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Cardioprotective Effect of Succinate Against Ischemia/Reperfusion Injury

MASATO SAKAMOTO[1], KOICHIRO TAKESHIGE[2], HISATAKA YASUI[1], and KOUICHI TOKUNAGA[1]

[1] Division of Cardiovascular Surgery, Research Institute of Angiocardiology and [2] Department of Biochemistry, Faculty of Medicine, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-82, Japan

Abstract: We investigated the protective effects of succinate, which is a respiratory substrate and a potential antioxidant, on myocardial ischemia/reperfusion injury with the whole heart. Isolated rat hearts were loaded with 25-min normothermic global ischemia followed by 30-min reperfusion in a working heart model. Succinate administered either before reperfusion or added to the cardioplegic solution improved the postischemic cardiac function significantly. The hearts arrested with succinate-supplemented cardioplegic solution replenished high-energy phosphates and maintained the total adenine nucleotides during the reperfusion period, whereas those arrested with succinate-nonsupplemented cardioplegic solution replenished the high-energy phosphates less, and also lost total adenine nucleotides during that period. We thus conclude that succinate administered before reperfusion may decrease the degree of mitochondrial damage during reperfusion and thereby reduce the amount of myocardial ischemia/reperfusion injury.

Key Words: reperfusion injury, high-energy phosphate, free radical, cardioplegia

Introduction

There is now accumulating evidence that oxygen free radicals contribute to myocardial damage incurred by ischemia and reperfusion.[1,2] The mitochodria contain high concentrations of unsaturated fatty acids, which are sensitive to free radicals. Therefore, the mitochondria are among the most vulnerable organelles to oxygen free radicals. We have previously reported the mechanism of the reduced nicotinamide adenine dinucleotide (phosphate) [NAD(P)H]-dependent lipid peroxidation of the mitochondria in vitro using the submitochondrial particles of the bovine heart.[3–5] In this system, succinate strongly inhibited the NAD(P)H-dependent lipid peroxidation by reducing ubiquinone.[3] Ubiquinone is one of the components of the mitochondrial respiratory chain with an antioxidant effect.[6,7] Although the oxidized form of ubiquinone converts to polar substances by a reaction with lipid peroxides, it is rereduced in the presence of an appropriate electron donor and replenishes the antioxidant effect.[4]

Ischemia followed by reperfusion is known to impair the intracellular antioxidant system.[8] This impairment may be one of the main causes in myocardial ischemia/reperfusion injury.[8] If mitochondrial lipid peroxidation is responsible for the injury, succinate which inhibits mitochondrial lipid peroxidation in vitro may thus prevent such an injury. We show in the present study, using an isolated rat-heart model, that succinate actually improves the postischemic energy metabolism and the postischemic cardiac functions in the whole heart exposed to an ischemia/reperfusion insult. These beneficial effects of succinate also appear to occur during the reperfusion period.

Materials and Methods

Isolated Working Rat-Heart Models

Seventy-five male WKA rats weighing 250–300 g were used in the present study. The method for the isolated working rat heart model was previously reported by our colleagues.[9] The rats were anesthetized with intraperitoneal pentobarbital in a dose of 100 mg/rat. Heparin in a dose of 2 mg/rat was injected intravenously. The heart was quickly excised and immersed into a cold perfusion medium on ice. The main pulmonary artery was incised to drain coronary effluent. The aorta and the left atrium were then cannulated. After 10 min of a non-working Langendorff-mode perfusion with a perfusion pressure of 80 cmH_2O, the heart was converted into a working

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mode perfusion with a left atrial filing pressure of 16 cmH_2O, so as to eject fluid against a hydrostatic pressure of 80 cmH_2O. After 10 min of the working mode, both the coronary and aortic flow rates (ml/min) were measured by a timed collection. The cardiac output was determined by the sum of the aortic and coronary flow rates. The aortic pressure was monitored via the side arm of the aortic cannula with a pressure transducer (Statham model P231D, Gould Oxnard, CA, USA) and was continuously recorded (Recti-Horiz-8K, NEC San-ei, Tokyo, Japan). The heart rate was determined from the pressure record. At the end of the 20 min of preischemic perfusion, the aortic and left atrial lines were clamped, and 6 ml of cardioplegic solution was injected into the aortic root through the side branch of the aortic cannula. Thus complete and global ischemia was induced and maintained for the next 25 min. During ischemia, the heart was kept in a temperature-controlled, water-jacketed chamber. The temperature in the chamber was strictly maintained at 37°C. Reperfusion was started with the perfusion in the Langendorff mode for 5 min, followed by the working mode for 25 min. Recovery of cardiac functions was examined at 30 min of reperfusion. The indexes of the cardiac functions were expressed as a percentage of the preischemic control value.

Perfusate and Cardioplegic Solution

As a standard perfusion medium, we used modified Krebs-Henseleit bicarbonate solution (KHB) containing Na^+ 143 mM; K^+ 5.9 mM; Ca^{2+} 2.5 mM; Cl^- 126.7 mM; HCO_3^- 25 mM; SO_4^{2-} 1.2 mM; $H_2PO_4^-$ 1.2 mM; and glucose 11 mM. This solution was gassed with 95% oxygen and 5% carbon dioxide, and fluid temperature was maintained at 37°C. Sodium succinate was added to this solution at various concentrations. The concentration of sodium ion and the osmotic pressure in the solution containing succinate was adjusted by sodium chloride and glucose. As a standard cardioplegic solution, we used Kyushu University Cardioplegic Solution, which both was developed and is used clinically at our institution.[10] The composition of the solution was as follows: K^+ 20 mM; Na^+ 80 mM; Cl^- 90 mM; HCO_3^- 10 mM; and glucose 140 mM. The sodium concentration and the osmotic pressure were also adjusted with sodium chloride and glucose when succinate was added.

Myocardial Metabolites

For the analysis of myocardial metabolites, the heart was rapidly frozen with a Wollenberger clamp precooled in liquid nitrogen. The frozen sample was then analyzed to measure the contents of adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), and creatine phosphate (CrP). These phosphate compounds were measured by high-performance liquid chromatography[11,12] (Shimadzu, Kyoto, Japan). The total number of adenine nucleotides (TAN) was determined as the sum of ATP, ADP, and AMP. High-energy phosphate (HEP) was determined as 2ATP + ADP + CrP. The values of metabolite content were expressed as μmol/g dry wt.

Experimental Design

Protocol 1 (Fig. 1)
In order to estimate the dose-dependency of succinate in the myocardial protection against the ischemia/reperfusion insult, we examined the cardiac function recovery at 30 min of reperfusion after the normothermic global ischemia for 25 min, by administering succinate into the perfusate at a concentration of 0, 1, 3, 5, 10, or 20 mM.

Protocol 2 (Table 1)
We next estimated the influence of the timing of succinate administration on the myocardial protective effects, and the effect of succinate-supplemented cardioplegic solution under the consideration of clinical application. Five groups were studied. In group 1 (n = 6), KHB without succinate was used for the perfusion during both the preischemic and reperfusion periods; in group 2 (n = 6), KHB added with succinate was used during the preischemic period alone; in group 3 (n = 5), KHB added with succinate was used during the reperfusion period alone; in group 4 (n = 7), KHB added with succinate was used during both the

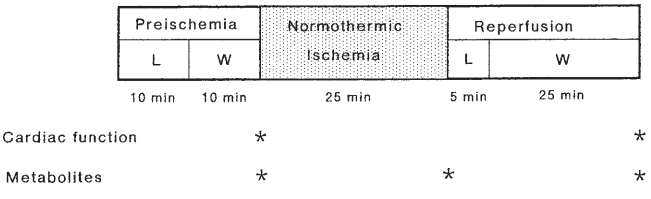

Fig. 1. Experimental protocol. *L*, Langendorff mode; *W*, Working mode. *Asterisks* denote sampling points

Table 1. Succinate supplement for each group in protocol 2

	Preischemic perfusate	Reperfusion perfusate	Cardioplegic solution
Group 1	−	−	−
Group 2	+	−	−
Group 3	−	+	−
Group 4	+	+	−
Group 5	−	−	+

preischemic and reperfusion periods. The hearts of groups 1 to 4 were arrested with the cardioplegic solution without succinate. In group 5 ($n = 6$), KHB without succinate was used for perfusion during both the preischemic and reperfusion periods, but the hearts were arrested with the succinate-supplemented cardioplegic solution. To estimate the myocardial energy state in the hearts of group 1 and group 5, the contents of myocardial metabolites was measured. The hearts for the analysis of myocardial metabolites at the end of the reperfusion period were the same hearts used to estimate the cardiac function recovery. The hearts for analyzing myocardial metabolites before ischemia and at the end of the ischemic period were sampled in the other experiments. Since the hearts of groups 1 and 5 were treated by the same method before ischemia, the hearts for the preischemic myocardial metabolites were sampled at the end of the preischemic period.

All animals used in this study recieved humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society of Medical Research, and the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by National Institute of Health (NIH publication No. 80-23, revised 1978).

Statistics

All data are presented as the mean ± SEM. The intergroup comparisons were made first, by one-way analysis of variance. Only when the variance was significant, the unpaired Student's *t*-test with Bonferroni correction was employed for multiple comparisons. Intragroup comparisons were made by the unpaired Student's *t*-test. Differences were considered significant when $P < 0.05$.

Results

Dose-dependency of Succinate Contained in the Perfusate

There were no significant differences in the preischemic control values of cardiac functions among the groups perfused with the KHB containing various concentrations of succinate (data not shown). The curves of the percent recovery in the aortic flow and the cardiac output showed that succinate was effective in preventing the ischemia/reperfusion damage when it was added at a concentration of 3 mM or more (Fig. 2). The percent recovery in the aortic flow and the cardiac output at a concentration of 10 mM was 77.8% ± 2.1% and 79.6% ± 1.9%, respectively. These values were significantly better than those in the succinate-free group, in which

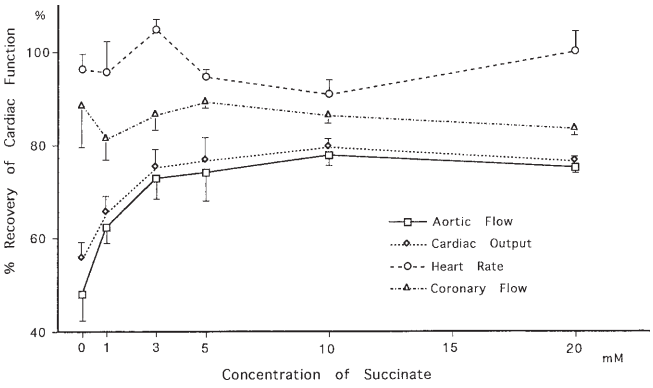

Fig. 2. Percent recovery of cardiac functions after ischemia at various concentrations of succinate

the aortic flow and the cardiac output were 47.9% ± 5.5% and 55.6% ± 3.6% ($P < 0.01$), respectively. The heart rate recovered completely at any concentration of succinate. The postischemic coronary flow decreased by approximately 15% compared with the preischemic value, but there were no significant differences among the groups perfused with the KHB containing various concentrations of succinate. From these results, we concluded that the optimal concentration of succinate was 10 mM. Thus, in the experiment in protocol 2, succinate was added to KHB or cardioplegic solution at this concentration.

Optimal Timing for Succinate Administration and the Effect of Succinate-Supplemented Cardioplegic Solution

The timing of succinate administration did not influence the postischemic recovery of the heart rate or coronary flow (Fig. 3). The preischemic administration of succinate, which was performed in groups 2 and 4, significantly improved the postischemic recovery in the aortic flow from 47.9% ± 5.5% (group 1) to 75.0% ± 3.4% (group 2) ($P < 0.01$ *vs* group 1), or to 77.8% ± 2.3% (group 4) ($P < 0.01$ *vs* group 1), and in the cardiac output from 55.6% ± 3.6% (group 1) to 75.3% ± 2.9% (group 2) ($P < 0.05$ *vs* group 1), or to 79.6% ± 1.9% (group 4) ($P < 0.01$ *vs* group 1). Although succinate was added to the perfusate not only in the preischemic period but also in the postischemic period in group 4, no further improvement in cardiac functions was observed in group 4 compared with group 2. In addition, the hearts arrested with the cardioplegic solution supplemented with succinate at a concentration of 10 mM (group 5) showed significantly better postischemic recoveries of aortic flow and cardiac output, which were

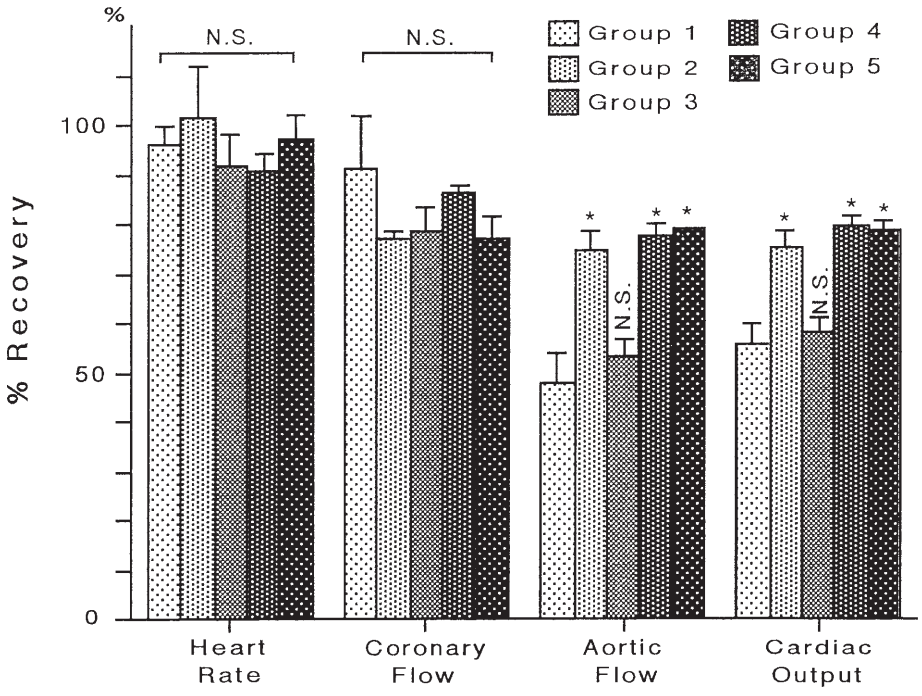

Fig. 3. Postischemic recovery following succinate administration. *$P <$ 0.01

Table 2. Contents of myocardial metabolites

	ATP	TAN	CrP	HEP
Preischemia				
Groups 1 and 5	29.1 ± 0.6	38.6 ± 0.3	45.0 ± 3.6	110.7 ± 4.5
End of ischemia				
Group 1	12.0 ± 1.2*	29.5 ± 1.7*	12.8 ± 1.7*	46.9 ± 2.8*
P	NS	NS	NS	NS
Group 5	12.1 ± 0.6*	28.1 ± 2.2*	11.7 ± 0.3*	47.3 ± 2.2*
30 min of reperfusion				
Group 1	17.4 ± 0.9*‡	24.3 ± 1.0*§	44.5 ± 1.8‡	84.8 ± 2.5*‡
P	<0.01	<0.01	<0.01	<0.01
Group 5	20.6 ± 0.5*‡	28.1 ± 0.6*	57.4 ± 3.0†‡	104.8 ± 3.4‡

A unit of each content is μmol/g dry wt
ATP, Adenosine triphosphate; TAN, adenine nucleotides; CrP, creatine phosphate; HEP, high-energy phosphate; NS, not significant
P-value in the table is that compared between Group 1 and Group 5
*,†,‡,§ indicate the *P*-values for within-group comparisons:
* $P < 0.01$ compared to preischemia; † $P < 0.05$ compared to preischemia; ‡ $P < 0.01$ compared to the end of ischemia; § $P < 0.05$ compared to the end of ischemia

79.2% ± 1.3% ($P < 0.01$ *vs* group 1) and 78.9% ± 1.6% ($P < 0.01$ *vs* group 1), than those in group 1. When succinate was administered only during the postischemic period (group 3), no improvement in the postischemic cardiac functions could be observed.

Myocardial Metabolites

ATP, TAN, CrP, and HEP were all decreased significantly by ischemia (Table 2, Fig. 4). All of them except TAN recovered significantly during the reperfusion period, but the ATP level still remained lower than that of the preischemic period. In the within-group comparison, differences of group 1 and group 5 were found in the change of TAN, CrP, and HEP during the reperfusion period. In group 1, the myocardial content of TAN decreased significantly, that of CrP replenished to the preischemic level, and that of HEP incompletely replenished during the reperfusion period. While in group 5 no significant decrease of the content of TAN

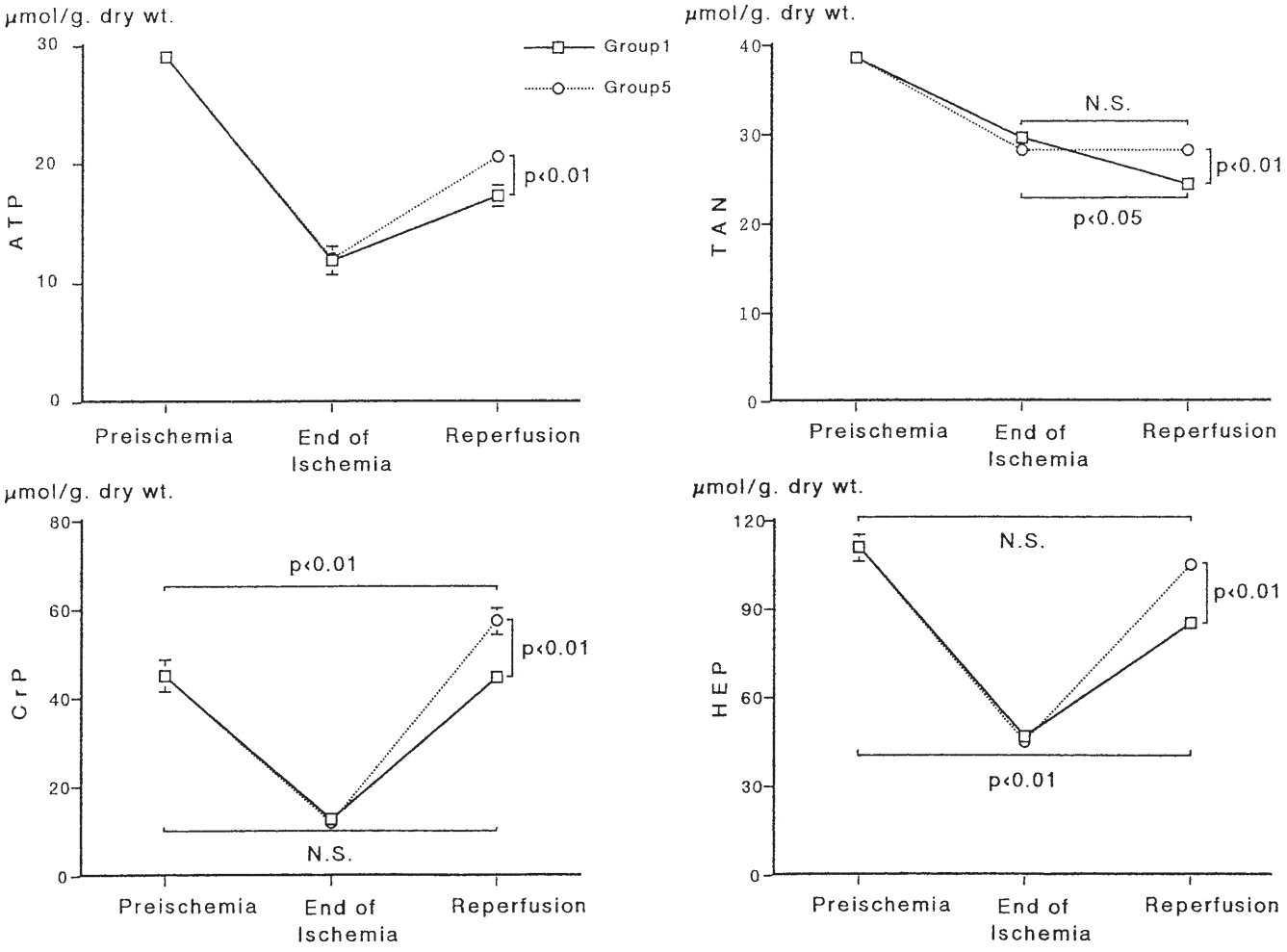

Fig. 4. Myocardial energy state of the hearts of group 1 and group 5 at preischemia, the end of ischemia, and the end of reperfusion

was recognized, the content of CrP replenished to a significantly higher level than the preischemic level, while that of HEP completely recovered.

In the between-group comparisons, no differences were found for any indexes at the end of the ischemic period, but at the end of the reperfusion period all indexes of group 5 were significantly higher than those of group 1.

Discussion

In this study we investigated the effects of succinate, which inhibits mitochondrial lipid peroxidation in vitro,[3,13,14] on ischemia/reperfusion injury of the whole heart. We found that the myocardial dysfunction caused by normothermic ischemia for 25 min followed by reperfusion for 30 min was significantly suppressed with succinate. The administration of succinate either to the perfusion medium or to the cardioplegic solution thus improved the recovery of the myocardial functions and energy metabolism. The improvement of the postischemic cardiac functions was dependent on the dose of succinate added to the perfusion medium. The administration of succinate to the cardioplegic solution accelerated the replenishment of the high-energy phosphates especially during the reperfusion period, because we could not find any difference in the tissue contents of the high-energy phosphates by the end of the ischemic period (Fig. 4).

In the within-group comparison of TAN, TAN decreased significantly during the reperfusion period in group 1, while in group 5 a decrease of the content of TAN during the reperfusion period was not found. Regarding the between-group comparisons, the hearts of group 5 had significantly more TAN than those of group 1 at 30 min of the reperfusion period. These facts suggest that the energy demand exceeds the energy supply during the reperfusion period, and that the myocardium consumes the energy from the degradation of adenine

nucleotides in group 1; the energy supply from the mitochondria is thus better in the hearts of group 5 than in those of group 1. This hypothesis was supported by the change of CrP. The content of CrP replenished to the level of the preischemic period during the reperfusion period in group 1, whereas it replenished to over the preischemic level in group 5. These findings suggest that the energy-producing system of the mitochondria, which is the phosphate donor to CrP, is thus preserved better in group 5 than in group 1 during the ischemia/reperfusion period. Furthermore, in the between-group comparison, all energy metabolism indexes of group 5 were significantly higher than those of group 1 at 30 min of reperfusion, although we could not find any difference at the end of ischemia. This result suggests that the administered succinate protects the myocardium through the preservation of the mitochondrial function during the whole ischemia/reperfusion period, but not through the preservation of the high-energy phosphate during the ischemic period.

When the myocardium is being precipitated in the ischemic condition, iron and the reduced substrates including NADH are accumulating.[15,16] NADH is the main electron donor to the electron transport system of the mitochondrion, and can also sustain lipid peroxidation in cooperation with ferric ion and ADP.[3] Once lipid peroxidation occurs in the mitochondria, NADH-ubiquinone (CoQ) reductase is impaired, as NADH-CoQ reductase is vulnerable to lipid peroxidation.[5] The impairment of NADH-CoQ reductase after ischemia and reperfusion is supported by the report of Marubayashi et al., in which they found that the oxidized form of ubiquinone was predominant in the reperfused liver after 90 min of ischemia.[17] The impairment of NADH-CoQ reductase may thus cause a leakage of electrons from the electron transport system of mitochondria, which forms superoxide radicals and thus exacerbases the impairment of mitochondria by lipid peroxidation, and inhibits the rereduction of the oxidized form of ubiquinone.[3,4,5] Ubiquinone is not only one of the components of the mitochondrial electron transport system, but also one of the important intracellular antioxidants. Its antioxidant effect is stronger as the reduced form than as the oxidized form.[6,7] When the rereduction of the oxidized form of ubiquinone is inhibited, the oxidized form is converted to a polar substance after the reaction with lipid peroxides and thus loses its antioxidant effect.[5] This leads to the deterioration of the intracellular antioxidant system. Thus, the reperfusion after ischemia provides a suitable condition for lipid peroxidation in the mitochondrion of the myocyte.

We have previously shown in in vitro studies that succinate increases the antioxidant effect of ubiquinone by reducing it, and strongly inhibits the NAD(P)H-dependent lipid peroxidation of mitochondria.[3] Succinate-CoQ reductase is more tolerant toward lipid peroxidation than NADH-CoQ reductase.[5] The administered succinate can be expected to inhibit the lipid peroxidation of mitochondria by the reduction of oxidized ubiquinone even at the condition after ischemia following reperfusion where NADH-CoQ reductase is impaired, and thus is effective against ischemia/reperfusion injury. We have also confirmed that succinate did not show any protective effect on ubiquinone-depleted submitochondrial particles, and that an inverse correlation has been found between the rate of peroxidation and the level of reduced ubiquinone.[3,4] We therefore investigated the myocardial protective effect of succinate against the ischemia/reperfusion insult. As mentioned above, we found that succinate had preserved the function of mitochondria well, and suppressed the impairment of the myocardial function induced by ischemia following reperfusion. However, we could not find any difference in the myocardial content of malondialdehyde (MDA), which is the side product of the reaction of lipid peroxidation, measured by TBA method and HPLC (data not shown). The measurement of MDA, which has been shown to be a nonspecific index of lipid peroxidation,[18] may be adequate as an index of lipid peroxidation of purified organelle, but inadequate for a whole organ which contains various impurities.

The beneficial effects of succinate against oxygen toxicity have been also confirmed by in vivo experiments. Block has reported that pretreatment with succinate reduces the occurrence of seizure of rats caused by hyperbaric oxygen.[19] Ronai et al. reported that pretreatment with succinate inhibits the lipid peroxidation of mouse liver mitochondria induced by irradiation.[20] Furthermore, in the field of myocardial protection it has been reported that some intermediate metabolites of the TCA cycle and the various amino acids, which convert to succinate as the end product during ischemia, have a myocardial protective effect.[21,22] Many of these reports have laid special emphasis on substrate phosphorylation during ischemic time or on the supplement of the TCA cycle intermediates as being the basic mechanism of the beneficial effects of those additives. Succinate is the end product of those intermediates or amino acids, and has no potential to produce ATP by substrate phosphorylation or to supply intermediates to the TCA cycle. This report may thus provide new and important information on the efficacy of these substances.

References

1. Hess ML, Manson NH (1984) Molecular oxygen: friend and foe. The role of the oxygen free radical system in the calcium paradox,

the oxygen paradox and ischemia/reperfusion injury. J Mol Cell Cardiol 16:969–985
2. Marklund S (1988) Role of toxic effects of oxygen in reperfuion damage. J Mol Cell Cardiol 20 (Supple 2):23–30
3. Takayanagi R, Takeshige K, Minakami S (1980) NADH- and NADPH-dependent lipid peroxidation in bovine heart submitochondrial particles. Dependence on the rate of electron flow in the respiratory chain and an antioxdant role of ubiquinol. Biochem J 192:853–860
4. Takeshige K, Takayanagi R, Minakami S (1980) Lipid peroxidation and the reduction of ADP-Fe^{3+} chelate by NADH-ubiquinone reductase preparation from bovine heart mitochondria. Biochem J 192:861–866
5. Narabayashi H, Takeshige K, Minakami S (1982) Alteration of innermembrane components and damage to electron-tranfer activities of bovine heart submitochondrial particles induced by NADPH-dependent lipid peroxidation. Biochem J 202:97–105
6. Booth RFG, Galanopoulou DG, Quinn PJ (1982) Protection by ubiquinone and ubiquinol against lipid peroxidation in egg yolk phosphatidylcholine liposomes. Biochem Int 5:151–156
7. Mellors A, Tappel AL (1966) The inhibition of mitochondrial peroxidation by ubiquinone and ubiquinol. J Biol Chem 241: 4353–4356
8. Shlafer M, Meyers CL, Adkins S (1987) Mitochondrial hydrogen peroxide generation and activities of glutathione peroxides and superoxides dismutase following global ischemia. J Mol Cell Cardiol 19:1195–1206
9. Kohno H, Shiki K, Ueno Y, Tokunaga K (1987) Cold storage of the rat heart for transplantation. J Thorac Cardiovasc Surg 93:86–94
10. Kinoshita K, Oe M, Tokunaga K (1991) Superior protective effect of low calcium, magnesium-free potassium cardioplegic solution on ischemic myocardium: clinical comparison with St. Thomas' Hospital solution. J Thorac Caardiovasc Surg 101:432–445
11. Kamiike W, Watanabe F, Hashimoto T, Tagawa K, Ikeda Y, Nakao K, Kawashima Y (1982) Changes in cellular levels of ATP and its catabolites in ischemic rat liver. J Biochem 91:1349–1356
12. Juengling E, Kammermeier H (1980) Rapid assay of adenine nucleotides or creatine compounds in extracts of cardiac tissue by paired-ion reverse-phase high-performance liquid chromatography. Anal Biochem 102:358–361
13. Meszaros L, Tihany K, Horvath I (1982) Mitochondrial substrate oxidation-dependent protection against lipid peroxidation. Biochim Biophys Acta 713:675–677
14. Bindoli A, Cavallini L, Jocelyn P (1982) Mitochondrial lipid peroxidation by cumene hydriperoxide and prevention by succinate. Biochim Biophys Acta 681:496–503
15. Aust SD, White BC (1985) Iron chelation prevents tissue injury following ischemia. Adv Free Radical Biol Med 1:1–17
16. Hearse DJ, Braimbridge MV, Jynge P (1981) Ischemia and reperfusion: The progression and prevention of tissue injury. In: Hearse DJ, Braimbridge MV, Jynge P (eds) Protection of the ischemic myocardium. Raven, New York, pp 21–49
17. Marubayashi S, Dohi Y, Yamada K, Kawasaki T (1984) Changes in the level of endogenous coenzyme Q homologs, α-tocopherol and glutathione in rat liver after hepatic ischemia and reperfusion, and the effect of pretreatment with Q_{10}. Biochim Biophys Acta 797:1–9
18. Kosugi K, Kikugawa K (1989) Potential thiobarbituric acid-reactive substances in peroxidized lipids. Free Radical Biol Med 7:205–207
19. Block ER (1977) Effect of superoxide dismutase and succinate on the development of hyperbaric oxygen toxicity. Aviat Space Environ Med 48:645–648
20. Ronai E, Tretter L, Szabados G, Horvath I (1987) The inhibitory effect of succinate on radiation-enhanced mitochondrial lipid peroxidation. Int J Radiat Biol 51:611–617
21. Rousou JA, Engelman RM, Anisimowicz L, Lemeshow S, Dobbs WA, Breyer RH, Das DK (1986) Metabolic enhancement of myocardial preservation during cardioplegic arrest. J Thorac Cardiovasc Surg 91:270–276
22. Rosenkranz ER, Okamoto F, Buckberg GD, Robertson JM, Vinten-Johansen J, Bugyi HI (1986) Safety of prolonged aortic clamping with blood cardioplegia. III. Aspartate enrichment of glutamate-blood cardioplegia in energy-depleted hearts after ischemia and reperfusion injury. J Thorac Cardiovasc Surg 91:428–435