Korea J. of Toxicology
Vol. 4, No. 1, pp. 23-35, 1988

ROLE OF CALCIUM IN REPERFUSION DAMAGE OF ISCHEMIC MYOCARDIUM; INFLUENCE ON OXYGEN RADICAL PRODUCTION[1]

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ABSTRACT: *The role of calcium in the production of oxygen radical which causes reperfusion damage of ischemic heart has been examined. The reperfusion damage was induced in isolated Langendorff perfused rat hearts by aortic clamping for 60 min followed by reperfusion with oxygenated Krebs-Henseleit solution with or without 1.25 mM $CaCl_2$.*

On reperfusion of the ischemic hearts with the calcium containing solution, the release of cytosolic enzymes (LDH and CPK) increased abruptly. These increased release of enzymes were significantly inhibited by additions of oxygen radical scavengers (SOD, 5,000 U; catalase, 12,500 U) into the reperfusion solution. In the hearts isolated from rats pretreated with allopurinol(20 mg/kg orally, 24 hr and 2 hr prior to the experiments), the levels of enzymes being released during reperfusion were significantly lower than that of the control. However, in the hearts perfused with the calcium-free but oxygenated solution, the increase in the release of cytosolic enzymes during reperfusion was neither inhibited by oxygen radical scavengers nor by allopurinol pretreatment. For providing the evidence of oxygen radical generation during the reperfusion of ischemic hearts in situ, the SOD-inhibitable reduction of exogenously administered ferricytochrome C was measured. In the hearts perfused with the calcium containing solution, the SOD-inhibitable ferricytochrome C reduction increased within the first minute of reperfusion, and was almost completely inhibited by allopurinol pretreatment. When the heart was perfused with the calcium free solution, however, the reduction of ferricytochrome C was not only less than that in the calcium containing condition, but also was not so completely inhibited by allopurinol pretreatment. By ischemia, xanthine oxidase (XOD) in the ventricular tissue was changed qualitatively, but not quantitatively. In the heart made ischemic with the

1: This work was supported by the research grants from the Korea Science & Engineering Foundation (1987), the Basic Research Grant from College of Medicine, SNU(1987) and the Clinical Research Grant of Seoul National University Hospital (1988)

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calcium containing condition, the oxygen radical producing O-form of XOD increased, while the D- and D/O-form decreased. However, in the ischemic heart reperfused with the calcium free condition, the D/O-form of XOD was elevated without significant increase in O-form of the enzyme.

It is suggested from these results that the calcium may play a contributing role in the genesis of reperfusion damage by promoting the conversion of xanthine oxidase from the D/O-form to the oxygen radical producing O-form in the ischemic myocardium.

Keywords: *Ischemic myocardium, Reperfusion injury, Calcium, Oxygen radical, Xanthine oxidase*

INTRODUCTION

Upon reperfusion of the heart after prolonged ischemia, loss of cellular basement membrane, hypercontracture of cardiomyocyte with sarcolemmal destruction, mitochondrial swelling and eventual cellular necrosis could be developed ("Reperfusion Damage") (Hearse, 1977). One mechanism by which reperfusion of ischemic heart causes myocardial cellular damage may involve oxygen free radicals which are produced from various extra- and intramyocardial cellular sources during the reperfusion of ischemic heart(Hess and Manson, 1984; Simpson and Lucchesi, 1987). Alternatively, there are many supports for the possibility that a number of aspects of ischemic damage and reperfusion injury may be attributed to the uncontrolled oxygen or energy-dependent calcium fluxes(Hearse *et al.*, 1978; Jennings and Ganote, 1976; Park *et al.*, 1975; Shen and Jennings, 1972). It has been reported that calcium channel blockers(Ferrari *et al.*, 1986; Nayler *et al.*, 1985; Sashida and Abiko, 1986) and low calcium perfusion(Kooman *et al.*, 1983 ; Kuroda *et al.*, 1986; Nayler, 1981) prevented the development of ischemic-reperfusion damage in animal heart. Basically, however, the exact role of calcium in the genesis of cellular damage during the reperfusion of ischemic heart has not been clarified yet.

As one of the oxygen radical sources in ischemic-reperfused heart, xanthine oxidase (XOD) system has been focused in several animal species(Chambers *et al.*, 1985; Hearse *et al.*, 1986; Lim and Kim, 1988). Among the three different forms of biologically existing XOD, NAD-dependent dehydrogenase form(D-form), which does not produce oxygen radical, exists in large part in normal animal tissues(Battelli *et al.*, 1972; Kaminski and Jezewska, 1979). However, it has been demonstrated that a brief period of hypoxia causes the enzyme to be converted to an O_2-dependent oxidase form(O-form) which produces oxygen free radicals(Granger *et al.*, 1981, 1986). Furthermore, it has been reported, in the ischemic intestine, that the conversion of XOD could be modified either by protease inhibitors or by Ca^{++}-calmodulin inhibitors (Granger *et al.*, 1986; Roy and McCord, 1983). This may indicate the possibility that XOD conversion could be brought about by calcium-dependent proteolysis.

In the present study, we tried to test the hypothesis that calcium might be involved in the development of reperfusion injury of ischemic heart by participating in oxygen

radical production through the action on xanthine oxidase. Generation of superoxide anion and XOD conversion were examined in isolated, ischemic-reperfused rat heart under the conditions of either the presence or the absence of calcium.

MATERIALS AND METHODS

Adenosine diphosphate(ADP), adenosine monophosphate(AMP), catalase, creatine phosphate(CP), dithiothreitol(DTT), ferricytochrome C(type III), glucose-6-phosphate dehydrogenase(G-6-PDH), hexokinase, lactate dehydrogenase(LDH), nicotinamide adenine dinucleotide(NAD), nicotinamide adenine dinucleotide phosphate(NADP), reduced nicotinamide adenine dinucleotide(NADH), sodium pyruvate, superoxide dismutase(SOD), xanthine were purchased from Sigma Chemical Co.(St. Louis, MO., U.S.A.). Allopurinol(Zyloric) was obtained from Samll Pharmaceutical Co., and other chemicals were of reagent grade.

Preparation of isolated heart and induction of reperfusion damage

Sprague-Dawley rats of either sex, weighing 150-200 g were heparinized intraperitoneally(100 I.U.). Sixty minutes after heparinization, the heart was quickly removed and perfused retrogradely through the aorta at a constant perfusion pressure of 100 cmH_2O(Langendorff preparation). The perfusion solution was Krebs-Henseleit bicarbonate buffer(K-H solution) containing(mM) NaCl, 118; $NaHCO_3$, 27.2; KCl, 4.8; $MgSO_4 \cdot 7H_2O$, 1.2; KH_2PO_4, 1; $CaCl_2$, 1.25; and glucose, 10; and was saturated with a 95% O_2-5% CO_2 gas mixture yielding a pH value of 7.4 at 37°C. The heart was kept in a humidified chamber maintained at 37°C during perfusion. After a 15 min equilibration period, the heart was subjected to global ischemia by a complete stopping of perfusion for 1 hr. During the ischemic period, the heart was immersed in nitrogen saturated hypoxic K-H solution in which glucose was replaced by equimolar concentration of mannitol. After a period of ischemia, the heart was reperfused for 20 min with oxygenated normal K-H solution. For maintaining the heart in calcium-free condition, the heart was perfused with calcium-free K-H solution containing 0.1 mM EDTA for 15 min prior to ischemia. Immersion of the heart during ischemic period and post-ischemic reperfusion were also done with calcium-free K-H solution.

Indices of myocardial cellular damage

Release of cytosolic enzymes, creatine phosphokinase(CPK) and lactic dehydrogenase(LDH), into the coronary effluent was measured as the indicator of myocardial cellular damage. Coronary effluents were collected at indicated time intervals during reperfusion period. After having measured the volume, the samples were kept in ice until the assays were completed(within 8 hrs).

CPK activity was assayed by UV-spectrophotometry(Forster *et al.*, 1974). The reaction mixture contained imidazole, 100 mM(pH 6.9); glucose, 20 mM; $MgCl_2$, 10 mM; ADP, 1 mM; AMP, 10 mM; CP, 20 mM; NADP, 0.7 mM; cysteine HCl, 10 mM; hexokinase, 0.94 U/ml; and G-6-PDH, 0.48 U/ml. The reaction was started by the addition of 0.05 ml of coronary effluent sample into 2.95 ml of the reaction mixture. The rate of change of optical density at 25°C and 340 nm was recorded by UV-Spec-

trophotometer(Perkin-Elmer, Model 139).

LDH activity was assayed by UV-spectrophotometry(Bergmeyer and Bernt 1974). A 0.5 ml aliquot of the sample was added into 2.5 ml of the reaction mixture containing 48 mM phosphate buffer(pH 7.5), 0.6 mM pyruvate and 0.18 mM NADH. The rate of change of optical density was measured by UV-spectrophotometer at 25°C and 340 nm.

To examine the possibility of oxygen radical involvement and the role of xanthine oxidase in reperfusion damage, cardioprotective effects of oxygen radical scavengers and allopurinol were evaluated. Superoxide dismutase(SOD), which eliminates superoxide anion, and catalase, which degrades H_2O_2, were administered through the aortic cannula by using an infusion pump. The infusion was done from 2 min before making the ischemia and throughout the reperfusion period at a rate of 0.5 ml/min. The scavengers were also added to the solution used to immerse the heart during ischemia. Total amount of scavengers administered were 5,000 U for SOD and 12,000 U for catalase. Allopurinol, a specific xanthine oxidase inhibitor, was administered orally(20 mg/kg) two times at 24 hr and 2 hr prior to the experiment. Almost complete inhibition of myocardial xanthine oxidase activity was confirmed in the preliminary experiments with this dose of allopurinol.

Measurement of superoxide anion production

Reduction of exogenously administered ferricytochrome C was used to examine superoxide anion production during the reperfusion of ischemic myocardium(Salin and McCord, 1974). Starting with reperfusion, ferricytochrome C solution either containing SOD(100 U/ml) or not was infused through the aortic cannula at a rate of 0.5 ml/min. The coronary effluent was collected at an interval of 30 sec. Immediately after the volume determination, optical density was measured at 418 nm with UV-spectrophotometer(Cam Spec, Model 301). The extent of ferricytochrome C reduction during reperfusion was calculated by using the difference of molar extinction coefficient($\Delta E_{418} = 7.0 \times 10^4 M^{-1} cm^{-1}$) between reduced ferricytochrome C and oxidized ferricytochrome C. The total amount of ferricytochrome C in the effluent was estimated after full reduction of ferricytochrome C by addition of a few crystals of sodium dithionite. Superoxide anion production was estimated from SOD-inhibitable portion of ferricytochrome C reduction, which was calculated by subtraction of the ferricytochrome C reduction with SOD from that without SOD.

Assay of xanthine oxidase activity

Heart removed from Langendorff apparatus was quickly forzen and powderized in liquid nitrogen. The powdered frozen tissue was homogenized in a 5 vol of the homogenation solution(Tris-HCl, 100 mM; EDTA, 1 mM; DTT, 10 mM; pH, 8.1) with a Polytron tissue disintegrator(Brinkman, U.S.A.). The homogenate was centrifuged for 20 min at 1,000 g and 4°C. The supernatant was removed and recentrifuged for 60 min at 27,000 g and 4°C. The resulting supernatant was fractionated with 3.8 M ammonium sulfate solution. The fraction precipitating in the range of 1.6-2.4 M ammonium sulfate was obtained by refrigerated centrifugation for 10 min at 20,000 g. The pellet was dissolved in 50 mM Tris-HCl buffer(pH 8.0) and used for the assay of xanthine oxidase activity. The standard incubation mixture for the enzyme assay con-

tained 50 mM Tris-HCl buffer(pH 8.0), 60 uM xanthine and the enzyme preparation(0.3-0.5 mg protein / ml) with or without 167.5 uM NAD. The enzyme activity was measured by monitoring the formation of uric acid at 290 nm and of NADH at 340 nm with UV-spectrophotometer(Perkin-Elmer, Model 139). In some experiments, 1.2 U/ml of lactate dehydrogenase and 0.5 mM sodium pyruvate were added to oxidize NADH and thereby preventing the inhibition of the D-form of enzyme(DellaCorte and Stirpe, 1972). The molar absorption coefficient for NADH at 340 nm($E_{340} = 6.22 \times 10^3\ M^{-1}\ Cm^{-1}$) and that for the conversion of xanthine to uric acid at 290 nm(E_{290}, x⟶urate $= 0.85 \times 10^4\ M^{-1}\ Cm^{-1}$) were used for calculation of the enzyme activities. When uric acid formation was measured at 290 nm under the presence of NAD but without LDH and pyruvate, changes in optical density due to the simultaneously produced NADH($E_{290,\ NADH} = 2.10 \times 10^3\ M^{-1}\ Cm^{-1}$) was taken into account. Protein concentration in the enzyme preparation was determined by the method of Lowry *et al.*, (1951).

Separation and determination of the activities of D, D/O and O forms of xanthine oxidase was done according to Kaminski and Jezewska(1979). (I) : The enzyme activity measured at 290 nm in the presence of O_2 only comprises the oxidase activities of forms O and D/O. (II) : The enzyme activity measured at 290 nm in the presence of O_2, NAD and in the absence of LDH and pyruvate consists of dehydrogenase activities (partially inhibited by NADH) of forms D and D/O and the activity of O-form. (III) : The enzyme activity measured at 290 nm in the presence of O_2, NADH, LDH and pyruvate consists of the uninhibited dehydrogenase activities of forms D and D/O and the activity of form O. (IV) : The enzyme activity measured at 340 nm in the presence of O_2, NAD and in the absence of LDH and pyruvate consists of dehydrogenase activities(partially inhibited by NADH) of forms D and D/O. Consequently, the separate enzyme activities can be calculated as D-form = (III) - (I), O-form = (II) - (IV) and D/O = (I) - [(II)-(IV)].

RESULTS

Influence of calcium on the protective effect oxygen radical scavengers and allopurinol

In the control heart perfused with oxygenated K-H solution for 2 hrs, LDH and CPK were not released regardless of the presence or the absence of calcium. In the presence of calcium, 60 min of ischemia did not increase the enzyme release either. However, upon reperfusion following the ischemia, the enzyme release increased dramatically and reached to a maximum value at 3 min of reperfusion(CPK, 6.2 U / min / g wet wt; LDH, 4.8U/min/g wet wt). This increase in the release of enzyme was prevented significantly by adding SOD and catalase to the perfusion medium. On the other hand, in the absence of calcium, the release of enzyme was induced considerably by 60 min of ischemia itself, and it was more markedly increased upon starting the reperfusion. This increased enzyme release in the absence of calcium was hardly inhibited by either SOD or catalase(Fig. 1 and Fig. 2). In the heart isolated from allopurinol pretreated rat, the extent of CPK and LDH release during the post-ischemic reperfusion period was significantly lower with the presence of calcium than with the absence of calcium. In the presence of calcium, the enzyme release from the allopurinol treated heart was also significantly lower than that from the control heart. This effect of allopurinol was com-

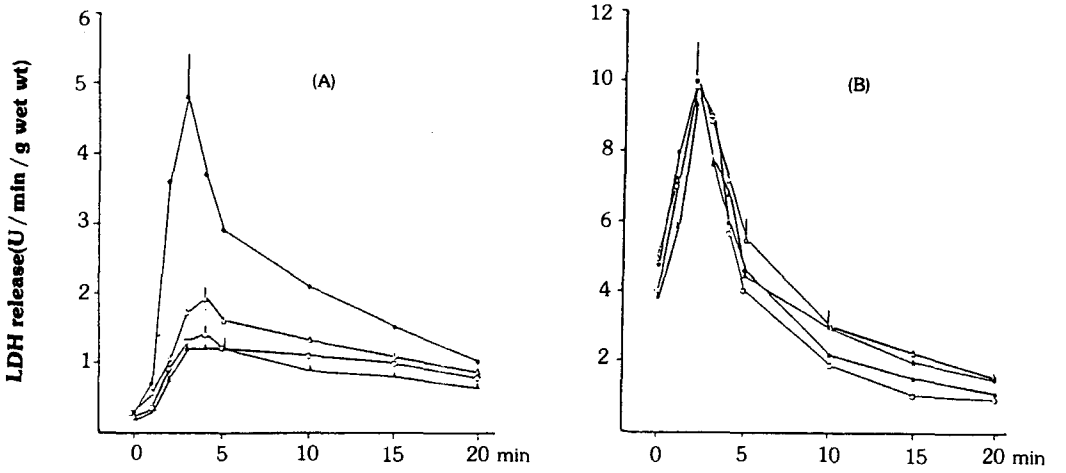

Fig. 1. Effects of oxygen radical scavengers and allopurinol on lactic dehydrogenase release in ischemic-reperfused rat hearts. Isolated rat hearts were subjected to 60 min of global ischemia followed by 20 min of oxygenated reperfusion. Oxygen radical scavengers were infused for 2 min before global ischemia and for 20 min throughout reperfusion. Allopurinol was administered two times orally(20 mg / kg) at 24 hr and 2 hr before the experiment. The global ischemia and reperfusion was made under the condition of presence(panel A) or absence(panel B) of calcium. Each point represents the mean ± S.E.M. of six experiments.
●, untreated hearts; △, SOD(5,000 U); ▲, Catalase(12,500 U); ○, Allopurinol.

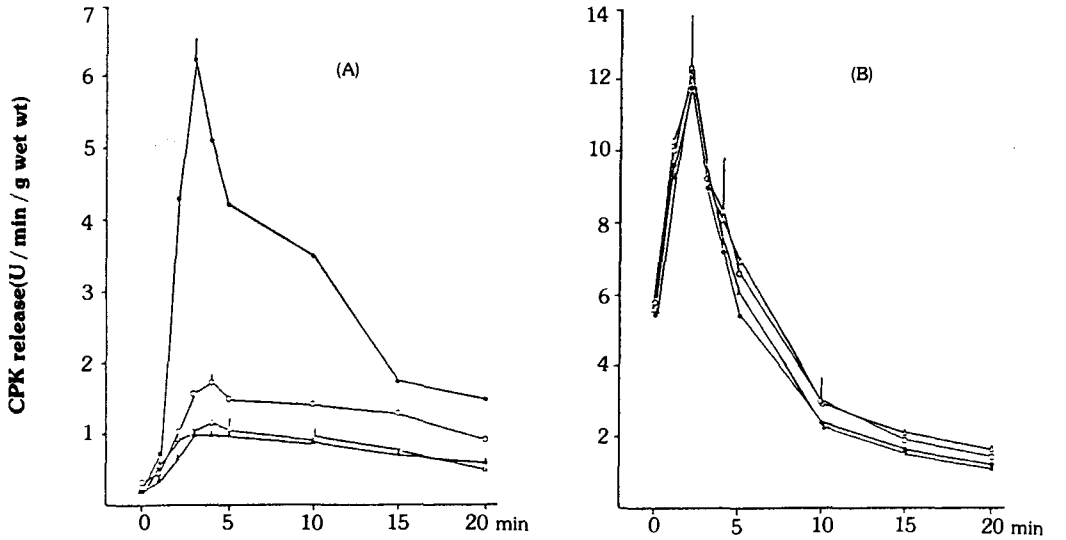

Fig. 2. Effects of oxygen radical scavengers and allopurinol on creatine phosphokinase release in ischemic-reperfused rat hearts. Methods of reperfusion, and scavenger and allopurinol administration were the same as in Fig. 1. Each point represents the mean ± S.E.M. of six experiments. Panel(A); Calcium containing condition, Panel(B); Calcium free condition. ●, untreated hearts; △, SOD(5,000 U); ▲, Catalase(12,500 U); ○, Allopurinol.

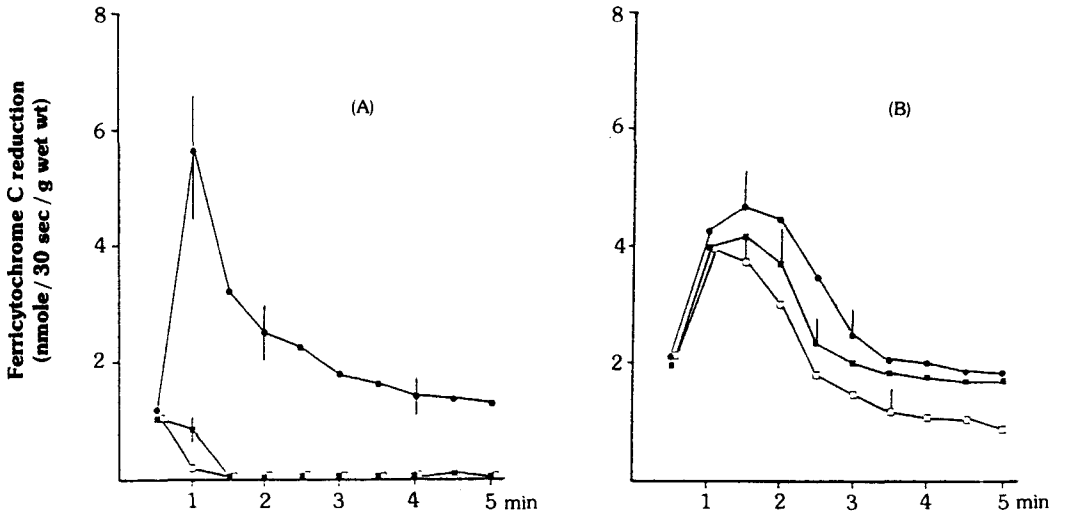

Fig. 3. Effect of SOD and allopurinol on ferricytochrome C reduction in ischemic-reperfused rat hearts. Isolated rat hearts were subjected to 60 min of global ischemia followed by 20 min of oxygenated reperfusion under the condition of the presence(panel A) or the absence(panel B) of calcium. Ferricytochrome C(100 μM) was infused at a rate of 0.5 ml/min during reperfusion. SOD (100 U) was mixed with ferricytochrome C and infused at the same rate. Allopurinol (20 mg/kg) was administered orally at 24 hr and 2 hr before the experiment. Each point represents the mean ± S.E.M. of six experiments. ($^*p < 0.05$). ●, Control; ■, Allopurinol; □, SOD.

parable to that of oxygen radical scavengers. (Fig. 1 and Fig. 2). SOD, catalase and allopurinol neither induced the enzyme release in the control hearts nor affected the analytical methods for the enzyme assay.

Influence of calcium on superoxide anion production

To examine the superoxide anion production in the heart subjected to post-ischemic reperfusion, reduction of exogenously administered ferricytochrome C was measured in the coronary effluent during the reperfusion. In the presence of calcium, the rate of ferricytochrome C reduction increased abruptly to a maximum value of 5.6 nmole/30 sec/g wet wt at 1 min after starting the reperfusion. This increase in the reduction of ferricytochrome C was almost completely inhibited by the addition of SOD as well as by the allopurinol pretreatment. On the other hand, in the absence of calcium, although the ferricytochrome C reduction increased at the same extent as that in the presence of calcium, large part of the cytochrome reduction was inhibited neither by SOD nor by allopurinol (Fig. 3). Thus, in the presence of calcium, almost every reduction of ferricytochrome C during the reperfusion was XOD-dependent and SOD-inhibitable (Fig. 4), while in the absence of calcium, a considerably large portion of the reduction was XOD-independent and SOD-uninhibitable (Fig. 5).

Influence of calcium on xanthine oxidase activity in ischemic myocardium

Myocardial content of total XOD was not significantly different between the normal

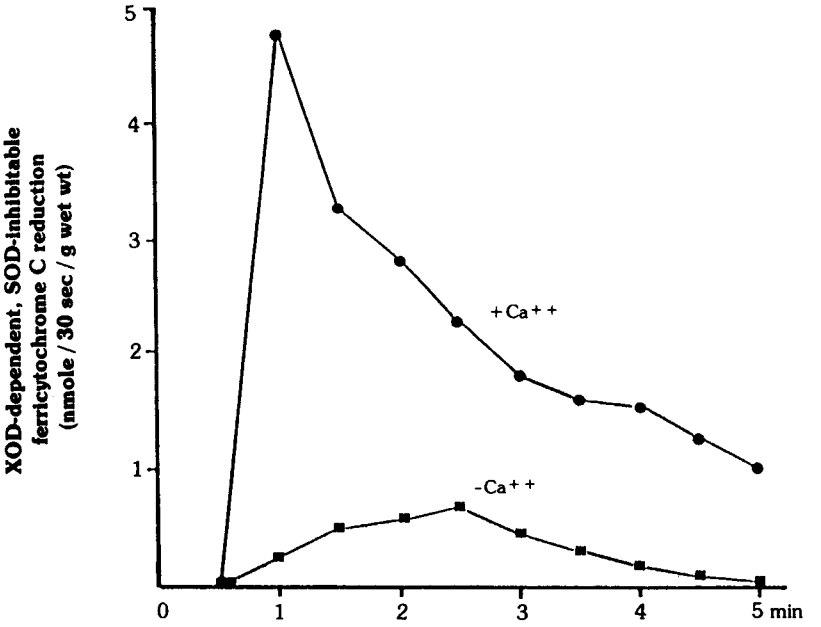

Fig. 4. Effect of calcium on xanthine oxidase-dependent, SOD-inhibitable ferricytochrome C reduction in ischemic-reperfused rat hearts. Curves were replotted from the data of Panel A and B of Fig. 3.

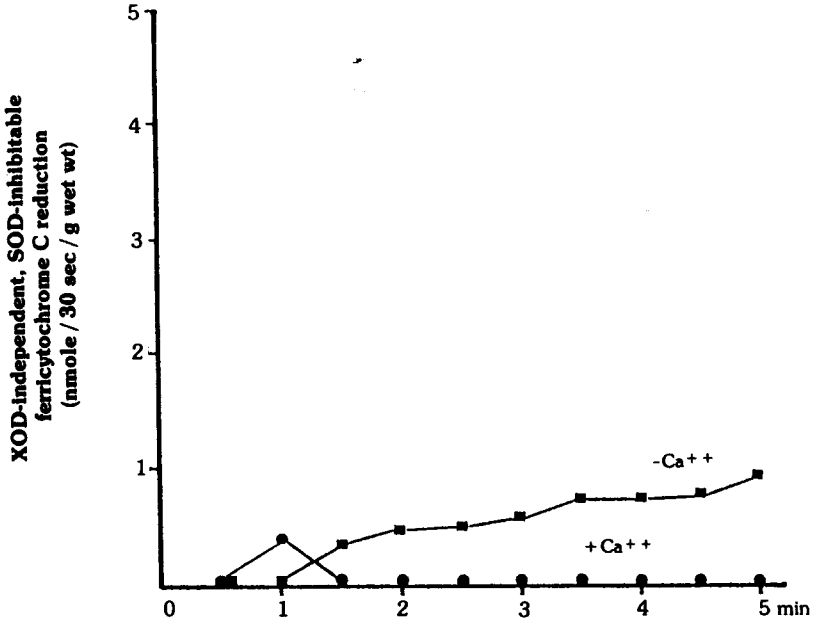

Fig. 5. Effect of calcium on xanthine oxidase-independent, SOD-inhibitable ferricytochrome C reduction in ischemic-reperfused rat hearts. Curves were replotted from the data of Panel A and B of Fig. 3.

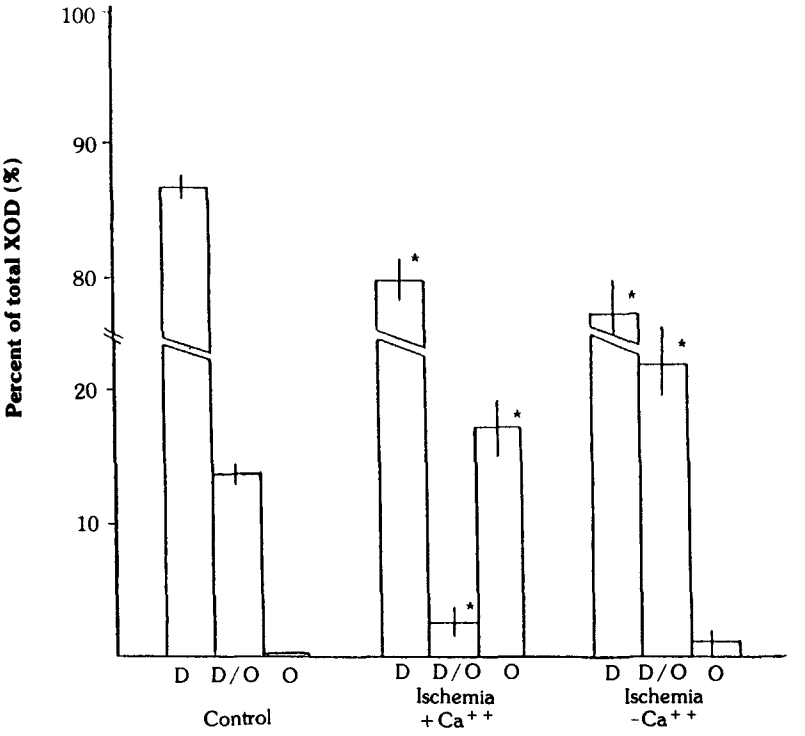

Fig. 6. Xanthine oxidase activity in ischemic myocardium. The tissue preparation for measuring xanthine oxidase activity in ischemic myocardium was done immediately after 60 min of global ischemia made under the condition of the presence or the absence of calcium. D-, D/O- and O-type of XOD were determined by method of Kaminski & Jezewska(1979) as described in the Method and Material. Values are mean ± S.E.M. of six experiments($^*p < 0.05$).

control hearts and the ischemic hearts regardless of the presence or the absence of calcium. In normal control hearts, proportions of D-, D/O- and O-form of XOD were 86%, 13% and 1% of total content, respectively. Compared with the control hearts, in the presence of calcium, ischemic hearts showed lower activities of D-form(80%) and D/O-form(2.6%), but higher activity of O-form(17%). On the other hand, in the absence of calcium, D/O-form(22%) was increased, while O-form was slightly but not significantly increased comparing with the normal (Fig. 6).

This result suggests that the increase in O-form of XOD in the ischemic myocardium of rat may be calcium dependent.

DISCUSSION

In the present study, it is suggested that calcium may be involved in the ischemia-induced conversion of XOD and may contribute to the genesis of myocardial damage by promoting oxygen radical generation during the reperfusion of ischemic myocardium.

Xanthine oxidase has been proposed as a possible source of oxygen free radical in

ischemic-reperfused myocardium of some animal species. It has been supported by observations that allopurinol, a specific competitive inhibitor of XOD, prevents functional and biochemical alterations in the ischemic-reperfused hearts of dog and rat (Chambers *et al.*, 1985; Hearse *et al.*, 1986; Kim and Akera, 1987; Lim and Kim, 1988; Manning *et al.*, 1984). In the early studies, it was reported that allopurinol protected heart by preventing the loss of purine bases and then by salvaging the high energy phosphate in the ischemic myocardium(Dewall *et al.*, 1971). But currently, allopurinol is suggested to inhibit the O-form of XOD and thereby prevent the production of oxygen free radical. The present result indicates that allopurinol prevents the release of intracellular enzymes and the generation of superoxide anion (SOD-inhibitable ferricytochrome C reduction) from the ischemic-reperfused heart of rat and strongly supports the hypothesis of XOD involvement in the reperfusion damage.

Although intracellular calcium overload is a common feature of irreversibly damaged cells in ischemic-reperfused myocardium(Katz and Reuter, 1979; Nayler, 1981), and although calcium channel blockers(Ferrari *et al.*, 1986; Nayler *et al.*, 1985; Sashida and Abiko, 1986) and low calcium perfusion(Koomen *et al.*, 1983; Kuroda *et al.*, 1980; Nayler, 1981) are reported to protect some cellular damages in ischemic and reperfused hearts, the presently available evidence for the exact role of calcium in the development of ischemic-reperfusion injury is very circumstantial. In the present study, the increase of the intracellular enzyme release upon reperfusion was significantly prevented by adding SOD and catalase as well as by allopurinol pretreatment in the presence of calcium, but not in the absence of calcium. This suggests possible involvement of calcium with XOD-linked oxygen radical generation and the resultant myocardial injury in ischemic-reperfused heart of rat. This is also supported by the observation that the extent of XOD-dependent, SOD-inhibitable ferricytochrome C reduction is much higher in calcium containing heart than in calcium-free heart.

Since McCord and Roy(1982) have reported the existence of oxygen radical producing O-form of XOD in large amount in ischemic intestine, several other investigators(Chambers *et al.*, 1985; Hearse *et al.*, 1986; Lim and Kim, 1988) observed higher content of O-form in ischemic myocardium of dog and rat. We observed also in the present study that the proportion of O-form to the total XOD was much higher in ischemic heart than in normal control heart, while that the myocardial content of total XOD was similar in both normal and ischemic heart. However, the exact mechanism of the increase of O-form in the ischemic tissue is not clarified yet. In an *in vitro* system, the NAD-dependent D-form, which does not produce oxygen radical, is known to be converted to the O-form by physical and chemical alterations of experimental conditions including pH and temperature changes, anoxia, metal ions and sulfhydryl modifying agents. Also, this *in vitro* conversion of XOD is known to be prevented by proteolytic enzyme inhibitors and SH-donors such as dithiothreitol and mercaptoethanol (Battelli *et al.*, 1972; DellaCorte and Stirpe, 1972; Granger *et al.*, 1981; Waud and Rajagopalan, 1976). Additionally, Roy and McCord(1983) suggested that in the ischemic intestine, the D-form of XOD might be converted to the O-form by calcium-calmodulin dependent proteolysis. They observed that the conversion was prevented by soy-bean trypsin inhibitor as well as by stelazine, a calcium-calmodulin inhibitor. In the present study, sixty minutes of ischemia caused an increase of O-form in the

presence of calcium, but not in the absence of calcium. This is also thought to provide an evidence of calcium-dependent conversion of XOD in the ischemic heart of rat. Furthermore, in the calcium presenting ischemic heart the proportion of D/O-form was decreased in parallel with the increase of the O-form, while in the calcium-free ischemic myocardium, there was an increase in the D/O-form only. This suggests that calcium-dependent conversion to O-form from D/O-form rather than from D-form may occur in the ischemic heart of rat. Since the D/O-form of XOD is an intermediate form which uses both NAD and O_2 as electron acceptors, and since the affinity of this enzyme is considerably greater for NAD than for O_2(Kaminski and Jezewska 1979), the D/O-form in normal heart or in ischemic hearts of which NAD content is not so severely depressed, may act like D-form. It has been reported that a significant decrease in NAD was not noted until after 90 min or longer of ischemia in dog heart (Jennings *et al.*, 1981).

The extent of SOD-inhibitable ferricytochrome C reduction was lower in the absence of calcium than in the presence of calcium, and most of the reduction in calcium-free heart was not dependent on XOD. This may indicate that, in ischemic heart of rat, there is another possible source of oxygen free radical in addition to that generated by XOD. Other than XOD, neutrophile leukocytes, mitochondria and catecholamines are proposed as probable sources, but their relative importances in ischemic-reperfusion injury of heart are yet to be determined.

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