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Original Contribution

EFFECT OF ENDOGENOUS NITRIC OXIDE ON ENERGY METABOLISM OF RAT HEART MITOCHONDRIA DURING ISCHEMIA AND REPERFUSION

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Abstract—The effects of ischemia and postischemic reperfusion on the functions of the heart and its mitochondria were studied with special attention to the effect of nitric oxide (NO) by treatment of rat hearts with the nitric oxide synthase (NOS) inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) or its noninhibitory isomer N^G-nitro-D-arginine methyl ester (D-NAME). NO generated during reperfusion caused increase in coronary flow (CF), but had no effect on the left ventricular pressure (LVP) or heart rate (HR). The ATP level of the heart decreased during ischemia and was not completely restored by introduction of oxygen during reperfusion due to damage of complexes I and II of the respiratory chain of mitochondria by NO. Inhibition of the respiratory chain resulted in generation of hydrogen peroxide, and NO and NO-derived species generated after production of NO caused further damage of various proteins in mitochondria, such as complexes I and II of the respiratory chain and pyruvate dehydrogenase (PDH). These results suggested that NO generated on reperfusion was the primary cause of mitochondrial dysfunction by damage of complexes I and II of the respiratory chain, with consequent increase of CF in the heart. © 1998 Elsevier Science Inc.

Keywords—Nitric oxide, Ischemia-reperfusion, Mitochondria, Heart, Free radicals

INTRODUCTION

Nitric oxide (NO) is generated in various mammalian tissues, and acts as an intercellular messenger associated with various physiological and pathological events [1]. However, NO is toxic, inhibiting various enzymes and proteins, such as NADPH oxidase [2], creatine kinase [3], the calcium-dependent proteinase calpain [4] and protein kinase C [5]. In addition, NO generated by ischemia-reperfusion injures various organs such as the heart, brain and lungs [6–9]. However, its effects are not always consistent: for instance, it causes cardiac muscle contraction [10–14], but reduces infarction after ischemia-reperfusion of rat heart [7,15].

As NO is reported to dissipate the membrane potential and inhibit complex I- and complex II-dependent respiration in isolated mitochondria [16–18], inhibition of bioenergy transduction in mitochondria should be directly associated with its inhibitory effect on heart function during ischemia-reperfusion. However, most effects of NO have been studied by direct exposure of either the heart, purified enzymes or isolated mitochondria to exogenous NO [2–15], and the effects of endogenous NO in myocardial ischemia-reperfusion injury have not been examined in detail [15,19].

Therefore, we studied the effects of endogenous NO on the cardiac function of rats during ischemia-reperfusion, with special attention to energy transduction in mitochondria. The effect of endogenous NO was studied by examining the actions of the NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) and its noninhibitory isomer N^G-nitro-D-arginine methyl ester (D-NAME).

EXPERIMENTAL PROCEDURES

Materials

L-NAME, D-NAME, ethylene glycol bis (*β*-aminoethyl ester) N,N,N′,N′-tetraacetic acid (EGTA), *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamide (TMPD) and thiamine pyrophosphate were obtained from Nacalai Tesque, Inc. (Kyoto), ADP, ATP, NAD^+ and lactate dehydrogenase (LDH) were from Oriental Yeast, Co.

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(Osaka). Nagarse was purchased from SERVA Feinbiochemica (Heidelberg). Fatty acid-free bovine serum albumin (BSA), coenzyme A (CoA), antimycin A and horseradish peroxidase were obtained from Sigma Chemical Co. (St. Louis). Other reagents were obtained locally and were of the highest grade available.

Perfusion of the heart

Hearts were removed from male Wistar rats (250–300 g) and perfused through the aorta with Krebs-Henseleit buffer at 80 mmHg in a Langendorff apparatus. A latex balloon (Technical Supply Co., Osaka) was inserted into the left ventricle, and the left ventricular pressure (LVP) and heart rate (HR) were monitored. After perfusion for about 20 min, consistent values for the LVP and HR were attained. Perfusion was continued for 20 min further (preperfusion), and then ischemia was induced by stopping the perfusion. After ischemia for 20 min, perfusion was restarted (reperfusion) for 20 min, and the LVP, HR and coronary flow (CF) were measured continuously. For examinations of the effects of L-NAME and D-NAME, these compounds were added to the perfusate just after consistent HR and LVP values had been attained.

Determinations of ATP and lactate in the heart

Just after the periods of preperfusion, ischemia and reperfusion, hearts were removed from the apparatus, and quickly frozen in liquid nitrogen. Then they were homogenized in a Polytron in 0.6 M perchloric acid, and the extracts were neutralized with saturated KH_2CO_3, and stored at -80°C for analysis [20]. The ATP concentration in the neutralized supernatant was determined from the optical absorbance at 260 nm after high-performance liquid chromatography (HPLC) in a Shimadzu LC-10AD on a Shimadzu Shim-pack CLC-ODS column. Lactate was determined from the absorbance at 340 nm due to oxidation of NADH mediated by LDH [21].

Isolation of rat heart mitochondria

Mitochondria were isolated from ischemic or reperfused rat heart by the method of Palmer et al. [22] with a slight modification. After ischemia or reperfusion for a defined period, the hearts were finely minced, and suspended in buffer A consisting of 100 mM KCl, 2 mM EGTA, 0.2% fatty acid-free BSA and 50 mM Mops (pH 7.4) at 1 g of mince/10 ml of buffer A. The suspensions were homogenized in a Polytron, and Nagarse (subtilisin) was added at a final concentration of 5 mg/g wet weight of mince. The homogenates were incubated for 3 min on ice, diluted 2-fold with buffer B consisting of 100 mM KCl and 50 mM Mops (pH 7.4), and then promptly centrifuged at 12,000 $\times$ *g* for 5 min. The resulting pellets were homogenized in buffer A in a Potter-Elvehjem homogenizer, and centrifuged at 500 $\times$ *g* for 5 min. The supernatants were centrifuged at 5,000 $\times$ *g* for 10 min, and the pellets were suspended in buffer B and centrifuged at 12,000 $\times$ *g* for 10 min. This procedure was repeated. The mitochondria obtained as pellets were suspended in buffer B. The mitochondrial protein concentration was determined with a BCA protein assay kit (Pierce, Rockford) in the presence of 1% SDS with BSA as a standard.

Mitochondrial respiration

The respiration of mitochondria (0.5 mg protein/ml) was monitored polarographically with a Clark-type electrode (Yellow Spring, YSI 5331) as change in the oxygen concentration of the mitochondrial suspension in medium consisting of 100 mM KCl, 5 mM potassium phosphate, 1 mM EGTA and 5 mM Mops (pH 7.4) in a total volume of 2.2 ml at 25°C. As substrates for complex I-, II-, and IV-dependent respirations, 5 mM glutamate/malate, 5 mM succinate plus rotenone (1 μg/mg protein), and 5 mM ascorbate plus 0.5 mM TMPD, respectively, were used.

H^+-ATPase activity

The H^+-ATPase activity of mitochondria (1 mg protein) was determined as the amount of the reaction product, inorganic phosphate (Pi), derived from the hydrolysis of 5 mM ATP at 30°C in medium consisting of 70 mM KCl, 50 mM sucrose, 2 mM $MgCl_2$, and 50 mM Hepes (pH 7.4). Oligomycin (3 μg/mg protein) and the weakly acidic uncoupler SF6847 (50 nM) were added when necessary. The reaction was terminated by addition of ice-cold 8% trichloroacetic acid, and the amount of Pi produced was determined by the method of Fiske and Subbarow [23].

Hydrogen peroxide production

Hydrogen peroxide production by mitochondria was determined by dual wavelength spectrophotometry as the amount of a complex of horseradish peroxidase and hydrogen peroxide in mitochondrial suspensions (0.1 mg protein/ml) with a wavelength pair of 417 nm and 402 nm ($\Delta\varepsilon$:50 $mM^{-1}cm^{-1}$) in the presence of 5 mM succinate and 10 mM Pi [24].

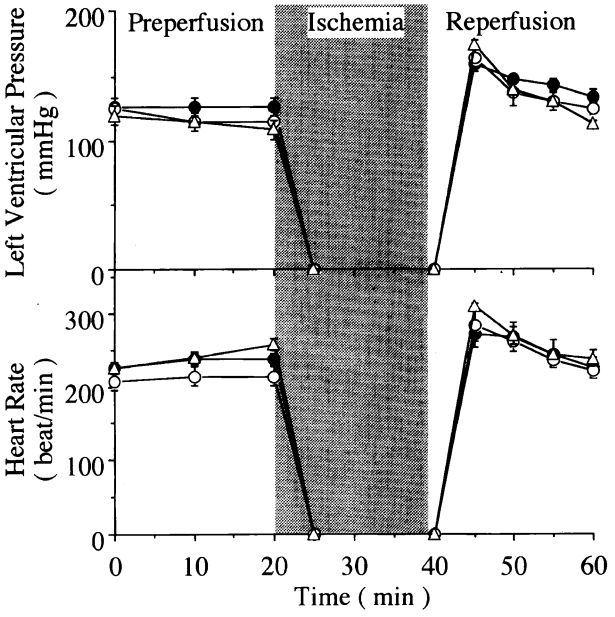

Fig. 1. Changes of left ventricular pressure (LVP) and heart rate (HR) during ischemia and reperfusion. Rat hearts were perfused from the aorta with Krebs-Henseleit buffer at 80 mmHg in a Langendorff apparatus. Hearts were perfused until constant LVP and HR values were attained. Perfusion was then continued for 20 min (preperfusion) without additions (closed circle) or with L-NAME (1 mM, open circles) or D-NAME (1 mM, open triangles) added to the perfusate. After stopping perfusion for 20 min (ischemia), aerobic perfusion was started again for 20 min (reperfusion). Values are means ± SE for 6 hearts.

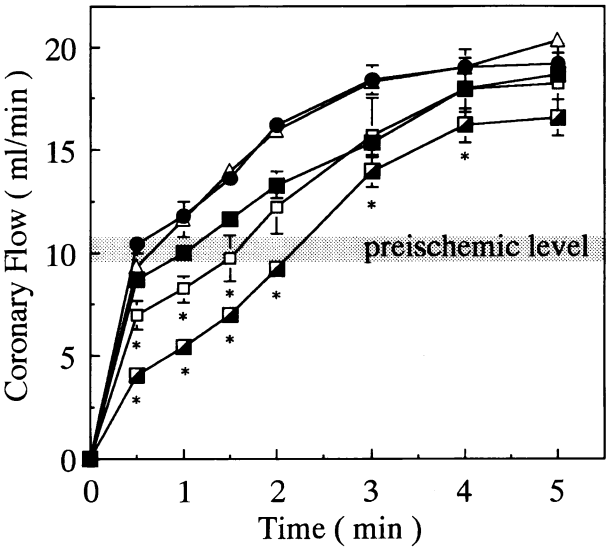

Fig. 2. Effects of L-NAME and D-NAME on coronary flow (CF) during reperfusion immediately after ischemia. Experimental conditions were as for Fig. 1. Closed circles, no addition; open triangles, with 1 mM D-NAME; closed squares, open squares and half-closed squares, with 0.1 mM, 0.5 mM and 1 mM L-NAME, respectively. Values are means ± SE for 6 hearts. Values significantly different from that of the control are shown by (*).

Pyruvate dehydrogenase activity

The pyruvate dehydrogenase (PDH) activity of mitochondria (0.1 mg protein/ml) was determined from the rate of increase in the optical absorbance at 340 nm due to production of NADH from NAD^+ in medium consisting of 2 mM $MgCl_2$, 1 mM NAD^+, 0.2 mM thiamine pyrophosphate, 0.2 mM CoA, 1 mM dithiothreitol, 2 mM pyruvate, 0.1% Triton X-100, and 50 mM Tris-HCl (pH 7.8) at 25°C [25].

All data are expressed as means ± SE. The Bonferroni/Dunn post hoc t-test was used to analyze differences in values in different groups. A level of $p < .05$ was taken as statistically significant.

RESULTS

Effects of L-NAME on Cardiac Functions During Ischemia-Reperfusion

The left ventricular pressure (LVP) and heart rate (HR) before induction of ischemia were 126 ± 7 mmHg and 240 ± 8 beats/min, respectively, and 1 mM L-NAME did not affect these values appreciably (115 ± 5 mmHg, 214 ± 13 beats/min, respectively). As shown in Fig. 1, both the LVP and HR, which decreased to zero soon after induction of ischemia, were promptly restored on reperfusion to slightly higher levels than before ischemia, and then gradually decreased to their preischemic levels. L-NAME and D-NAME at up to 1 mM had no effects on these changes. In contrast, as shown in Fig. 2, the coronary flow (CF) terminated on onset of ischemia and increased time-dependently during reperfusion. After 30 s of reperfusion, the CF was restored to the preischemic level (10 ± 1 ml/min), and then continued to increase, attaining a constant level of about twice the preischemic level after 3 min. L-NAME added during preperfusion suppressed increase of the CF concentration dependently, especially in the early stage of reperfusion: the CF values with 1 mM L-NAME at 30 s and 5 min after reperfusion were about 30% and 80%, respectively, of those before treatment. D-NAME had no apparent effect. These results suggest that NO generated by ischemia-reperfusion increased the blood flow by causing relaxation of the coronary vessels.

ATP level in the heart during ischemia and reperfusion

Ischemia-reperfusion should affect energy metabolism of the heart. Therefore, we next determined the amounts of ATP in the heart in ischemia and on reperfusion. As shown in Fig. 3, the amount of ATP in the heart decreased 40% after ischemia for 20 min, and this level was not affected by treatment with L-NAME or D-NAME at up to 1 mM. During ischemia, ATP is

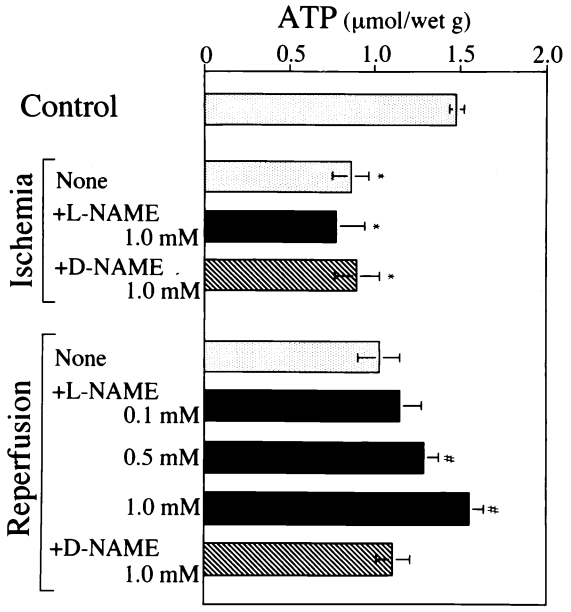

Fig. 3. Changes in ATP levels in the heart on ischemia and reperfusion. Hearts were perfused as described in the legend of Fig. 1. The amounts of ATP in the heart before ischemia (control), after 20 min ischemia and after 20 min reperfusion were determined. Values are means ± SE for 6 hearts. Values significantly different from that for control hearts and reperfused hearts without L- or D-NAME are shown by (*) and (#), respectively.

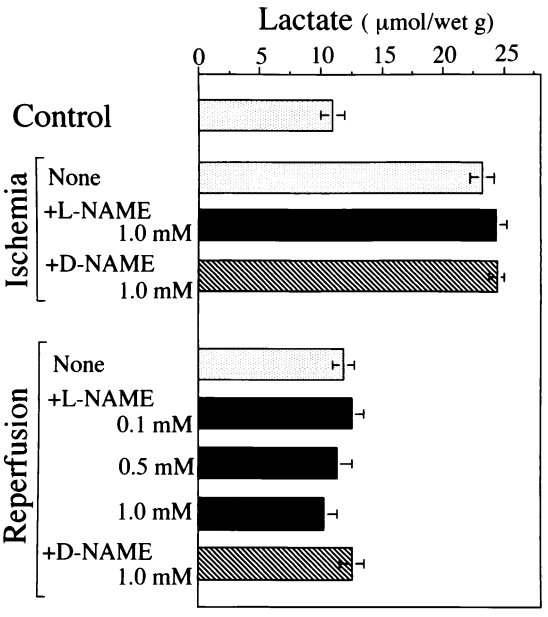

Fig. 4. Changes of lactate levels in hearts after ischemia and reperfusion with and without L-NAME or D-NAME. After ischemia and reperfusion for 20 min, the amount of lactate in cardiac cells was determined from the absorbance at 340 nm due to oxidation of NADH mediated by lactate dehydrogenase. Values are means ± SE for 4 hearts.

decomposed to AMP and then to xanthine and hypoxanthine [26,27]. The amount of AMP after ischemia for 20 min was 1.3-fold that before ischemia, and L-NAME and D-NAME did not affect its level (data not shown). In contrast, the amount of ATP was restored to 80% of its preischemic level after reperfusion for 20 min. L-NAME caused progressive increase in the ATP level, complete recovery to the preischemic level being attained with 1 mM L-NAME ($p < .01$), whereas D-NAME at up to 1 mM had no effect.

Lactate levels of the heart during ischemia and reperfusion

The CF is directly related with oxygen supply to the cardiac cells during reperfusion. As L-NAME suppressed the increase in the CF associated with recovery of the ATP level during reperfusion, it seemed possible that it might enhance anaerobic energy metabolism. Therefore, we determined change in the lactate level as a measure of glycolytic energy metabolism. The lactate level was increased more than 2-fold by ischemia for 20 min, and decreased to the preischemic level after reperfusion for 20 min (Fig. 4). These changes were not affected by treatment with L-NAME or D-NAME, indicating that NO probably does not affect glycolysis in the heart during ischemia-reperfusion.

Effect of NO on heart mitochondrial functions

As the glycolytic activity of the heart during ischemia-reperfusion was not affected by L-NAME, and as the decreased cardiac ATP level during ischemia was restored by treatment with L-NAME during reperfusion, it seemed possible that the NO generated during reperfusion inhibits oxidative phosphorylation in mitochondria. Therefore, we next examined the effect of NO on oxidative phosphorylation in heart mitochondria. For this we isolated mitochondria from individual hearts that had been subjected to ischemia and reperfusion. To avoid possible damage caused by Ca^{2+} released by ischemia such as activation of proteases, the Ca^{2+}-specific chelating agent EGTA was added to the medium for isolation.

Figure 5 shows results on oxygen uptake by mitochondria under various conditions. The rate of respiration initiated by succinate (plus rotenone) as a respiratory substrate was low in mitochondria isolated from preperfused rat heart, but increased more than 3-fold on addition of ADP due to ATP synthesis (state 3 respiration). When all the added ADP had been converted to ATP, the respiratory rate decreased to the original level (state 4 respiration). State 4 respiration was released about 4-fold by addition of the protonophoric uncoupler SF6847 (uncoupled respiration) as a result of maximum electron flow through the respiratory chain due to dissipation of the proton motive force across the inner mitochondrial

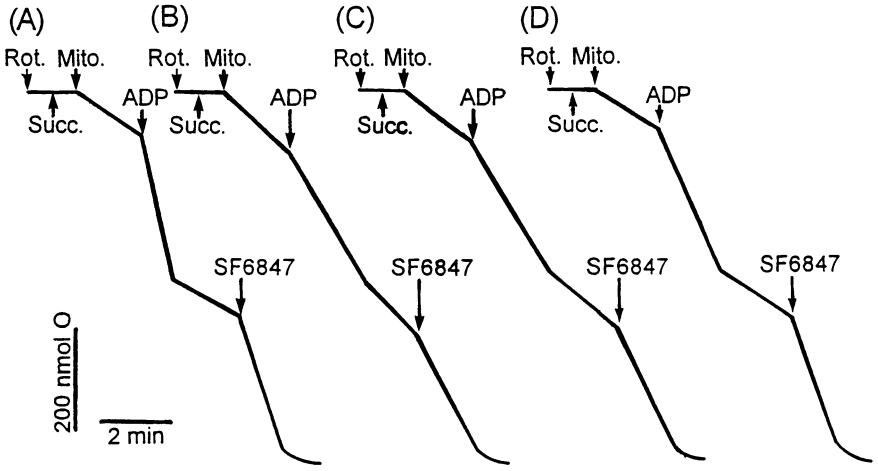

Fig. 5. Effects of ischemia and reperfusion on mitochondrial respiration. Mitochondria were isolated from rat heart after ischemia and reperfusion under the conditions described in the legend of Fig. 1. Respiration of mitochondria was monitored polarographically at 25°C and pH 7.4 with 5 mM succinate (plus 1 μg rotenone/mg protein) as substrate in a total volume of 2.2 ml. To the suspension of rat heart mitochondria (Mito., 0.5 mg protein/ml), 5 mM succinate (Succ.) plus rotenone (Rot., 1 μg/mg protein) was added to initiate respiration. Then 1 mM ADP and 50 nM SF 6847 were added as indicated. (A), control mitochondria isolated from preperfused heart; (B), ischemic mitochondria; (C), reperfused mitochondria; (D), reperfused mitochondria with 1 mM L-NAME added before the onset of ischemia.

membrane (Fig. 5A). The ratio of the rate of state 3 respiration to that of state 4 respiration, referred to as the respiratory control index (RCI), and the ratio of the rate of uncoupled respiration to that of state 4 respiration, referred to as the respiratory control ratio (RCR), are measures of ATP synthesis efficiency and intactness of mitochondria, respectively.

Changes in the respiratory rates of ischemic and reperfused mitochondria and the effect of L-NAME on the respiratory rates of reperfused mitochondria are shown in Fig. 5B-D, and the values of respiratory rates, RCI and RCR under various conditions are summarized in Table 1. Ischemia and reperfusion caused decrease in state 3 respiration and increase in state 4 respiration. The RCI value was decreased from 3.3 to 2.0, and that of RCR from 3.7 to 2.2 by ischemia, showing that ischemia damaged the mitochondria; i.e., it caused decrease in state 3 respiration and decreases in state 4 and uncoupled

Table 1. Respiration of Mitochondria Isolated from Ischemic and Reperfused Rat Heart[a]

	Respiratory Rate (V_{OX}) (nmol O/min/mg protein)				
Mitochondria	state 4	state 3	uncoupled	RCI[b]	RCR[c]
Control	73 ± 3	241 ± 10	273 ± 11	3.3 ± 0.1	3.7 ± 0.1
Ischemic	82 ± 1	166 ± 4[e]	185 ± 7[e]	2.0 ± 0.1[e]	2.2 ± 0.1[e]
+L-NAME[d]	82 ± 2	170 ± 5[e]	186 ± 6[e]	2.1 ± 0.1[e]	2.3 ± 0.2[e]
+D-NAME[d]	83 ± 3	168 ± 6[e]	183 ± 4[e]	2.1 ± 0.1[e]	2.2 ± 0.1[e]
Reperfused	79 ± 2	170 ± 7[e]	199 ± 5[e]	2.4 ± 0.1[e]	2.6 ± 0.1[e]
+L-NAME[d]	74 ± 3	234 ± 4[f]	281 ± 3[f]	3.3 ± 0.1[f]	3.6 ± 0.1[f]
+D-NAME[d]	77 ± 4	178 ± 10[e]	189 ± 3[e]	2.3 ± 0.1[e]	2.5 ± 0.2[e]

[a] The effects of ischemia and reperfusion on the mitochondrial respiratory rate (V_{OX}) of rat heart mitochondria isolated after ischemia and reperfusion were examined. Mitochondria isolated from rat heart after preperfusion were used as a control. Succinate (5 mM) was used as a respiratory substrate in medium consisting of 100 mM KCl, 5 mM potassium phosphate, 1 mM EGTA and 5 mM Mops, pH 7.4. State 3 respiration and uncoupled respiration were induced by addition of 1 mM ADP and 50 nM SF6847, respectively. Values are averages for 6 separate experiments (±SE);

[b] V_{OX}(state 3)/V_{OX}(state 4);

[c] V_{OX}(uncoupled)/V_{OX}(state 4);

[d] 1 mM L-NAME or D-NAME was added before induction of ischemia;

[e] Significantly different from V_{OX} of control mitochondria ($p < .01$);

[f] Significantly different from V_{OX} of mitochondria isolated from reperfused heart without treatment with 1 mM L-NAME ($p < .01$).

respirations resulting in lower RCR and RCI values. Reperfusion after ischemia partially reversed the damage. L-NAME had no effect on ischemic mitochondria, but kept reperfused mitochondria as intact as control mitochondria, while D-NAME did not have any effect. Thus the damage of mitochondria by ischemia was not caused by NO. Possibly, the mitochondrial damage by ischemia was due to decrease in adenine nucleotides in the cells [28].

To examine which site(s) of the respiratory chain is damaged by ischemia-reperfusion, we next determined the complex I-, II- and IV-dependent state 3 respirations of mitochondria isolated from hearts after ischemia and reperfusion. As summarized in Table 2, ischemia and reperfusion significantly inhibited complex I-dependent respiration initiated with glutamate/malate as substrate, and had appreciable effects on complex II-dependent respiration with succinate as a substrate ($p < .01$). As L-NAME completely prevented the damages of complex-I and II ($p < .01$), NO should be associated with these damages of the respiratory chain. Ischemia and reperfusion caused no change in complex IV-dependent respiration with ascorbate/TMPD as substrate.

Inhibition of the mitochondrial respiratory chain is known to generate reactive oxygen species. In fact, as shown in Fig. 6, the low hydrogen peroxide production from preperfused mitochondria was increased about 7-fold by inhibition of complex II components of the respiratory chain by addition of antimycin A. Similarly, more than 8-fold and 6-fold higher levels of hydrogen peroxide were generated from mitochondria isolated from ischemic and reperfused hearts with succinate as substrate, respectively, than from intact mitochondria.

Table 2. Effects of Ischemia and Reperfusion on Mitochondrial Complex I, II, and IV-Dependent Respirations[a]

	State 3 Respiratory Rate (% of control)		
Mitochondria	complex I	complex II	complex IV
Control	100 ± 6	100 ± 5	100 ± 5
Ischemic	58 ± 9[c]	69 ± 2[c]	101 ± 2
Reperfused	60 ± 5[c]	70 ± 3[c]	101 ± 5
+L-NAME[b]	98 ± 8[d]	97 ± 2[d]	102 ± 5
+D-NAME[b]	64 ± 9[c]	74 ± 4[c]	99 ± 4

[a] Values for the state 3 respiratory rate of rat heart mitochondria isolated after ischemia and postischemic reperfusion were determined. State 3 respiration was initiated by addition of 1 mM ADP with 5 mM glutamate/malate, succinate and ascorbate/TMPD as respiratory substrates for complex I, II and IV-dependent respirations, respectively. Experimental conditions were as for Table I. The rates of state 3 respirations of control mitochondria were 217, 241, and 292 nmol O/min/mg protein for complex I, II, and IV-dependent respirations, respectively. Values are averages for 6 separate experiments (±SE);
[b] 1 mM L-NAME or D-NAME was added before ischemia;
[c] Significantly different from control value ($p < .01$);
[d] Significantly different from the value for mitochondria without additions isolated after reperfusion.

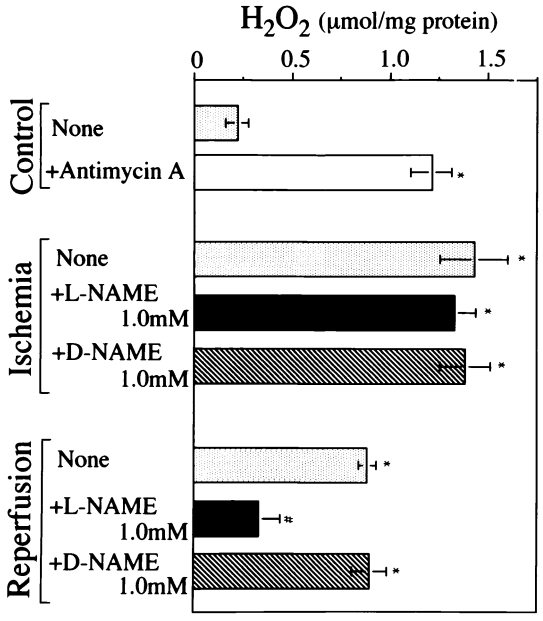

Fig. 6. Hydrogen peroxide production from mitochondria isolated from ischemic and reperfused heart. The amounts of hydrogen peroxide produced by mitochondria (0.1 mg protein/ml) isolated from rat hearts after 20 min ischemia and 20 min reperfusion were determined with horseradish peroxidase [24]. L-NAME was added at a final concentration of 1 mM just before the onset of ischemia. Respiration was induced with 5 mM succinate plus rotenone (1 μg/mg protein). The effect of 1 μM antimycin A on hydrogen peroxide production by control mitochondria isolated from the heart *after* preperfusion was also examined. Values are means ± SE for 4 preparations. Values significantly different from that of control mitochondria and of reperfused mitochondria without L-NAME are shown by (*) and (#), respectively.

L-NAME, but not D-NAME, completely inhibited the enhanced production of hydrogen peroxide from mitochondria isolated from reperfused heart, whereas it had no effect on hydrogen peroxide production from ischemic heart mitochondria. These results showed that production of hydrogen peroxide from mitochondria of reperfused heart was due to inhibition of the respiratory chain by NO and/or NO-derived species generated by reperfusion after ischemia, and that the mitochondrial damage in ischemia was not due to NO. This is consistent with our results on respiratory changes in ischemia and on reperfusion.

Table 3 summarizes the ATPase activities of mitochondria isolated from rat hearts after ischemia and reperfusion. Neither Mg^{2+}-dependent ATPase nor oligomycin sensitive ATPase (F_0F_1-ATPase) activity was affected by ischemia and reperfusion. Addition of the weakly acidic uncoupler SF6847 activated the Mg^{2+}-dependent and oligomycin sensitive ATPase activities of ischemic and reperfused mitochondria about 13-fold like those of control mitochondria. As these ATPase activities were not affected by ischemia-reperfusion, NO was concluded not to affect these ATPase activities.

Table 3. Effects of Ischemia and Reperfusion on ATPase Activity of Rat Heart Mitochondria[a]

Conditions	ATPase activity (μmol Pi/min/mg protein)		
	Mg^{2+}-dependent	Oligomycin sensitive	Uncoupler induced
Control	0.12 ± 0.01	0.12 ± 0.00	1.56 ± 0.15
Ischemic	0.13 ± 0.01	0.12 ± 0.01	1.62 ± 0.06
Reperfused	0.11 ± 0.00	0.10 ± 0.00	1.54 ± 0.10
+ L-NAME[b]	0.13 ± 0.00	0.12 ± 0.00	1.59 ± 0.01
+ D-NAME[b]	0.11 ± 0.00	0.10 ± 0.00	1.67 ± 0.09

[a] Mg^{2+}-dependent, oligomycin sensitive and uncoupler induced ATPase activities of mitochondria isolated from rat heart after preperfusion (control), and rat hearts after ischemia and reperfusion were determined by the amount of Pi formed by hydrolysis of 5 mM ATP at 30°C. Oligomycin-sensitive ATPase activity was determined by addition of 3 μg oligomycin/mg protein, and uncoupler induced ATPase activity was determined with 50 nM SF6847. Values are averages for 6 separate experiments (±SE);

[b] L-NAME or D-NAME at 1 mM was added to the perfusate before induction of ischemia.

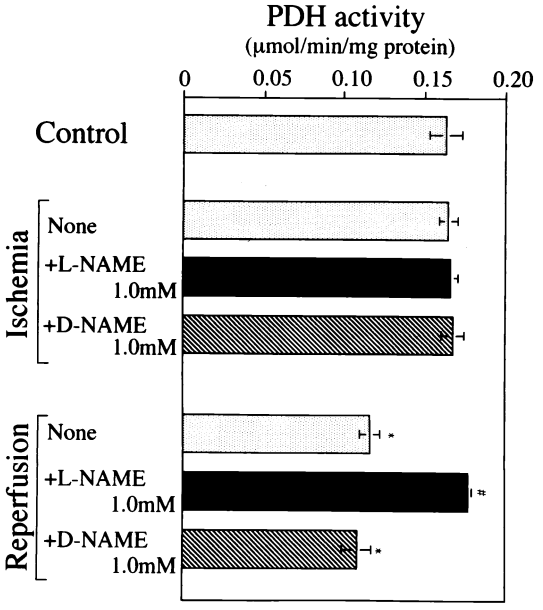

Fig. 7. PDH activity of mitochondria isolated from ischemic and reperfused rat heart. Mitochondria were isolated from ischemic and reperfused rat heart as described in the legends of Fig. 1. The PDH activity of mitochondria (0.1 mg protein/ml) was determined in a total volume of 2 ml of reaction mixture consisting of 2 mM $MgCl_2$, 1 mM NAD^+, 0.2 mM thiamine pyrophosphate, 0.2 mM CoA, 1 mM dithiothreitol, 2 mM pyruvate, 0.1% Triton X-100, and 50 mM Tris-HCl (pH 7.8) at 25°C from the optical absorbance at 340 nm due to formation of NADH from NAD^+ coupled with conversion of pyruvate to acetyl-CoA. The effects of 1 mM L-NAME and D-NAME were examined by their additions to the perfusate before ischemia. Values are means ± SE for 4 preparations. Values significantly different from those of control mitochondria isolated from the heart after preperfusion and reperfused mitochondria without L- or D-NAME are shown by (*) and (#), respectively.

The pyruvate dehydrogenase (PDH) complex present in the mitochondrial matrix space catalyzes conversion of pyruvate to acetyl-CoA. As shown in Fig. 7, ischemia did not have any effect on the PDH activity, whereas reperfusion reduced it to about 60% of the control value ($p < .01$). L-NAME added during preperfusion maintained the PDH activity at the level before induction of ischemia ($p < .01$), showing that NO generated by reperfusion inhibited the PDH activity.

DISCUSSION

We studied the effects of ischemia and reperfusion on the functions of the heart and the activities of energy transduction in mitochondria with special attention to the effects of endogenous NO. Although reperfusion is reported to increase the NO concentration, the exact amount of NO in the reperfused heart does not seem to have been determined [29,30], and the effect of NO has usually been examined by the effect of the NOS inhibitor L-NAME, which is effective at 0.1–0.5 mM [30,31]. In this study, we also examined the participation of NO by the effects of L-NAME under various conditions, in comparison with those of the non-inhibitory isomer D-NAME. For studies on the effects of endogenous NO on energy transduction, we used mitochondria isolated from individual rat hearts after ischemia and reperfusion. There are several reports on the effects of exogenous NO on mitochondria isolated from intact rat heart [16–18]. Results have shown that the inhibitory effect of exogenous NO on cells and mitochondria depends on the oxygen tension, being more significant at low oxygen concentrations (30–60 μM) than at high oxygen concentrations (220–260 μM) [18]. Moreover, the lifetime of NO at low oxygen tensions is much longer than that at high oxygen tensions [32]. As the oxygen concentration is about 50 μM in the cytosol and less in mitochondria, endogenous NO should have marked effects on the functions of cells and mitochondria [18].

NO is generated by cNOS which is activated by Ca^{2+} [10,11]. Ischemia increases intracellular Ca^{2+} in cardiac myocytes and mitochondria [33,34], and oxygen, which is necessary for NO production, is supplied by reperfusion, so NO generation in the cytoplasm of heart cells should take place during postischemic reperfusion, but not during ischemia [9]. The marked increase in the CF during reperfusion should be due to relaxation of the coronary vessels caused by NO generated in endothelial cells and vascular smooth muscle cells [33,34].

NO is known to have high affinity for heme-proteins, and has been shown to reduce cytochrome a_3 in the mitochondrial inner membrane [18]. Therefore, NO generated on reperfusion may bind to the heme moieties of cytochromes of components of the mitochondrial respi-

ratory chain resulting in inhibition of respiration. Inhibition of complex I- and II-dependent respiration is known to generate active oxygen species from mitochondria [35–37]. In fact, we showed that the production of hydrogen peroxide from intact mitochondria treated with antimycin A, an inhibitor of complex II-dependent respiration, was 7-fold that without antimycin A treatment. Moreover, hydrogen peroxide production by mitochondria isolated from reperfused heart, in which complex I and II-dependent respirations were inhibited, was sixfold that of intact mitochondria.

Active oxygen species are generated not only by the respiratory chain of mitochondria, but also by xanthine and/or hypoxanthine in the presence of oxygen mediated by xanthine oxidase [26,27]. Active oxygen species and NO are reported to react with each other rapidly yielding the highly oxidizing and potent biological oxidant peroxynitrite anion ($ONOO^-$) [38]. Exogenous $ONOO^-$ is reported to inhibit complex I- and complex II-dependent respirations and to inactivate succinate dehydrogenase and NADH dehydrogenase, but to have no effect on complex IV-dependent respiration or cytochrome c oxidase in rat heart mitochondria [18,38,39]. Therefore, NO-derived species should also damage mitochondria and cardiac cells. In this study, we showed that endogenous NO and/or NO-derived species generated on postischemic reperfusion of the heart damaged components of complex I- and II, but not complex-IV, like exogenous NO [16–18,38,39]. However, we found that mitochondrial ATPase activities were insensitive to endogenous NO, whereas they are inhibited by exogenous NO [18]. This discrepancy was possibly because the amounts of exogenous NO used in previous studies were greater than the amount generated by reperfusion.

As the phospholipid bilayer membrane is permeable to NO, it could affect enzymes in the matrix space of mitochondria. In fact, we found that NO generated on reperfusion decreased the mitochondrial PDH activity, although this activity was not affected by ischemia. Activation of PDH in postischemic heart by dichloroacetate or pyruvate is reported to enhance glucose oxidation and repress glycolysis, resulting in improvement of cardiac function during reperfusion [40,41]. As PDH catalyzes the conversion of pyruvate to acetyl-CoA, and supplies electrons to NADH and $FADH_2$ of the mitochondrial respiratory chain via the tricarboxylic acid cycle, repression of PDH activity should increase the lactate level or accelerate fatty acid oxidation by inhibiting acetyl-CoA production from glucose. However, the activated glycolytic energy transduction induced by ischemia, as evaluated by the lactate level, was decreased to the preischemic level during reperfusion, and the ATP level reduced by ischemia was greatly restored by reperfusion. Therefore, on onset of reperfusion, ATP could be mainly supplied by oxidative phosphorylation by the damaged mitochondria. Possibly this could be feasible by activation of fatty acid metabolism to compensate for repressed ATP production caused by inhibition of glucose metabolism due to NO-induced inactivation of PDH. Further studies are in progress to examine this possibility.

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ABBREVIATIONS

L-NAME—N^G-nitro-L-arginine methyl ester
D-NAME—N^G-nitro-D-arginine methyl ester
LVP—left ventricular pressure
HR—heart rate
CF—coronary flow
RCI—respiratory control index;
RCR—respiratory control ratio
TMPD—*N,N,N',N'*-tetramethyl-*p*-phenylenediamide
NOS—NO synthase
PDH—pyruvate dehydrogenase