piece was exposed first to polyclonal rabbit IgG antibody against brain (b)-NOS, inducible (i)-NOS or e-NOS overnight at 4°C and then with horseradish peroxidase-linked anti-rabbit IgG goat IgG antibody (1: 10,000 dilution) for 1 h at room temperature. The antibodies were diluted with PBS containing 0.1% Tween 20. The Western blots were visualized with 3,3′-diaminobenzidine tetrahydrochloride in PBS containing 0.03% H_2O_2. The affinity-purified polyclonal antibodies, which were developed in rabbits against the specific protein fragments of human b-NOS, i-NOS and e-NOS as immunogen, were obtained from Transduction Laboratories (Lexington, KY, USA).

2.1.3. Electron paramagnetic resonance (EPR) spectrometry

2.1.3.1. Measurement of active oxygen radicals generated in mitochondria. The mitochondria preparations (50 μl) (30–35 protein mg/ml) were diluted 4-fold (200 μl) in the MOPS-sucrose medium used for the intramitochondrial Ca^{2+} measurements with fura-2. The suspension was exposed to a high Ca^{2+} concentration medium (1 μM) for 30 min at 24°C. The mitochondrial preparations were centrifuged at $8000 \times g$ for 1 min, and then the reaction was started by exposure to a low Ca^{2+} concentration medium (100 nM) (200 μl) containing the spin trapping reagent 5,5-dimethyl-1-pyrroline-1-oxide (DMPO, 8.8 M) (Irani et al., 1997). In another experiment, the same mitochondrial preparations (200 μl) in normal medium at pH 7.4 were centrifuged and then resuspended in an acidic medium (pH 6.5) containing 8.8 M DMPO. These mitochondrial reaction suspensions were transferred to an aqueous quartz flat cell (EPR cuvette, LC-12, JEOL, Tokyo) with an effective sample volume of 130 μl, which was then placed in the cavity of an X-band EPR spectrometer (JES-TE200, JEOL). EPR spectra were recorded at room temperature after 45 s. The operation conditions for the EPR were as follows: Power: 4.03 mW, Sweep time: 2 min, Resonance frequency: 9.41 GHz, Modulation frequency and modulation width: 100 Hz, 0.079 mT, Time constant: 0.1 s. The EPR parameter, hyperfine coupling constant (hfcc), was calculated from the splitting of MnO ($\Delta H_{3-4} = 8.69$ mT). The signal intensity of spin adduct of DMPO-OH was evaluated from the peak height of the first of four signals (quartet). 1 μM 4-hydroxyl-2,2,6,6,-tetramethylpiperidine-*N*-oxyl (TEMPOL) solution was used as a primary oxygen radical standard of EPR absorption. Pretreatment with Cu, Zn-SOD (200 units/ml) quenched these signals.

2.1.3.2. Measurement of NO radicals generated in mitochondria. Mitochondria preparations diluted 4-fold in MOPS-sucrose medium (pH 7.4, 200 μl) were centrifuged at $8000 \times g$ for 1 min and then resuspended in an acidic medium (pH 6.5) containing 40 μl of an NO· spin trapping reagent, the $(MGD)_2$–Fe complex (*N*-(dithiocarbamoyl)-*N*-methylglucamine sodium salt (MGD) (153 mM) and reduced iron ($FeSO_4$) (3 mM) in water degassed with nitrogen) (Akaike et al., 1996). The three-line EPR signal of the $(MGD)_2$–Fe complex could be observed readily following pretreatment with Cu, Zn-SOD (20 units/ml). The magnetic field was calibrated by using tetracyanoquinodimethane lithium salt (TCNQ-Li) as a marker. The signal intensity of the $(MGD)_2$–Fe complex was evaluated from the peak height of the first of three signals. The spin concentrations of spin adducts of NO· were measured using 1 μM 4-hydroxyl-2,2,6,6,-tetramethylpiperidine-*N*-oxyl (TEMPOL) as a primary standard of EPR absorption.

2.2. Langendorff guinea-pig heart preparation and examination procedures

The procedures for the ischemia-reperfusion cardiac injury model were described previously (Hotta et al., 1994, 1998; Koike et al., 1996); we have summarized the technique and procedures here. Hartley strain guinea pigs of either sex, weighing between 300–350 g, were anesthetized with diethyl ether and heparinized (250 IU, i.p.). The hearts were rapidly excised and the aortas were cannulated. The Langendorff heart preparations were perfused with Krebs–Henseleit solution (KH solution, pH 7.4, at 37°C) containing (in mM) NaCl 115, $NaHCO_3$ 25, KCl 4.7, $CaCl_2$ 2.0, $MgCl_2$ 1.2, KH_2PO_4 1.2 and glucose 10. The solution was pre-saturated with a gas mixture containing 95% O_2 and 5% CO_2, and the hearts were perfused with this solution at a constant pressure of 75 cm H_2O. The perfused hearts were paced at a driving rate of 3–4 Hz stimulation via a 3 M KCl-agar electrode. A latex balloon was inserted into the left ventricle and inflated enough to measure the left ventricular developed pressure at an end-diastolic pressure of 10 mm Hg. The hearts were exposed to a 40-min period of global ischemia by clamping the perfusion flow line, and were then reperfused for 40 min. Test drugs were introduced into the perfusate line 2 min before the start of ischemia. At all concentrations, none of the test drugs had an effect on the LVDP or coronary flow during the aerobic perfusion period before the onset of ischemia. The recovery of the LVDP from ischemia under each drug action was determined and compared with the preischemic LVDP level, as a percentage.

2.3. Immunohistochemistry

After a 20-min perfusion of the Langendorff heart preparations with KH solution, the left ventricle was cut into several pieces and fixed with 0.5% glutaraldehyde in KH solution for 2 h at 4°C. Cubes about 3 mm^3 were cut from these pieces, left for 2 h in the same fixative, washed with PBS, and then repeatedly frozen and thawed. These specimens were sectioned while frozen into slices 10–20 μm thick, permeabilized with 3% H_2O_2 for 30 min, washed for 30 min with PBS, exposed overnight at 4°C

and 1 h at room temperature to 3.3% heat-treated normal goat serum (Sigma, St. Louis, MO). The specimens were then washed with PBS, and incubated under the same conditions with rabbit anti-human e-NOS antibody (25 μg/ml, Transduction Lab.) or rabbit anti-human b-NOS antibody (25 μg/ml, Transduction Lab.), after which they were washed thoroughly with PBS and reincubated with peroxidase-linked goat anti-rabbit immunoglobulin G (IgG) (1: 300, dilution) and then washed with PBS. Peroxidase activity was visualized using 3,3′-diaminobenzidine and H_2O_2. The specimens were treated with 1% osmium tetroxide in PBS for 2 h at 4°C and processed for electron microscopy (H700, Hitachi, Tokyo). Ultrathin sections were stained with lead citrate for 15 min at room temperature. As controls, the sections were treated exactly as above except that the goat serum was substituted for the primary antibodies.

2.4. Chemicals

L-arginine (L-Arg), manganese superoxide dismutase (Mn-SOD), copper and zinc superoxide-dismutase (Zn, Cu-SOD), catalase, $FeSO_4$ and allopurinol were obtained from Wako (Osaka, Japan). N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME), 3-[2-Hydroxy-1-(1-methyl-ethyl)-2-nitrosohydrazino]-1-propananine (NOC 5), *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP), *N*-morpholino sydnonimine hydrochloride (SIN-1), fura-2 AM, *O*,*O*′-Bis (2-aminophenyl) ethyleneglycol-*N*,*N*,*N*′,*N*′-tetraacetic acid-tetraacetoxymethyl ester (BAPTA-AM), *N*-(dithio-carbamoyl)-*N*-methylglucamine sodium salt (MGD) and peroxynitrite anion ($ONOO^-$) in 0.3 M NaOH solution were obtained from Dojindo Laboratories (Kumamoto, Japan). H_2O_2 was obtained from Mitsubishi Glass (Tokyo). 5,5-dimethyl-1-pyrroline-1-oxide (DMPO), tetracyano-

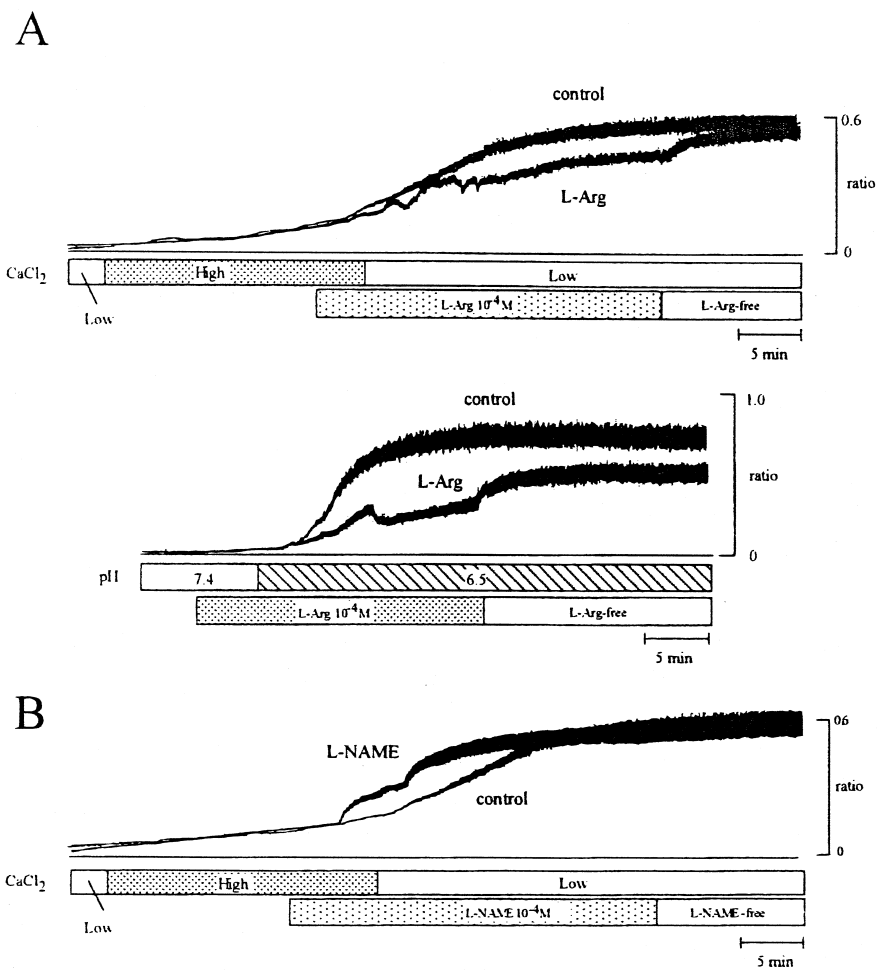

Fig. 1. Recordings of intramitochondrial Ca^{2+} (A, B) in fura-2-loaded mitochondria preparations affected by changing the Ca^{2+} concentration in the perfusate. The mitochondrial Ca^{2+} signal ($[Ca^{2+}]_m$) gradually increased with extremely high Ca^{2+} (1 μM), and perfusion with low Ca^{2+}-content solution (100 nM) rapidly increased the intramitochondrial Ca^{2+} level (A, top trace). Perfusate acidification in a low Ca^{2+}-content solution (100 nM) produced a more rapid Ca^{2+} signal elevation than that with changes in Ca^{2+} contents (A, bottom trace). L-Arg (10^{-4} M) reduced the increase in the intramitochondrial Ca^{2+} signal by changing the Ca^{2+} content of the perfusate. (B) In contrast, a sudden elevation of $[Ca^{2+}]_m$ was induced by the NO · -synthase inhibitor L-NAME (10^{-4} M), and the $[Ca^{2+}]_m$ elevation induced by the external Ca^{2+} concentration change occurred more rapidly than in the normal condition.

Table 1
The effect of various drugs on the changes in mitochondrial Ca^{2+} concentration elicited by perfusion with a medium with a low Ca^{2+} content (A) or pH (B)
The data are ratios (R340/380) of the change in mitochondrial Ca^{2+} concentration under the influence of each drug and are the means ± S.E.M. of 3 ~ 5 preparations.

Drug	(A) Low Ca^{2+}	(B) pH 6.5
control	0.69 ± 0.06 (4)	0.86 ± 0.03 (4)
L-Arg	0.47 ± 0.03 (5)[a]	0.15 ± 0.04 (4)[b]
NOC 5	0.24 ± 0.05 (3)[b]	0.02 ± 0.01 (3)[b]
SNAP	0.33 ± 0.04 (4)[b]	0.02 ± 0.01 (3)[b]
SIN-1	0.38 ± 0.05 (3)[b]	0.05 ± 0.02 (3)[b]
$ONOO^-$	0.34 ± 0.06 (3)[b]	0.06 ± 0.02 (3)[b]
catalase	0.17 ± 0.04 (3)[b]	0.03 ± 0.02 (3)[b]
Mn-SOD	0.19 ± 0.03 (3)[b]	0.03 ± 0.02 (3)[b]
L-NAME	0.72 ± 0.03 (3)[b]	0.87 ± 0.01 (3)[b]

[a] $P < 0.05$, [b] $P < 0.01$ significantly different from control.

quinodimethane lithium salt (TCNQ-Li) and 4-hydroxyl-2,2,6,6,-tetramethylpiperidine-*N*-oxyl (TEMPOL) were purchased from Labotec (Tokyo).

2.5. Statistical analysis

The results of the experiments are expressed as means ± S.E.M. Student's *t*-test was used for statistical analysis of the results.

3. Results

3.1. Effects of L-Arg and L-NAME on the Ca^{2+}-uptake of mitochondria

In the super-perfused mitochondrial preparations, an appreciable increase in the mitochondrial Ca^{2+} signal ($[Ca^{2+}]_m$) was induced by a decrease of the Ca^{2+} content in the perfusate or a decrease in pH from 7.4 to 6.5 in a low Ca^{2+}-concentration perfusate, as shown in our previous study (Hotta et al., 1998). When the mitochondrial preparations were immersed in a high Ca^{2+} concentration (1 μM ~) perfusate, $[Ca^{2+}]_m$ underwent only a steady, gradual elevation from the ratio (R340/380) 0.01 to 0.11 ($n = 5$) during the perfusion period of 30 min. As shown in Fig. 1A, lowering the Ca^{2+} concentration in the perfusate to a physiological low level (100 nM) produced a rapid and intense increase in $[Ca^{2+}]_m$, to the ratio 0.69 ($n = 5$). These increases in $[Ca^{2+}]_m$ were abolished by infusion of Ca^{2+}-chelating agent 10^{-5} M *O*,*O*′-Bis(2-aminophenyl)ethyleneglycol-*N*,*N*,*N*′,*N*′-tetraacetic acid-tetraacetoxymethyl ester (BAPTA-AM).

As shown Table 1, pretreatment with the NO · precursor L-Arg (10^{-4} M) depressed the Ca^{2+}-uptake to 68.1% ($n = 5$) of the maximum Ca^{2+} elevation (as drug-free 100%) induced by perfusate exchange (0.69 ± 0.06 ($n = 4$) → 0.47 ± 0.03 ($n = 5$), $P < 0.05$). Additionally, the $[Ca^{2+}]_m$ elevation induced by acidification of the perfusate was reduced to 18.3% by the prior infusion of L-Arg (0.86 ± 0.03 ($n = 4$) → 0.15 ± 0.04 ($n = 4$), $P < 0.01$). In contrast, the administration of the NO-synthase inhibitor L-NAME (10^{-4} M) suddenly elevated the $[Ca^{2+}]_m$, and the Ca^{2+} concentration leveled off at a low level of 0.3, as shown in Fig. 1B. Ca^{2+}-uptake into mitochondria induced by the subsequent exchange of perfusate to one with a low concentration of Ca^{2+} was more rapid than that in the drug-free preparations. Thereafter, the maximum Ca^{2+} level was 104.3% (0.69 ± 0.06 ($n = 4$) → 0.72 ± 0.03 ($n = 3$), not significant) when the Ca^{2+} concentration was lowered and 101.2% (0.86 ± 0.03 ($n = 5$) → 1.01 ± 0.01 ($n = 3$), not significant) when the pH was lowered (as 100%, with drug-free).

3.2. Immunoblot analysis of mitochondrial proteins from guinea-pig myocardial cells with anti-NOS antibodies

Mitochondrial proteins obtained from guinea-pig myocardial cells were examined by Western blotting for

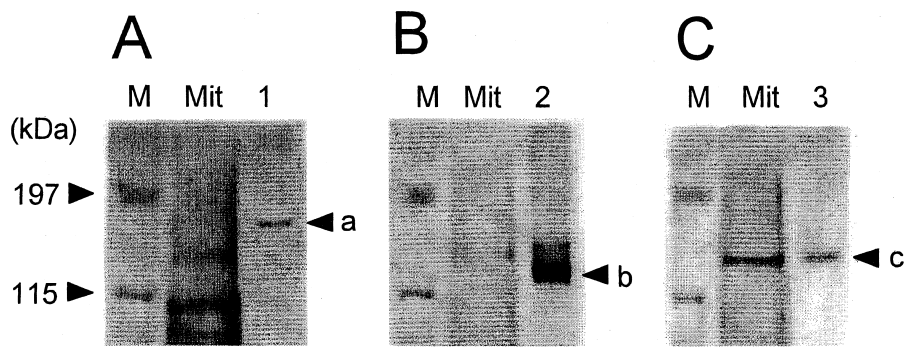

Fig. 2. Immunoblot analysis of mitochondrial proteins from guinea-pig myocardial cells with anti-NOS antibodies. Mitochondria from guinea-pig myocardial cells were purified and the mitochondrial protein was extracted as described in Section 2. The mitochondrial protein was subjected to SDS-PAGE (8% gel) and electrically blotted to an Immobilon PVDF membrane. The PVDF membrane was treated with polyclonal rabbit antibodies against b-NOS (A), i-NOS (B) and e-NOS (C). Lane M contained the prestained SDS-PAGE standards (myosin, 197 kDa; β-galactosidase, 115 kDa). Lane Mit, mitochondrial protein (15 μg/lane); Lane 1 in (A), the positive control for b-NOS (rat pituitary tumor cell lysate, 10 μg/lane); Lane 2 in (B), the positive control for i-NOS (mouse macrophage cell lysate, 10 μg/lane); Lane 3 in (C), the positive control for e-NOS (human endothelial cell lysate, 10 μg/lane).

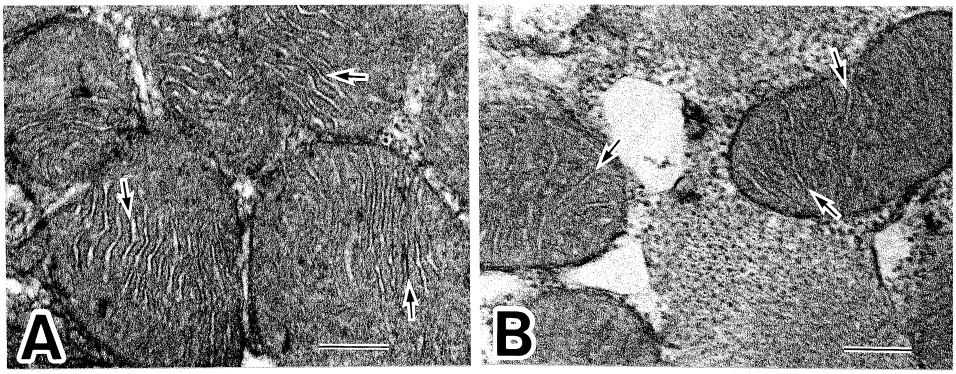

Fig. 3. Indirect immunolabeling for e-NOS (A) in cardiac muscle cell mitochondria together with the control experiment where heated normal serum was used instead of the primary antibody (B). Reaction substances representing antibody binding sites are present on several regions at the cristae (arrows in A). Negligible labeling is seen in the control experiment (arrows in B).

cross-reactivity with polyclonal antibodies raised against three NOS isoforms, b-NOS (Fig. 2A), i-NOS (Fig. 2B) and e-NOS (Fig. 2C). Among the bands detected as reactive to anti-NOS antibody for each mitochondrial lane, a specific dense band was recognized (Fig. 2C) that corresponded to standard human e-NOS. The molecular weight of the protein was ~ 150 kDa.

3.3. Electron microscopic studies

Ultrastructural immunohistological study of the guinea-pig heart revealed partial disruption of the muscle cells and mitochondria. Peroxidase activity, which indicates the presence of antibody binding sites, was detected at the cristae of several mitochondria in the specimens incubated with anti-e-NOS antibody (Fig. 3A), but not in the specimens incubated with anti-b-NOS antibody. In the control experiment, the peroxidase activity was found to be negligible (Fig. 3B).

3.4. Effects of active oxygen generator, NO· precursor and NO· synthase inhibitor on mitochondrial Ca^{2+}-elevation

Fig. 4A and B show a typical change in fura-2 Ca^{2+} signals of mitochondria induced by either external Ca^{2+} concentration changes or acidification, demonstrating a marked increase in $[Ca^{2+}]_m$. Prior infusion of L-Arg (10^{-4} M), oxidizing peroxynitrite anion $ONOO^-$ (10^{-4} M), the NO· donor of SNAP (10^{-4} M) and the antioxidative enzyme Mn-SOD (200 units/ml) clearly depressed this increase in $[Ca^{2+}]_m$. The pretreatment with 10^{-4} M L-Arg reduced the $[Ca^{2+}]_m$ influx by 31.9% or 81.7% relative to the influx in the drug-free state (considered as 100%)

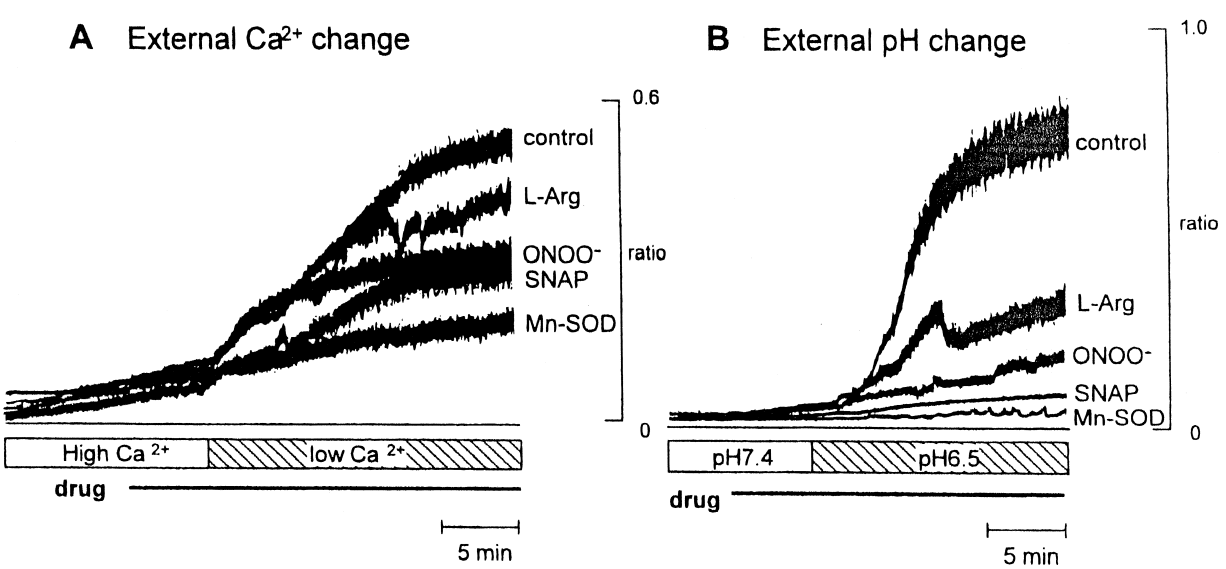

Fig. 4. Effects of the NO-precursor L-Arg, the NO· donor SNAP, the oxidizing peroxynitrite anion $ONOO^-$, and the antioxidative enzyme Mn-SOD on the mitochondrial Ca^{2+}-uptake induced by external Ca^{2+} concentration changes (A) or acidification (B). The elevation of $[Ca^{2+}]_m$ (A and B) was depressed with L-Arg at 10^{-4} M, $ONOO^-$ at 10^{-4} M, SNAP at 10^{-4} M and Mn-SOD at 200 units/ml.

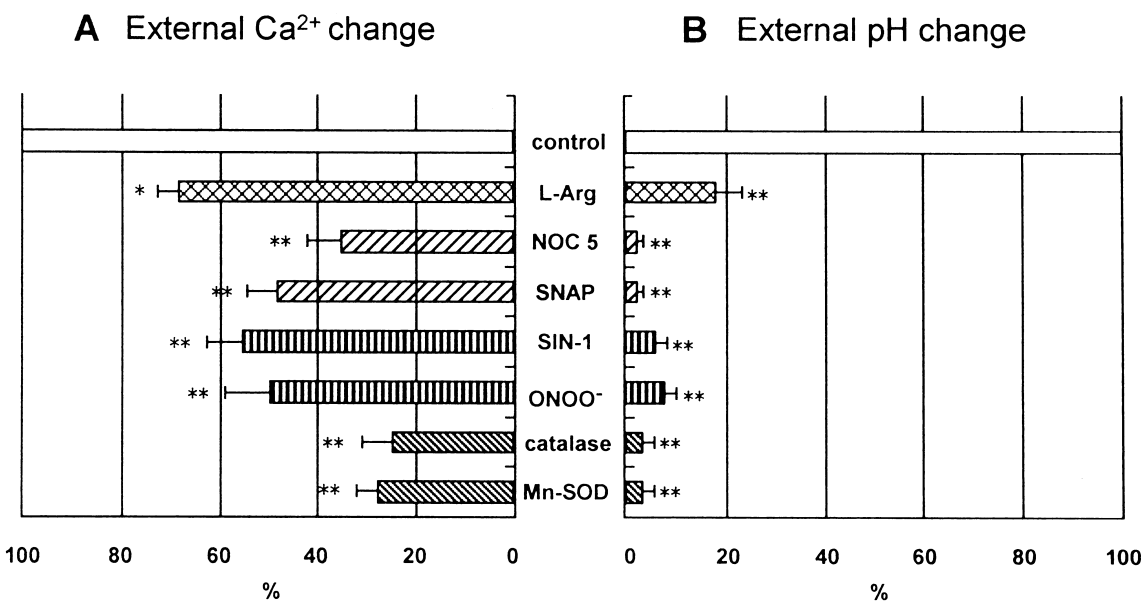

Fig. 5. Comparison of inhibition of Ca^{2+}-uptake into mitochondria in the presence of various drugs related to NO· and $\cdot O_2^-$. The mitochondrial Ca^{2+} level was elevated by an external Ca^{2+} concentration change or external acidification. The $[Ca^{2+}]_m$ elevation (A and B) was reduced by the NO· precursor L-Arg at 10^{-4} M, the NO· donors SNAP at 10^{-5} M, NOC 5 at 10^{-5} M, $ONOO^-$ at 300 μM, the NO· plus $ONOO^-$ generator SIN-1 at 10^{-5} M, the antioxidative enzyme, Mn-SOD at 200 units/ml and catalase at 200 units/ml. $^*P < 0.05$; $^{**}P < 0.01$, significantly different from the control (drug-free) values. Data are the mean values of 3–5 preparations; Vertical lines represent S.E.M.

achieved with the perfusate Ca^{2+} or pH changes, respectively.

In light of the changes in influx (up or down) shown in Fig. 4A and B, the suppression caused by the agents used

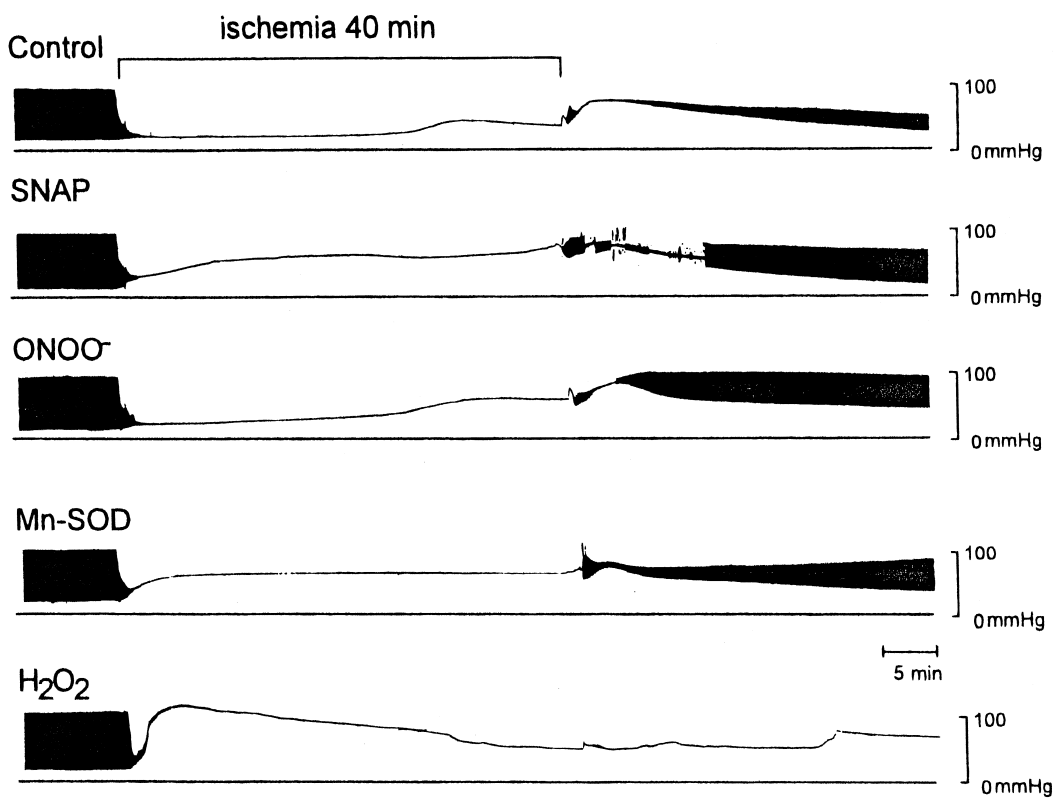

Fig. 6. Beneficial effects on LVDP of the NO·-donor SNAP, $ONOO^-$ and Mn-SOD during ischemia/reperfusion injury in Langendorff heart preparations. SNAP, $ONOO^-$ and Mn-SOD enhanced the recovery of LVDP after reperfusion.

seemed to be regulated by characteristic mechanisms. The R340/380 ratios of Ca^{2+} concentrations in drug-treated and drug-free mitochondria are presented in Table 1. The inhibitory effect of each drug on the rapid Ca^{2+} influx into the mitochondria was different among the active oxygen generators, NO· precursor, and NOS inhibitor, as shown in Fig. 5A, B and Table 1. The pretreatment with SNAP and NOC 5, which spontaneously generate NO·, suppressed the $[Ca^{2+}]_m$ elevations by 55.2%, 65.2% (Ca^{2+}) and 97.6%, 97.6% (pH), respectively. SIN-1 (10^{-5} M), which simultaneously generates NO· as well as $\cdot O_2^-$, and peroxynitrite ($ONOO^-$) inhibited the increase in $[Ca^{2+}]_m$ by 44.9% (Ca^{2+}) and 93.9% (pH), respectively. $ONOO^-$ (3×10^{-4} M) itself also inhibited the increase by 50.7% (Ca^{2+}) and 92.1% (pH). Pretreatment with the $\cdot O_2^-$-scavenger Mn-SOD (200 units/ml) or catalase (200 units/ml) completely inhibited the $[Ca^{2+}]_m$ elevation caused by lowering the Ca^{2+} concentration or pH. The decrease in the $[Ca^{2+}]_m$ elevation caused by these drugs was correlated with the external Ca^{2+} concentration changes and acidification.

3.5. Effects of NO·-donors, $ONOO^-$ and Mn-SOD, and the detrimental effect of H_2O_2 during ischemic-reperfusion injury Langendorff heart preparations

Fig. 6 shows a typical change in contractility (LVDP) in drug-free and drug-treated Langendorff heart preparations exposed to ischemia-reperfusion. SNAP, $ONOO^-$ and Mn-SOD each enhanced the recovery of LVDP after reperfusion. In contrast, the administration of an active oxygen radical generator, 0.3% H_2O_2, depressed the recovery of LVDP (7.5%). The LVDP recovery percentages obtained at 30 min after reperfusion with various drugs are presented in Fig. 7. The LVDP suppressed by reperfusion was restored by each of the drugs tested as follows: L-Arg 10^{-4} M $39.6 \pm 4.4\%$ (not significant, $n = 5$), SNAP 10^{-5} M $57.0 \pm 5.2\%$ ($P < 0.05$, $n = 5$), NOC 5 10^{-5} M $55.0 \pm 3.7\%$ ($P < 0.05$, $n = 5$), $ONOO^-$ 300 μM $57.5 \pm 5.5\%$ ($P < 0.05$, $n = 5$) and SIN-1 10^{-5} M $61.6 \pm 5.1\%$ ($P < 0.05$, $n = 5$), Mn-SOD 200 units/ml $64.0 \pm 3.8\%$ ($P < 0.01$, $n = 5$) and catalase 200 units/ml $54.8 \pm 3.3\%$ ($P < 0.05$, $n = 5$) compared to the drug-free value (control) $38.6 \pm 5.7\%$ ($n = 6$). Treatment with each of these drugs which inhibited the Ca^{2+} influx into mitochondria enhanced by the external Ca^{2+} change or acidification (Fig. 5) improved the cardiac contractility reduced by ischemia/reperfusion in guinea-pig Langendorff hearts (Fig. 7).

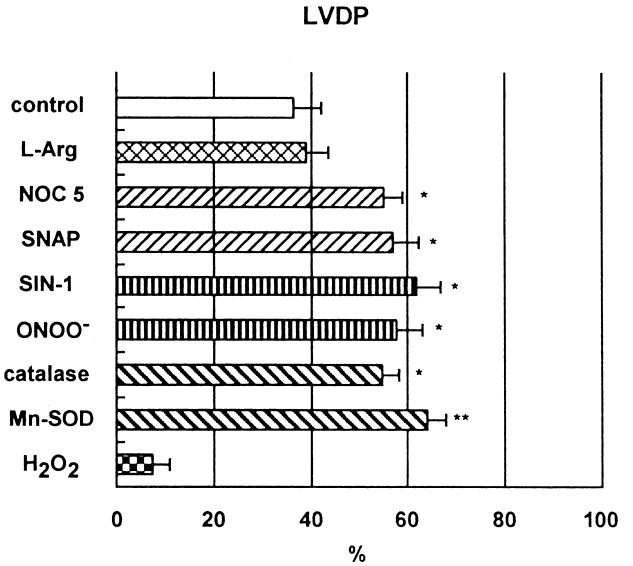

Fig. 7. Post-ischemia recovery of LVDP by reperfusion in the presence of various drugs. The LVDP suppressed by reperfusion was restored by the drugs shown (L-Arg 10^{-4} M, SNAP 10^{-5} M, NOC 5 10^{-5} M, $ONOO^-$ 300 μM, SIN-1 10^{-5} M, Mn-SOD 200 units/ml and catalase 200 units/ml). All of the drugs tested inhibited the enhanced Ca^{2+} influx into mitochondria induced by changing the external Ca^{2+} concentration or acidification of the perfusate. $^*P < 0.05$; $^{**}P < 0.01$, significantly different from the control (drug-free) values. Data are the mean values of 3–6 preparations; Vertical lines represent S.E.M.

3.6. Detection of mitochondrial NO· and $\cdot O_2^-$ generation by spin trapping EPR spectroscopy

The ROS spin trap agent DMPO demonstrated that the mitochondria displayed a 1:2:2:1 quartet signal (hfcc; $a_H = a_N = 1.49$ mT) indicative of the DMPO-OH adduct (Fig. 8A). The DMPO-OH adduct can be formed by direct trapping of the hydroxyl radical (·OH) (Fig. 8Ab and e). Therefore, as shown in Fig. 8Ac and f, superoxide dismutase (SOD) quenched the observed signal. Although this result indicates that the observed signal was attributable to trapping of ·OH generated from the reaction between $\cdot O_2^-$ and H_2O_2 (i.e., the Haber–Weiss reaction), it does not rule out the possibility that H_2O_2 is generated within the mitochondria, probably by the rapid dismutation of $\cdot O_2^-$. The amount of DMPO adduct detected in mitochondria was evaluated from the signal intensity (517 nM in Fig. 8Ab and 564 nM in Fig. 8Ae). At a low Ca^{2+} content (Fig. 8Ab) and pH 6.5 (Fig. 8Ae), clear signals were obtained compared to those obtained at a high Ca^{2+} content (Fig. 8Aa) and pH 7.5 (Fig. 8Ad). The results showed that $\cdot O_2^-$ radical was generated within the mitochondria when the perfusate had a low Ca^{2+} content or low pH.

Mitochondrial NO· production was measured as the adduct formed with the spin-trap $(MGD)_2$–Fe^{2+} complex by EPR spectroscopy (Fig. 8B). NOC 5 demonstrated a remarkable tree-signal as shown in Fig. 8Bc. An EPR spectrum of $[NO–(MGD)_2–Fe^{2+}]$ complex ($a_N = 1.30$ mT, $g_{iso} = 2.04$) detected showed that the mitochondrial preparation after the change of perfusate from pH 7.4 to 6.5 did not generate sufficient $[NO–(MGD)_2–Fe^{2+}]$ complex to be detectable by EPR spectroscopy (Fig. 8Ba). However, EPR spectra corresponding to $[NO–(MGD)_2–Fe^{2+}]$ com-

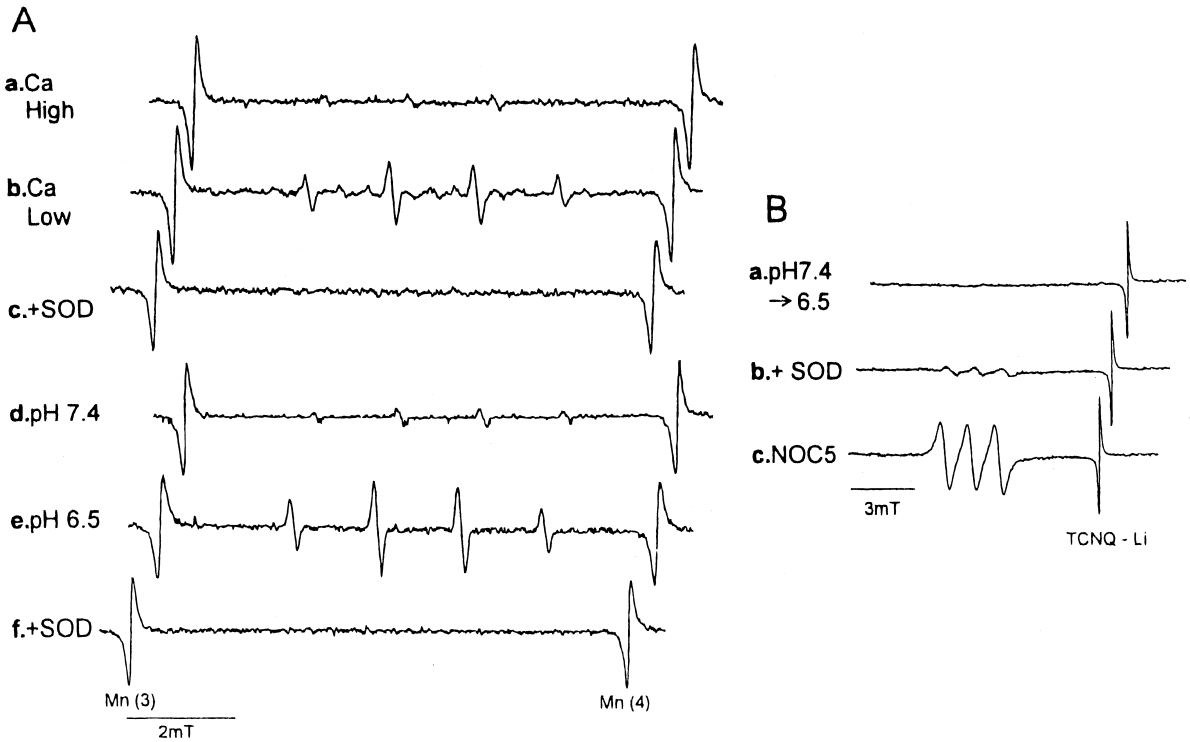

Fig. 8. EPR spectra of spin adducts generated from mitochondrial suspensions obtained by the use of spin traps, DMPO (A) and MGD_2–Fe complex (B). (A-a) EPR spectrum of DMPO recorded beginning at 30 min after the addition of a high Ca^{2+} concentration (1 mM)-perfusate. (b) The EPR spectrum was recorded at 45 s after the change to a normal Ca^{2+} concentration (100 nM)-perfusate. (c) The same conditions as in (b) were used except for the presence of Zn, Cu-SOD (200 units/ml). (d) The EPR spectrum recorded with pH 7.4 solution before acidification of the perfusate. (e) The EPR spectrum was recorded at 45 s after the change to pH 6.5 perfusate. (f) The same conditions as in (e) were used except for the presence of Zn, Cu-SOD (200 units/ml). (B-a) The EPR spectrum of $[NO–(MGD)_2–Fe^{2+}]$ complex was recorded at 45 s after the change of perfusate from pH 7.4 to pH 6.5. (b) The EPR spectrum of $[NO–(MGD)_2–Fe^{2+}]$ complex was recorded at 45 s after acidification of the perfusate in mitochondria suspensions pretreated with Zn, Cu-SOD (20 units/ml). (c) The EPR spectrum was recorded after 3 min in the presence of a NO donor, NOC 5 (3 mM).

plex could be observed after the inclusion of SOD (Fig. 8Bb). The amount of NO · formed in the mitochondria was very close to the detection limits of EPR spectroscopy (313 nM). Therefore, the findings in the present study suggest that NO · generation correlated with the formation of $\cdot O_2^-$.

4. Discussion

We showed, in superfused mitochondrial preparations, that a high Ca^{2+} concentration (more than 1 μM) in the perfusate gradually increased the uptake of Ca^{2+} into mitochondria, as described by Gunter et al. (1994). We also found that reperfusion with a physiological perfusate with a low Ca^{2+} content (100 nM) rapidly further increased the mitochondrial Ca^{2+} signal ($[Ca^{2+}]_m$) (Fig. 1A). The resting cytosolic free Ca^{2+} concentration in the heart cell has been estimated to be in the nM range (Allishire et al., 1987; Marban et al., 1990). The elevation of $[Ca^{2+}]_m$ was similar to that seen upon reperfusion after global ischemia in Langendorff heart preparations (Hotta et al., 1998). In addition, an appreciable increase in $[Ca^{2+}]_m$ was induced by a decrease in pH from 7.4 to 6.5 in a low Ca^{2+}-content perfusate (Fig. 1A). We therefore suspect that, under pathologic conditions, the cytosolic acidic pH alters the mitochondrial Ca^{2+} concentration.

The marked $[Ca^{2+}]_m$ elevations induced by the change in Ca^{2+} content or acidification of the perfusate were reduced by pretreatment of the mitochondria with the NOS substrate, L-arginine (L-Arg) (Fig. 1A). In contrast, the NOS blocker L-NAME caused a sudden $[Ca^{2+}]_m$ elevation that reached a maximum more rapidly than that seen in control preparations (Fig. 1B). These results indicate that NOS is present in the myocardium mitochondria, suggesting that NOS is regulated in some physiological system in the heart. The mitochondrial presence of e-NOS was demonstrated by the immunoblot analysis of mitochondrial proteins isolated from guinea-pig hearts (Fig. 2) as well as in the electron microscopic studies. In the latter, e-NOS was found at the inner membrane, which may act as a barrier to many ions (Fig. 3). In several very recent morphological studies of mitochondria, n-NOS in rat skeletal muscle was shown to be membrane- and possibly mitochondria-associated (Kobzik et al., 1995), and immunochemical studies showed i-NOS or e-NOS associated with the inner membrane of rat liver and brain mitochondria (Bates et al., 1995; Ghafourifar and Richter, 1997;

Giulivi et al., 1998; Tatoyan and Giulivis, 1998) and with the outer membrane of avian ciliary ganglion mitochondria (Nichol et al., 1995). If the e-NOS of myocardial mitochondria is stimulated by Ca^{2+} and located in the matrix or inner side of the inner mitochondria membrane, this would provide a self-regulating system for mitochondrial Ca^{2+} homeostasis concerned with the formation of NO·.

In the present study, preinfusion with the nitric oxide (NO·) donors SNAP, SIN-1 and NOC 5 as well as with the NO· precursor L-Arg inhibited the $[Ca^{2+}]_m$ elevation caused by the external acidification or Ca^{2+} concentration change (Figs. 4 and 5 and Table 1). These donors, which spontaneously generate NO·, had beneficial effects on the LVDP in the ischemia-reperfusion injury of Langendorff hearts (Figs. 6 and 7). NO· has been reported to reduce infarct size and to preserve blood flow as protective mechanisms against ischemia-reperfusion injury in models of the rat mesenteric microcirculation (Kurose et al., 1994), the cat coronary circulation (Weyrich et al., 1992) and the rat cerebral circulation (Morikawa et al., 1992; Malinski et al., 1993; Prado et al., 1993). The overproduction of NO· within tissues has been implicated as contributing to the injury associated with ischemia-reperfusion of cultured neurons (Cazeville et al., 1993), the pig heart (Matheis et al., 1992), the rabbit heart (Zweier et al., 1987; Patel et al., 1993) and the rat limb and lung (Seekamp et al., 1993). The role of NO· in this injury has generally been thought to result from the formation of reactive NO species ($ONOO^-$), an efficient oxidant of thiols (Radi et al., 1991), with NO· and reactive oxygen species (ROS) such as $\cdot O_2^-$.

The antioxidative enzymes Mn-SOD and catalase also suppressed the $[Ca^{2+}]_m$ elevations in mitochondria preparation induced by low pH or low Ca^{2+} concentrations. In contrast, the administration of the ROS generator 0.3% H_2O_2 elevated gradually the $[Ca^{2+}]_m$ to levels similar to those induced by a change in the perfusate Ca^{2+} content or acidification of the perfusate (data not shown). These observations indicated that ROS are essential as mediators of at least part of the injury in animal models of transient global ischemia-reperfusion. Free radicals were directly demonstrated in mitochondria preparations by electron paramagnetic resonance (EPR) spectroscopy with the spin trapping agent DMPO (Irani et al., 1997) (Fig. 8A). The concentration of generated $\cdot O_2^-$ and/or H_2O_2, the predominant precursors of the highly reactive OH radical, was estimated to be 564 nM with perfusate acidification and 517 nM with Ca^{2+} content change. Perfusate acidification, which generated a higher free radical concentration, produced a more rapid $[Ca^{2+}]_m$ elevation than did the change in the Ca^{2+} content of the perfusate (Fig. 4A,B). Therefore, these phenomena are referred to as mitochondrial injury by the oxygen species and Ca^{2+} balance. There is compelling evidence that mitochondrial ROS production and mitochondrial Ca^{2+} handling are of central importance in mitochondrial damage. Mitochondrial Ca^{2+} uptake stimulates $\cdot O_2^-$ production (Chacon and Acosta, 1991), and Ca^{2+} can increase the ROS production by mitochondria of hypoxic heart tissue (Paraidathathu et al., 1992). These results suggest that both effects of $\cdot O_2^-$ production and Ca^{2+} uptake are well correlated with their combined effects, resulting in mitochondrial damage.

In our experiment with $ONOO^-$, pretreatment with SIN-1, a generator of NO· and peroxynitrite anion ($ONOO^-$), and $ONOO^-$ itself reduced the $[Ca^{2+}]_m$ elevations. These compounds, in ischemia/reperfusion-injured Langendorff heart preparations, had marked beneficial effects on cardiac contractility, as did the NO·-precursors, NO·-donors, and antioxidative enzymes (Figs. 6 and 7). These findings, at the organelle level, contribute to the elucidation of the mechanism of ischemia/reperfusion injury of the heart: NO· reduces mitochondrial injury, implicating superoxide anion radical ($O_2^-\cdot$) generation during the reperfusion of ischemic myocardium ($NO\cdot + O_2^-\cdot \rightarrow ONOO^-$). The paradoxical findings of cardioprotective effects of NO· generators are attributed to the inhibition of Ca^{2+}-overloading of mitochondria. As shown in Fig. 8Bb, the EPR spectra corresponding to the spin trap [NO–$(MGD)_2$–Fe^{2+}] complex (Akaike et al., 1996) of mitochondrial NO· production could be observed after the inclusion of the $\cdot O_2^-$-scavenger SOD. The amount of NO· formed in the mitochondria was very close to the limits of detection of EPR spectroscopy (313 nM). This finding therefore suggests that NO· generation under pathological conditions coincides with the formation of $\cdot O_2^-$. Darley-Usmar et al. (1995), Liu et al. (1997), and Keller and Lefer (1999) proposed that the balance between $\cdot O_2^-$ (ROS) and NO· (NOS) generation is a critical determinant in the etiology of many human diseases, including ischemia-reperfusion and cancer.

The results obtained from many mitochondrial studies (Kroemer et al., 1998) indicate that mitochondria play a major role in the regulation of both physiological and pathological cell death. Since mitochondria are an important cellular Ca^{2+} buffer, and Ca^{2+} handling by mitochondria is important for the organelles themselves and for cell proliferation, apoptosis and necrosis, the possible involvement of e-NOS in these events must be investigated. The apoptotic decay of cells participates in acute pathologies as well as in infection induced by ischemia/reperfusion damage of the heart (Gottlieb et al., 1994; Umansky, 1999). Gottlieb et al. (1994) found evidence of apoptosis unique to reperfusion injury but not in ischemic injury alone. The hallmark of apoptosis, nucleosomal ladders of DNA fragments, was detected in ischemic/reperfused rabbit myocardial tissue but not in normal or ischemic rabbit hearts. Although the causes and mechanisms of apoptosis are not clearly understood, oxidative stress, NO· and its congeners, Ca^{2+}, proteases, nucleases, and mitochondria are important mediators of limiting events in the death process. Further investigation of mitochondrial function and its regulation should provide valuable information on the

normal physiology of cell death, and help in the design of novel cytoprotective drugs.

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