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Involvement of superoxide and nitric oxide in the genesis of reperfusion arrhythmias in rats

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Abstract

To assess the role of reactive oxygen species and nitric oxide (NO) in the genesis of reperfusion-induced arrhythmias, the effects of reactive oxygen species scavengers and NO synthase inhibitors on the incidence of ventricular fibrillation and irreversible ventricular fibrillation (mortality) were examined. Hearts of anesthetized rats were subjected to 4 min regional ischemia followed by 4 min reperfusion. The animals were treated i.v. with superoxide dismutase, a O_2^- scavenger, catalase, a H_2O_2 scavenger, dimethylthiourea, a ·OH scavenger, or N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-nitro-L-arginine (L-NNA), NO synthase inhibitors. Superoxide dismutase (430 and 4300 U/kg/min) reduced the mortality from 93% to 43% and 57%, respectively, whereas treatment with catalase or dimethylthiourea did not affect these arrhythmias. L-NAME (0.1 and 0.3 mg/kg/min) reduced the mortality from 93% to 50% and 43%, respectively. L-NNA (0.3 mg/kg/min) reduced the mortality from 93% to 50%. This reduction by the NO synthase inhibitors was abolished by administration of L-Arg. However, L-Arg blocked neither a small increase in systolic blood pressure nor a decrease in heart rate elicited by the NO synthase inhibitors. The combinated treatment of superoxide dismutase (4300 U/kg/min) with L-NAME (0.3 mg/kg/min) reduced the mortality from 93% to 7%. These results suggest that the genesis of reperfusion-induced arrhythmias observed in this model may be in part due to O_2^- and NO.

Keywords: Reperfusion arrhythmia; Superoxide dismutase; Catalase; Dimethylthiourea; N^G-Nitro-L-arginine; N^G-Nitro-L-arginine methyl ester

1. Introduction

Life-threatening ventricular arrhythmias, ventricular tachycardia and ventricular fibrillation, are known to occur during reperfusion after myocardial ischemia in both experimental animals and humans (Corr and Witowski, 1983; Tzivoni et al., 1983). Several mechanisms for reperfusion-induced arrhythmias have been proposed (Manning and Hearse, 1984), such as stimulation of adrenergic receptors, an increase in cyclic adenosine monophosphate, disturbances of lipid metabolism and disturbances in ionic homeostasis, particularly in calcium and potassium ions. Recently, reactive oxygen species, such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (·OH) have been suggested to be involved in the induction of reperfusion arrhythmias (Curtis and Riva, 1990). This hypothesis is supported by the findings that a variety of reactive oxygen species scavengers reduced the incidence of ventricular fibrillation during reperfusion (Bernier et al., 1986; Manning et al., 1988; Riva et al., 1987; Woodward and Zakaria, 1985) and that reactive oxygen species were detected in reoxygenated hearts by electron spin resonance (Arroyo et al., 1987; Zweier et al., 1987) and chemiluminescence (Henry et al., 1990; Ohoi et al., 1993c). On the other hand, Yamada et al. (1990) showed that the severity of reperfusion-induced arrhythmias was unrelated to the oxygen tension of the perfusate. Furthermore, the failure of reactive oxygen species scavengers to reduce reperfusion-induced arrhythmias has also been reported (Da-Luz et al., 1993; Hagar et al., 1991; Tosaki et al., 1993). Thus, the role of reactive oxygen species in reperfusion-induced arrhythmias remains controversial.

Recently, nitric oxide (NO) has been implicated to play a crucial role in a number of diverse physiological processes, including smooth muscle relaxation, platelet inhibition, neurotransmission and immune regulation (Moncada et al., 1991). It is still controversial whether NO is beneficial or deleterious for ischemic/reperfused hearts. Several

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investigators (Wang and Morgan, 1993; Pabla and Curtis, 1993, 1994, 1995; Pabla et al., 1995; Parratt, 1993) have shown that NO may function as an endogenous cardioprotective factor in ischemic/reperfused hearts, whereas others (Matheis et al., 1992; Schulz and Wambolt, 1993; Naseem et al., 1995; Depré et al., 1995) have shown that NO aggravated ischemia/reperfusion injury in the myocardium. Since NO has a single electron in its 2p-π antibonding orbital, it is one of the free radical species, like O_2^-, and highly reactive (Stamler et al., 1992). To assess the role of reactive oxygen species and NO in the genesis of reperfusion-induced arrhythmias, we examined in the present study the effects of reactive oxygen species scavengers and NO synthase inhibitors on the incidence of ventricular fibrillation and irreversible ventricular fibrillation in ischemic/reperfused rat hearts.

2. Materials and methods

2.1. Materials

The present study was performed using 424 male Sprague-Dawley rats (300–450 g; Charles River, Atsugi, Japan). The animals were maintained under artificial conditions at 23 ± 1°C, with a constant humidity of 55 ± 5%, a cycle of 12 h of light and 12 h of dark and had free access to food and tapwater, according to the Guidelines of Experimental Animal Care issued by the Prime Minister's Office of Japan.

Recombinant human copper-zinc superoxide dismutase (specific activity 4300 U/mg) was obtained from Nippon Kayaku (Tokyo, Japan). Catalase (bovine liver, specific activity 11 000 U/mg), N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-nitro-L-arginine (L-NNA) were purchased from Sigma (St. Louis, MO). Dimethylthiourea and Evans blue were purchased from Tokyo Chemical Product (Tokyo, Japan) and L-arginine hydrochloride (L-Arg) from Kokusan Chemical Works (Tokyo, Japan).

2.2. Surgical preparation

Animals were anesthetized with sodium pentobarbital (55 mg/kg i.p.), their tracheae were cannulated and ventilated with room air supplemented with 100% oxygen gas at a rate of 5 ml/min using a respirator (SN-480-7; Shinano Seisakusho, Tokyo, Japan). The tidal volume was set at 10 ml/kg. Atelectasis was prevented by maintaining an expiratory pressure of 2 cm H_2O. Blood in the carotid artery was sampled and blood gases were measured with a gas analyzer (Model 288; Ciba-Corning, New York, NY). The pO_2 and pCO_2 of the blood were adjusted to 100–130 mm Hg and 35–40 mm Hg, respectively, by changing the breathing rate from 40 to 60 cycle/min. Electrocardiogram (ZR-601G; Nihon-kohden, Tokyo, Japan) was measured via electrodes placed on the left thorax (apex of the heart) and the right scapula and recorded on a thermal pen recorder (WT-645; Nihon-kohden). Heart rate measurement was triggered from electrocardiogram pulses with a cardiotachometer (AT-601G; Nihon-kohden). Blood pressure was measured with a pressure transducer (TP-400T; Nihon-kohden) through a cannula inserted into the carotid artery. The femoral vein was cannulated for administration of drugs. Rectal temperature was maintained at 36.5 ± 0.5°C with an infrared lamp.

The chest was opened and the heart was gently exteriorized after the pericardiac incision. A ligature (5-0; Kono Seisakusho, Ichikawa, Japan) was placed around the left coronary artery approximately 2 mm from its origin according to the method of Selye et al. (1960). Then, the heart was repositioned in the thoracic cavity. Both ends of the ligature were passed through a polyethylene tube (i.d. 0.4 mm, length 50 mm) and exteriorized out of the thoracic cavity. Both ends of the ligature were pulled, which occluded the left coronary artery. The tube and ligature were clamped together with a small hemostatic clamp. Reperfusion was initiated by removing the clamp from the tube and ligature and confirmed by a color change in ventricular surface from cyanosis to hyperemia. The ligature was left in place in order to measure the ischemic area at the end of experiments.

2.3. Duration of ischemia

In anesthetized rat hearts, reperfusion after 3–10 min of ischemia resulted in the highest incidence of reperfusion-induced ventricular fibrillation (Manning and Hearse, 1984). Furthermore, we observed in a previous study that most of the reperfusion-induced arrhythmias were initiated within the first 1 min of reperfusion (usually 10–30 s) (Ohoi et al., 1992). Thus, a 4-min period of ischemia followed by a 4-min period of reperfusion was employed in the present study.

2.4. Drug administration

Superoxide dismutase at rates of 130, 430 and 4300 U/kg/min, catalase at rates of 3300 and 33 000 U/kg/min, dimethylthiourea at rates of 5 and 50 mg/kg/min or saline was infused into the femoral vein from 1 min after coronary artery occlusion to 4 min after reperfusion. The doses of superoxide dismutase used were determined on the basis of those previously proved to be effective in suppressing reperfusion arrhythmias in this model (Ohoi et al., 1993a). The doses of catalase and dimethylthiourea were similar to those that most effectively suppressed reperfusion-induced arrhythmias (Manning et al., 1988) and stunning (Bolli et al., 1987), respectively. That is, the total dose infused for 7 min in the present study was similar to that of the bolus administration. Recently, several investigators have reported that low doses of L-NAME and L-NNA (e.g. 1 mg/kg i.p., by

Nowicki et al., 1991; 0.1 mg/kg i.v. bolus followed by 0.01 mg/kg/min i.v. infusion, by Ashwal et al., 1994) prevented the development of ischemia/reperfusion-induced cerebral infarction. Therefore, we used low doses of L-NAME and L-NNA: 0.03, 0.1 and 0.3 mg/kg/min L-NAME, 0.3 mg/kg/min L-NNA, combination of either 0.3 mg/kg/min L-NAME or L-NNA and 5 mg/kg/min L-Arg, and saline were infused into the femoral vein from 4 min before coronary artery occlusion to 4 min after reperfusion. Longer periods of treatment with L-NAME were chosen, since the agent requires a considerable period of time to exert its effect (Rees et al., 1990). These agents were dissolved in saline. The order of these treatments was randomized using a table of random numbers.

2.5. Measurement of ischemic area

After the experiment, 500 U/kg heparin was injected into the femoral vein. The heart was removed and perfused in a Langendorff manner with 10 ml of the Krebs-Henseleit solution at a constant perfusion pressure of 100 cm H_2O through the aortic cannula. The left coronary artery was occluded by the ligature remained around this artery. 5 ml of 75% ethanol was injected into the heart from a side arm of the canula inserted into the aorta for 1 min under 100–150 mm Hg of pressure. The heart was cut into 1.5-mm-thick transverse sections and these sections were blotted on a filter paper. Each section was divided into the two sections, one was the ischemic region which was not discolored by ethanol and the other, the non-ischemic region which was discolored. The wet weights of ischemic and non-ischemic areas were expressed as percentages of the total heart weight. We confirmed in a preliminary study that the ischemic area determined by ethanol injection was comparable to that determined by blue dye injection which was generally used by others (Pabla and Curtis, 1994; Hagar et al., 1991; Curtis and Hearse, 1989); 45.7 ± 1.3% for Evans blue group and 47.6 ± 2.0% for ethanol group ($n = 5$).

2.6. Selection of animals by the ischemic area

The data were analyzed by an observer blinded to the conditions of the experiment. In the rat hearts, the severity of reperfusion-induced arrhythmias is highly dependent on the size of ischemic area (Curtis and Hearse, 1989; Ohoi et al., 1992). Thus, for analysis of data, 287 rats whose ischemic areas were 40–55% were used, whereas 127 rats whose ischemic areas were < 40% or > 55% were deleted. This evaluation was based on the results of the antiarrhythmic effect of agents in a previous study (Ohoi et al., 1993a).

2.7. Criteria of arrhythmias and death

Classification of arrhythmias was carried out according to the Lambeth Conventions (Walker et al., 1988) with a slight modification (Tsuchihashi and Curtis, 1991). That is, ventricular fibrillation is defined as a signal from which individual QRS deflections vary in amplitude and coupling interval on a cycle-to-cycle basis. When ventricular fibrillation-induced hypotension (below 50 mm Hg) remained for > 2 min, animals were considered to be terminal. Rats that were deemed to be terminal never recovered normal cardiac rhythm during the experiment. Irreversible ventricular fibrillation was the only cause of death.

2.8. Statistics

The results are expressed as the mean ± S.E.M. Statistical significance was estimated using paired t-test for any difference from the initial in hemodynamics. One-way ANOVA followed by Bonferroni's multiple comparison test was used to compare the ischemic area between all groups. Fisher's exact test was used to compare the mortality between saline- and agent-treated groups. A probability of < 5% was considered significant ($P < 0.05$).

3. Results

3.1. Effects of superoxide dismutase, catalase and dimethylthiourea

Fig. 1 shows the effects of superoxide dismutase, catalase and dimethylthiourea on the incidence of ventricular fibrillation and mortality upon reperfusion in anesthetized rats. Superoxide dismutase (430 and 4300 U/kg/min) significantly reduced the incidence of ventricular fibrillation and mortality. Catalase (3300 and 33 000 U/kg/min) and dimethylthiourea (5 and 50 mg/kg/min) did not reduce the incidence of ventricular fibrillation and mortality.

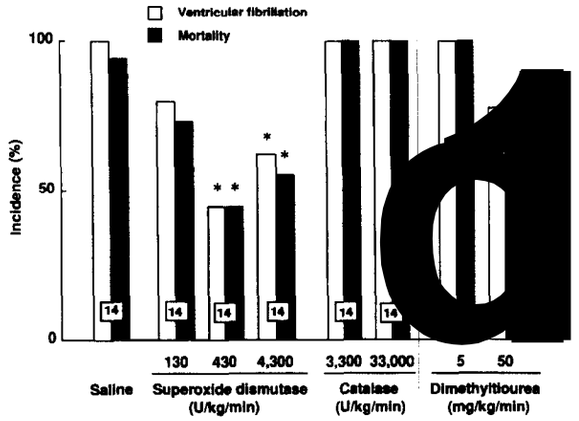

Fig. 1. Effects of superoxide dismutase, catalase and dimethylthiourea on the incidence of ventricular fibrillation and mortality upon reperfusion in anesthetized rats. Superoxide dismutase, catalase and dimethylthiourea were continuously infused into the femoral vein from 1 min after left coronary artery occlusion to the end of 4 min reperfusion. * $P < 0.05$ vs. saline.

Table 1
Systolic blood pressure and heart rate of coronary artery-occluded rats treated with either saline, superoxide dismutase, catalase or dimethylthiourea

Treatment	Number of rats	Systolic blood pressure (mm Hg)			Heart rate (beats/min)		
		Initial	Time after occlusion		Initial	Time after occlusion	
			1 min	4 min		1 min	4 min
Saline	14	111 ± 4	89 ± 5 [a]	99 ± 6	414 ± 5	420 ± 7	428 ± 9
Superoxide dismutase (U/kg/min)							
130	14	110 ± 3	87 ± 4 [a]	100 ± 6	427 ± 7	428 ± 7	431 ± 10
430	14	110 ± 4	89 ± 7 [a]	106 ± 8	404 ± 9	421 ± 11 [a]	424 ± 10 [a]
4300	14	112 ± 5	89 ± 6 [a]	107 ± 6	414 ± 9	429 ± 10	435 ± 9 [a]
Catalase (U/kg/min)							
3300	14	114 ± 5	89 ± 4 [a]	113 ± 8	424 ± 7	430 ± 9	449 ± 10 [a]
33000	14	110 ± 4	90 ± 4 [a]	105 ± 4	416 ± 8	429 ± 7 [a]	435 ± 5 [a]
Dimethylthiourea (mg/kg/min)							
5	14	109 ± 4	88 ± 7 [a]	100 ± 8	399 ± 11	402 ± 10	404 ± 10
50	14	106 ± 6	87 ± 7 [a]	85 ± 3 [a]	400 ± 13	400 ± 16	393 ± 17

Each value represents the mean ± S.E.M. Superoxide dismutase, catalase and dimethylthiourea were continuously infused into the femoral vein from 1 min after occlusion to the end of 4 min reperfusion. [a] $P < 0.05$ vs. initial.

Table 1 shows the effects of these agents on systolic blood pressure and heart rate at initial and 1 and 4 min of coronary artery occlusion. The coronary artery occlusion evoked an increase in heart rate and a decrease in systolic blood pressure, which recovered almost to the initial levels within 4 min. There were no differences in the ischemic area among these groups at the end of reperfusion (Table 2).

3.2. Effects of either L-NAME or L-NNA and the combination of either of them with L-Arg

Figs. 2 and 3 show the effects of L-NAME and the combination of L-NAME with L-Arg (L-NAME + L-Arg) and the effects of L-NNA and the combination of L-NNA with L-Arg (L-NNA + L-Arg), respectively, on the incidence of ventricular fibrillation and mortality upon reperfusion in anesthetized rats. The incidence of ventricular fibrillation and mortality were significantly reduced by L-NAME at a rate of 0.1 mg/kg/min and at rates of 0.1 and 0.3 mg/kg/min, respectively (Fig. 2). L-NAME (0.03 mg/kg/min) + L-Arg (5.0 mg/kg/min) did not reduce the incidence of VF and mortality. Similarly, L-NNA (0.3 mg/kg/min) significantly reduced the mortality, but did not reduce the incidence of ventricular fibrillation (Fig. 3). Similar effects of L-NNA on the incidence of ventricular fibrillation and mortality were detected when the animals were treated with a higher dose (1.0 mg/kg/min) of the agent (71.4% and 57.1%, respectively, $n = 7$). L-NNA (0.3 mg/kg/min) + L-Arg (5.0 mg/kg/min) did not reduce the incidence of ventricular fibrillation and mortality.

The effects of these agents on systolic blood pressure and heart rate at initial, preocclusion, and 1 and 4 min of occlusion were examined (Table 3). Systolic blood pressure was increased by the infusion of L-NAME (0.3 mg/kg/min) and L-NAME (0.3 mg/kg/min) + L-Arg

Table 2
Ischemic area of coronary artery-occluded rats treated with either saline, superoxide dismutase, catalase or dimethylthiourea

Treatment	Number of rats	Ischemic area (% of total heart wt)
Saline	14	47.8 ± 1.1
Superoxide dismutas (U/kg/min)		
130	14	44.6 ± 1.0
430	14	46.3 ± 0.9
4300	14	48.6 ± 1.2
Catalase (U/kg/min)		
3300	14	46.4 ± 1.4
33000	14	48.3 ± 1.5
Dimethylthiourea (mg/kg/min)		
5	14	47.5 ± 1.3
50	14	46.9 ± 1.1

Each value represents the mean ± S.E.M. There were no differences in ischemic area between saline- and drug-treated groups.

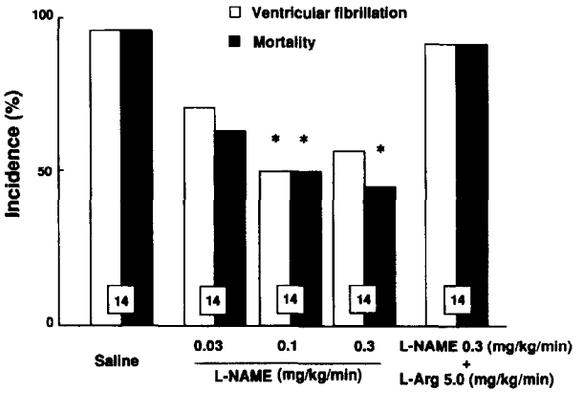

Fig. 2. Effects of N^G-nitro-L-arginine methyl ester (L-NAME) and the combination of L-NAME and L-arginine (L-Arg) (L-NAME + L-Arg) on the incidence of ventricular fibrillation and mortality upon reperfusion in anesthetized rats. L-NAME and L-NAME + L-Arg were continuously infused into the femoral vein from 4 min before left coronary artery occlusion to the end of 4 min reperfusion. * $P < 0.05$ vs. saline.

Table 3
Systolic blood pressure and heart rate of coronary artery-occluded rats treated with either saline, L-NAME or L-NAME + L-Arg, or with either saline, L-NNA or L-NNA + L-Arg

Treatment (mg/kg/min)		Number of rats	Systolic blood pressure (mm Hg)				Heart rate (beats/min)			
			Initial	Before occlusion	Time after occlusion		Initial	Before occlusion	Time after occlusion	
					1 min	4 min			1 min	4 min
Saline		14	105 ± 4	104 ± 4	83 ± 4 [a]	97 ± 4	401 ± 8	396 ± 7	406 ± 9	409 ± 11
L-NAME	0.03	14	93 ± 4	97 ± 5	79 ± 7 [a]	84 ± 6	404 ± 12	396 ± 12	400 ± 15	396 ± 17
	0.1	14	98 ± 6	102 ± 7	81 ± 8 [a]	93 ± 7	412 ± 14	405 ± 16	396 ± 17	398 ± 17
	0.3	14	109 ± 6	121 ± 5 [a]	98 ± 5 [a]	112 ± 6	408 ± 11	394 ± 13 [a]	394 ± 11 [a]	396 ± 13
L-NAME 0.3 + L-Arg 5.0		14	106 ± 5	118 ± 6 [a]	91 ± 9 [a]	104 ± 10	424 ± 12	422 ± 10	413 ± 12	409 ± 16
Saline		14	107 ± 4	107 ± 5	89 ± 6 [a]	104 ± 6	415 ± 13	411 ± 11	416 ± 12	415 ± 17
L-NNA 0.3		14	106 ± 3	115 ± 5 [a]	93 ± 7 [a]	111 ± 8	425 ± 10	416 ± 11	417 ± 12	413 ± 13
L-NNA 0.3 + L-Arg 5.0		14	108 ± 2	117 ± 3 [a]	95 ± 4 [a]	117 ± 4 [a]	413 ± 10	407 ± 10	414 ± 8	412 ± 6

Each value represents the mean ± S.E.M. Saline, L-NAME, L-NAME + L-Arg, L-NNA, and L-NNA + L-Arg were continuously infused into the femoral vein from 4 min before occlusion to the end of 4 min reperfusion. [a] $P < 0.05$ vs. initial.

(5.0 mg/kg/min) or by the infusion of L-NNA (0.3 mg/kg/min) and L-NNA (0.3 mg/kg/min) + L-Arg (5.0 mg/kg/min) and decreased at 1 min of occlusion. Systolic blood pressure was recovered thereafter. Heart rate was decreased by the infusion of L-NAME (0.3 mg/kg/min). There were no differences in the ischemic area among these groups at the end of reperfusion (Table 4).

3.3. Effect of the combination of superoxide dismutase with L-NAME

Effects of the combination of superoxide dismutase with a NO synthase inhibitor L-NAME on reperfusion-induced arrhythmias were studied. Superoxide dismutase (4300 U/kg/min), L-NAME (0.3 mg/kg/min) and superoxide dismutase (4300 U/kg/min) + L-NAME (0.3

Table 4
Ischemic area of coronary artery-occluded rats treated with either saline, L-NAME or L-NAME + L-Arg, or either saline, L-NNA or L-NNA + L-Arg

Treatment (mg/kg/min)		Number of rats	Ischemic area (% of total heart wt)
Saline		14	47.6 ± 1.0
L-NAME	0.03	14	48.2 ± 0.9
	0.1	14	47.7 ± 1.1
	0.3	14	47.0 ± 1.0
L-NAME 0.3 + L-Arg 5.0		14	48.5 ± 1.1
Saline		14	46.7 ± 1.2
L-NNA 0.3		14	46.2 ± 1.1
L-NNA 0.3 + L-Arg 5.0		14	48.5 ± 1.0

Each value represents the mean ± S.E.M. There were no differences in ischemic area between saline- and drug-treated groups.

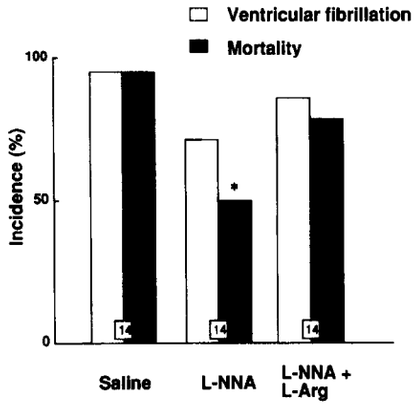

Fig. 3. Effects of N^G-nitro-L-arginine (L-NNA) and the combination of L-NNA and L-arginine (L-Arg) (L-NNA + L-Arg) on the incidence of ventricular fibrillation and mortality upon reperfusion in anesthetized rats. L-NNA and L-NNA + L-Arg were continuously infused into the femoral vein from 4 min before left coronary artery occlusion to the end of 4 min reperfusion. * $P < 0.05$ vs. saline.

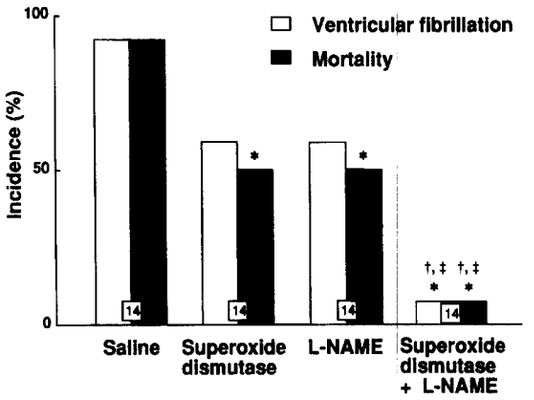

Fig. 4. Effects of treatment with superoxide dismutase and L-NAME on the ventricular fibrillation and mortality upon reperfusion in anesthetized rats. Superoxide dismutase (4300 U/kg/min), L-NAME (0.3 mg/kg/min) and superoxide dismutase (4300 U/kg/min) + L-NAME (0.3 mg/kg/min) were continuously infused into the femoral vein from 4 min before left coronary artery occlusion to the end of 4 min reperfusion. * $P < 0.05$ vs. saline, † $P < 0.05$ vs. superoxide dismutase, ‡ $P < 0.05$ vs. L-NAME.

Table 5
Systolic blood pressure and heart rate of coronary artery-occluded rats treated with either saline, superoxide dismutase, L-NAME or superoxide dismutase + L-NAME

Treatment	Number of rats	Systolic blood pressure (mm Hg)				Heart rate (beats/min)			
		Initial	Before occlusion	Time after occlusion		Initial	Before occlusion	Time after occlusion	
				1 min	4 min			1 min	4 min
Saline	14	102 ± 4	102 ± 5	86 ± 6 [a]	96 ± 6	393 ± 9	389 ± 7	405 ± 14	402 ± 12
Superoxide dismutase	14	98 ± 4	98 ± 5	85 ± 8 [a]	95 ± 7	383 ± 8	375 ± 10	381 ± 12	380 ± 12
L-NAME	14	110 ± 6	122 ± 6 [a]	103 ± 7 [a]	113 ± 7	400 ± 13	387 ± 16 [a]	391 ± 17	391 ± 15
Superoxide dismutase + L-NAME	14	99 ± 5	107 ± 8 [a]	87 ± 10 [a]	104 ± 9	388 ± 10	376 ± 11 [a]	373 ± 13 [a]	369 ± 12 [a]

Each value represents the mean ± S.E.M. Saline, superoxide dismutase (4300 U/kg/min), L-NAME (0.3 mg/kg/min) and superoxide dismutase (4300 U/kg/min) + L-NAME (0.3 mg/kg/min) were continuously infused into the femoral vein from 4 min before occlusion to the end of 4 min reperfusion.
[a] $P < 0.05$ vs. initial.

Table 6
Ischemic area of coronary artery-occluded rats treated with either saline, superoxide dismutase, L-NAME or superoxide dismutase + L-NAME

Treatment	Number of rats	Ischemic area (% of total heart wt)
Saline	14	47.8 ± 0.8
Superoxide dismutase	14	48.1 ± 1.1
L-NAME	14	47.5 ± 1.0
Superoxide dismutase + L-NAME	14	48.9 ± 1.1

Each value represents the mean ± S.E.M. There were no differences in ischemic area between saline- and drug-treated groups.

mg/kg/min) were continuously infused into the femoral vein from 4 min before coronary artery occlusion to the end of 4 min reperfusion. Fig. 4 shows the effects of superoxide dismutase, L-NAME and superoxide dismutase + L-NAME on the incidence of ventricular fibrillation and mortality upon reperfusion in anesthetized rats. Superoxide dismutase, L-NAME and superoxide dismutase + L-NAME significantly reduced the mortality. The incidence of ventricular fibrillation and mortality of the animals treated with superoxide dismutase + L-NAME were significantly lower than those of the animals treated with either superoxide dismutase or L-NAME alone.

Table 5 shows the effects of these agents on systolic blood pressure and heart rate at initial, preocclusion, and 1 and 4 min of occlusion. Systolic blood pressure was increased by the infusion of L-NAME and superoxide dismutase + L-NAME. Heart rate was decreased by the infusion of L-NAME and superoxide dismutase + L-NAME. There were no differences in the ischemic area among these groups at the end of reperfusion (Table 6).

4. Discussion

In the present study, superoxide dismutase, a scavenger of O_2^-, reduced the incidence of reperfusion-induced ventricular fibrillation and mortality, whereas catalase, a scavenger of H_2O_2, or dimethylthiourea, a scavenger of $\cdot OH$, did not reduce these arrhythmias in ischemic/reperfused hearts. The observations concerning the effects of superoxide dismutase, catalase and dimethylthiourea on ischemia/reperfusion-induced arrhythmias are in agreement with our previous reports (Ohoi et al., 1993a) and others (Woodward and Zakaria, 1985; Da-Luz et al., 1993). In contrast, others have shown that superoxide dismutase does not reduce reperfusion arrhythmias (Hagar et al., 1991; Tosaki et al., 1993), while catalase (Manning et al., 1988) and dimethylthiourea (Bolli et al., 1987) reduced reperfusion arrhythmias. Thus, a substantial role of reactive oxygen species remains controversial (Tosaki and Das, 1994; Euler, 1994), as described in the Introduction. The present results support the hypothesis that O_2^- induces the genesis of reperfusion-induced arrhythmias.

The major findings in the present study were that NO synthase inhibitors, L-NAME and L-NNA, reduced the incidence of ventricular fibrillation and mortality during reperfusion. Furthermore, this reduction in arrhythmogenesis by the NO synthase inhibitors was almost completely reversed by administration of L-Arg. The results suggest that reperfusion-induced arrhythmias in the rat in vivo are elicited, at least in part, related to NO.

Pabla and Curtis (1995) reported that L-NAME increased the incidence of ventricular fibrillation in isolated perfused rat hearts reperfused after 60 min of ischemia, but L-NAME did not increase it 5 or 35 min after ischemia. Furthermore, Pabla et al. (1995) suggested that cardiac cGMP functions as an endogenous protectant against reperfusion-induced ventricular fibrillation in hearts following 60 min ischemia. Their observations appear to be discrepant with ours. There is, however, a distinct difference in experimental conditions between the present study that L-NAME reduced reperfusion-induced arrhythmias and their study that L-NAME aggravated them (Pabla and Curtis, 1995; Pabla et al., 1995): the ischemic period of the present study was 4 min, on the other hand, that of their study was 60 min. Therefore, it seems likely that NO exerts deleterious effects on hearts reperfused after a brief ischemia, but it exerts protective effects on hearts reperfused after a sustained ischemia.

In agreement with the above hypothesis, conflicting

results have been reported concerning a role of NO in ischemia/reperfusion injury of the heart in vivo and in vitro. Several investigators reported observations which support the hypothesis that NO plays a deleterious role in ischemia/reperfusion injury. That is, Naseem et al. (1995) showed the improvement of myocardial function and reduction in the incidence of reperfusion arrhythmias by sustained inhibition of NO formation by L-NNA in isolated perfused rat hearts. Schulz and Wambolt (1993) reported that L-NAME and N^G-monomethyl-L-arginine (L-NMMA) prevented myocardial ischemia/reperfusion injury in isolated working rat hearts. Matheis et al. (1992) showed that L-NAME improved ischemia/reperfusion injury in in vivo piglets and suggested that the L-Arg-NO pathway contributes to the injury. In contrast, other investigators showed cardioprotective effects of NO in ischemic/reperfused heart. Johnson et al. (1990) have shown that acidified sodium nitrite, a releaser of NO, protected the cat myocardium from ischemia/reperfusion injury in vivo. Parratt (1993) reported the reduction in the severity of ischemia-induced ventricular arrhythmias by NO donors, such as glyceryl trinitrate and molsidomine. Wang and Morgan (1993) demonstrated the protective role of NO in myocardial reoxygenation injury in ferrets. Since NO is a free radical, the overall effect whether protective or injurious may depend on the source and half life of NO produced under specific experimental conditions. Thus, it would be premature to address the substantial role of NO in ischemia/reperfusion injury. In this context, the hypothesis of Lipton et al. (1993) that the action of NO is correlated with the redox state of nitrogen monoxide, NO ($NO^{\cdot}$) or nitrosonium ion (NO^+), should be noted. It seems likely, therefore, that the opposing effects of NO may be related to the experimental models, perfusion media and experimental conditions employed.

In the present study, we used NO synthase inhibitors at doses of 0.03–0.3 mg/kg/min, which were lower than those of other investigators (Patel et al., 1993; Vegh et al., 1992). Rees et al. (1990) have shown that hypertensive effects of L-NAME were observed at doses of > 0.3 mg/kg i.v. in rat, which was similar to the dose used in the present study. This suggests that L-NAME in this dose range is capable of inhibiting NO synthesis in endothelial cells under in vivo conditions. In contrast, high doses of NO inhibitors might reduce myocardial microvascular circulation and thereby aggravate myocardial function due to oligemia or ischemia caused by a profound inhibition of endogenous NO generation in vascular endothelial cells. In fact, L-NAME at doses of 30–100 mg/kg i.v. has been suggested to be capable of blocking microvascular circulation in rats (Rees et al., 1990). Recently, in the perfused rabbit heart, Depré et al. (1995) showed that nonvasoactive concentrations of L-NAME and L-NMMA protect the heart against ischemic damage. This is the reason why relatively low doses of NO synthase inhibitors were employed in the present study. As described in the Materials and methods, protection of ischemia/reperfusion-induced cerebral infarction was also achieved by L-NAME or L-NNA at doses similar to those in the present study.

The relatively low doses of L-NAME produced a small increase in systemic blood pressure and a decrease in heart rate. It is noted that these changes were not reversed by treatment with 5.0 mg/kg/min of L-Arg, 17-fold greater than the dose of L-NAME. This finding is in contrast with the complete reversal of reperfusion arrhythmias by L-Arg. A similar ineffectiveness of L-Arg on L-NAME-induced changes in hemodynamics of the in vivo rat has been documented (Rees et al., 1990); that is, changes in heart rate and blood pressure induced by 1 mg/kg of L-NAME was not reversed by treatment with 30 mg/kg L-Arg, 30-fold greater than the dose of L-NAME. These two findings suggest that the action of the NO synthase inhibitors on reperfusion arrhythmias differs from that on blood pressure and heart rate.

It is possible that reperfusion arrhythmias are influenced by restriction of coronary flow after reperfusion, particularly when agents have an ability to alter coronary flow and/or reactive hyperemia. It is not possible to deduce from the results of the present study whether coronary flow of the rat treated with NO synthase inhibitors is adequately restored following reperfusion. In a preliminary study, we examined the extent of reperfusion in the myocardium of rats treated with 0.3 mg/kg i.v. of L-NAME by Evans blue staining method and found that L-NAME treatment did not interfere the restoration of coronary flow after reperfusion. The perfusion areas after reperfusion in all the coronary artery-occluded animals were also confirmed by gloss examinations of a change in ventricular surface color from cyanosis to hyperemia and the myocardial slices on the basis of the presence of clots when reperfusion was inadequate. Although we observed thorough reperfusion of the ischemia-induced region of the animal under the present experimental conditions, these methods are not sufficient to prove this completely. It should be noted, however, that the dose of L-NAME in the present study was relatively low as compared with that of other investigators. This dose (0.3 mg/kg i.v.) of NO synthase inhibitors has been demonstrated to exert relatively minor effects on blood pressure and heart rate in normal rats (Rees et al., 1990). This suggests that a sustained constrictive effect will not be seen during reperfusion in L-NAME- or L-NNA-treated animals under the present experimental conditions.

In the present study, L-NAME at 0.3 mg/kg/min, the highest dose in the present study, induced bradycardia. Heart rate is considered to be an important determinant of the susceptibility to reperfusion-induced arrhythmias. As bradycardia during ischemia protects reperfusion-induced arrhythmias (Bernier et al., 1989), the decrease in heart rate by L-NAME at 0.3 mg/kg/min may contribute to the protection of L-NAME against reperfusion-induced arrhythmias.

We observed the additive effects of superoxide dismutase and L-NAME on reperfusion arrhythmias in the present study. That is, the reperfusion arrhythmias were abolished almost completely by treatment with a combination of superoxide dismutase and L-NAME. The result suggests that both O_2^- and NO play a critical role in the reperfusion arrhythmias of the in vivo rat under the present experimental conditions. We did not further address the exact role of these two factors in reperfusion arrhythmias. An increase in NO production during reperfusion has been demonstrated in the heart in vivo (Morita et al., 1994) and in vitro (Pabla and Curtis, 1995). In the same model as in the present study, generation of O_2^- was observed (Ohoi et al., 1993b). Thus, it may be concluded that O_2^- and NO may act separately or concertedly to induce reperfusion arrhythmias in the present study.

Recently, Beckman et al. (1990) suggested that O_2^- and NO rapidly react with each other, yielding peroxynitrite ($ONOO^-$), and that this reactive oxygen species is strongly active in multiple oxidative pathways (Beckman et al., 1990; Yu, 1994). Although we have not substantiated the production of $ONOO^-$ under the present experimental conditions, it is possible that $ONOO^-$ plays a role in reperfusion arrhythmias in the in vivo rat heart.

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