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Role of Xanthine Oxidase in Reperfusion Injury in Ischemic Myocardium[1]

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= Abstract =The present studies were conducted to confirm the hypothesis that xanthine oxidase-linked cytotoxic oxygen free radicals play an important role in producing the reperfusion injury of ischemic myocardium. The reperfusion injury was induced in isolated, Langendorff preparations of rat hearts by 60 min of global ischemia with aortic clamping followed by 20 min of reperfusion with oxygenated Krebs-Henseleit solution. Upon reperfusion of ischemic hearts, the release of creatine phosphokinase, lactic dehydrogenase and a lipid peroxidation product, malondialdehyde into the coronary effluent was abruptly increased. The increase of the enzymes and malondialdehyde was supprressed significantly in the presence of superoxide dismutase or catalse during the reperfusion period. In the hearts removed from rats pretreated with a specific xanthine oxidase inhibitor, allopurinol(20 mg/kg, orally 24 hrs and 2 hrs before study), the increased release of the enzymes and malondialdehyde was also significantly depressed. This effect of allopurinol was comparable to that of oxygen radical scavengers. When ferricytochrome C was infused to the hearts starting with reperfusion, the SOD-inhibitable reduction of ferricytochrome C increased during the first minute of reperfusion. Hydrogen peroxide measured in the coronary effluents also increased markedly with the similar time course as ferricytochrome C reduction. In the hearts treated with allopurinol, however, both the ferricytochrome C reduction and H_2O_2 were not increased significantly. Myocardial content of xanthine oxidase was determined for D-form, O-form and D/O-form separately in the normal control hearts and the ischemic ones. Total enzyme content was similar in both hearts. Compared with the normal control, however, the ischemic hearts showed lower activities in D-form and D/O-form, and higher activity in the oxygen radical producing O-form. The proportion of O-form to the total enzyme was much higher in the ischemic heart than in the normal control heart. After 60 min of ischemia, myocardial content of total adenine nucleotides were reduced to 40% of the normal control value, while their catabolic products, hypoxanthine and xanthine were accumulated significantly. These results provide evidence that xanthine oxidase is involved in the production of oxygen free radicals during the reperfusion of ischemic heart, and plays a contributing role in the genesis of reperfusion injury.

Key words: . ***Xanthine oxidase, Oxygen free radical, Reperfusion injury, Myocardial ischemia***

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INTRODUCTION

In the early stages of myocardial ischemia, cellular damage is reversible. If adequate coronary flow is restored in this reversible period, any metabolic imbalances and contractile impairment are eventually normalized. As the duration and the severity of

ischemia increase, however, the restoration of cornary flow may worsen the myocardial cellular damage rather than improve the function. This phenomenon has been termed the reperfusion injury (Hearse 1977).

A number of factors has been suggested as important determinants of the myocardial cellular damage in the reperfusion injury; including myocardial high energy phosphate depletion(Jennings and Reimer 1981), release of endogenous catecholamines(Gaudel *et al.* 1979), intracellular calcium overload(Katz and Reuter 1979; Nyler 1981), activation of phospholipases(Chien *et al.* 1981) and accumulation of lysophosphatides(Pitts and Ohkuysen 1984). In addition to this, recently, reactive oxygen free radicals, such as superoxide anion, hydrogen peroxide and hydroxyl radical have been also suggested to play a crucial role in the development of myocardial cellular damage in the process of ischemia-reperfusion. The involvement of oxygen free radicals in the reperfusion injury has been supported by the observations that the exogenous scavengers of oxygen free radicals, superoxide dismutase and catalse significantly reduce the fuctional and biochemical indices of cellular damage in isolated or in vivo ischemic-reprerfused animal hearts(Guarnieri *et al.* 1980; Hess and Manson 1984; Shlafer *et al.* 1982). However, inspite of the cardioprotective effect of the oxygen radical scavengers, the presently available evidence for the oxygen radical involvement in the reperfusion injury seems to be very circumstantial. At present, there is little information about from where and by what mechanism oxygen radicals are produced in the ischemic-reperfused myocardium, and furthermore, the production of oxygen radicals has never been detected directly in myocardial tissues.

In recent studies, xanthine oxidase has been proposed as a possible source of oxygen radical in ischemic-reperfusion injury of various tissues (Granger *et al.* 1981; Granger *et al.* 1986; Hearse *et al.* 1986; McCord and Roy 1982). Xanthine oxidase is an oxidoreductase which catalyzes the oxidation of hypoxanthine and xanthine to uric acid in adenine nucleotide metabolism. In in vitro system, this enzyme has been well known to produce superoxide anion by utilizing molecular oxygen as an electron acceptor. Biologically existing xanthine oxidase consists of three different forms, that is, NAD-dependent dehydrogenase form(D-form), O_2-dependent, oxygen radical producing oxidase form(O-form) and both NAD- and O_2-dependent D/O-form. Among these three different forms, NAD-dependent D-form, which does not produce oxygen radical, exists in large part in normal animal tissues. (Battelli *et al.* 1972; Kaminski and Jezewska 1979).

The proposed theory about xanthine oxidase involvement in the ischemic-reperfusion injury is based largely on the observations that (1) allopurinol, a specific competitive inhibitor of xanthine oxidase is as effective as the oxygen radical scavengers in preventing the reperfusin injury(Dawall *et al.* 1971; Granger *et al.* 1981; Kim and Akera 1987; Myers *et al.* 1985), (2) normally existing D-form of xanthine oxidase is converted to oxygen raidcal producing O_2-dependent O-form in the ischemic tissues(Granger *et al.* 1981; McCord and Roy 1982; Parks *et al.* 1982), and (3) in the ischemic tissues, hypoxanthine and xanthine, which are substrates for xanthine oxidase, are accumulated as a consequence of continuous degradation of cellular ATP(Jennings *et al.* 1981). It was hypothesized from these observations that, if molecular oxygen is supplied into the ischemic tissues by reperfusion, O-form of xanthine oxidase could produce large amount of oxygen radical by utilizing the accumulated hypoxanthine and xanthine as substrate and the supplied oxygen as an electron acceptor.

In the present study, we applied and tested this hypothesis in the ischemic-reperfused heart of rat. For that purpose, we compared the myocardial contents of the different forms of xanthine oxidase as well as hypoxanthine and xanthine in normal heart and ischemic one. Additionally, we tried to confirm the involvement of xanthine oxidase-linked oxygen radical in the myocardial ischemic-reperfusion injury by detecting the production of oxygen radicals more directly in in situ heart.

MATERIALS AND METHODS

Adenosine diphosphate(ADP), adenosine monophosphate(AMP), catalase, creatine phosphate(CP), ferricytochrome C(Type VI), dithiothreitol(DTT), glucose-6-phosphate dehydrogenase, hexokinase, horse radish peroxidase, lactic dehydrogenase, myokinase, nicotineamide adenine dinucleotide(NAD), nicotineamide adenine dinucleotide phosphate(NADP), phosphenol pyruvate, pyruvate kinase, reduced nicotinamide adenine dinucleotide(NADH), superoxide dismutase(SOD), thiobarbituric acid, xanthine, xanth-

ine oxidase were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). 2'7'-dichlorofluorescin diacetate(DCFDA) was obtained from Eastman Kodak and other chemicals were reagent grade.

Experimental ischemic-reperfusion injury

Sprague-Dawley rats of either sex, weighing 150-200 g were heparinized intraperitoneally(100 IU). Forty-five minutes after heparinization, rat was stunned by a blow to the head. The heart was quickly removed and perfused through the aorta (Langendorff preparation) with Krebs-Henseleit bicarbonate buffer solution(K-H solution) containing(in mM) NaCl 118, $NaHCO_3$ 27.2, KCl 4.8, $MgSO_4$, $7H_2O$ 1.2, KH_2PO_4 1, $CaCl_2$ 1.25 and glucose 11.1 at a constant perfusion pressure of 100 CmH_2O. The buffer solution was saturated with a 95% O_2-5% CO_2 gas mixture yielding a final pH value of 7.4 at 37°C. The heart was kept in a humidified chamber maintained at 37°C during the perfusion. The pulmonary artery was cut at where it leaves the right ventricle to allow complete drainage of the coronary effluent. After 20 min of control perfusion for washing-out the residual blood and equilibration, the heart was subjected to global ischemia by completely stopping the perfusion for 60 min. During the ischemic period, the heart was immersed in glucose-free(substituted with mannitol) K-H solution gassed with 95% N_2-5% CO_2 and maintained at 37°C. Following the ischemia, the reperfusion injury was induced by 20 min of reperfusion with oxygenated K-H solution as described above. Normal control heart was continuously perfused with oxygenated K-H solution for 90 min. At the end of each experiment, the heart was removed and was weighed after having blotted surface fluid.

As an index of myocardial cellular damage, release of intracellular enzymes, creatine phosphokinase(CPK) and lactic dehydrogenase(LDH) into the coronary effluent was measured. The degree of lipid peroxidation in myocardial tissue was also estimated from a lipid peroxidation product, malondialdehyde(MDA) released into the coronary effluent. Coronary effluents were collected at the indicated time intervals as in the results and were kept in ice until further assay.

CPK activity was assayed UV-spectrophotometrically(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, cystine 10 mM, hexokinase 0.94 U/ml and glucose-6-phosphate dehydrogenase 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 at 25°C. The rate of change of optical density was measured at 340 nm with UV-spectrophotometer (Perkin-Elmer, Model 139).

LDH activity was assayed by using UV-spectrophotometer (Bergmeyer and Bernt 1974). 0.5 ml of the coronary effluent 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 at 25°C and 340 nm with UV-spectrophotometer.

MDA was measured by thiobarbituric acid method (Yagi 1982): 0.6 ml of 1:1 mixture of 0.67% thiobarbituric acid and glacial acetic acid was added into 2.4 ml of the coronary effluent sample. The reaction mixture was placed on boiling water bath for 60 min and then cooled to room temperature. After cooling, the absorbance was measured at 532 nm with UV-VIS spectrophotometer. The MDA released was expressed as nmole/min/g wet wt using the molar extinction coefficient of 1.52×10^5/M/Cm(Placer *et al.* 1966).

Assay of superoxide anion and hydrogen peroxide

The production of superoxide anion was estimated by measuring the reduction of ferricytochrome C(Salin and McCord 1974). Starting with the reperfusion, ferricytochrome C solution(100 μ M) was infused to the heart through the aortic cannula at a rate of 0.5 ml/min. Its reduction after passing through the heart was measured in the coronary effluent collected at 30 sec interval. Concentration of reduced ferrocytochrome C in the coronary effluent was determined by measuring the absorbance at a wavelength of 418 nm with spectrophotometer(Cam Spec, M301). After that, the remaining ferricytochrome C in each sample was then fully reduced by the addition of a few crystals of sodium dithionite. The extent of reduction that had occured during the perfusion was calculated by using the difference between the two readings and by the difference of molar extinction coefficient($\triangle E_{418} = 7.0 \times 10^4$/M/Cm) between oxidized ferricytochrome C and reduced ferrocytochrome C.

Hydrogen peroxide released into the coronary effluent was measured as another evidence for the

production of oxygen radical in the myocardium. The concentration of H_2O_2 was determined spectrofluorometrically by using 2'7'-dichlorofluorescin diacetate(DCFDA) and horse radish peroxidase-(HRP)(Black and Brandt 1974). 0.2 ml of coronary effluent collected at 1 min interval during the reperfusion period was mixed with 3 ml of a reaction mixture containing sodium phosphate buffer 25 mM(pH 7.2), DCFDA 1 μM and HRP 2.2 U/ml. The mixture was incubated for 20 min at 25°C with a shaking incubator. After incubation, the fluorescence was measured with spectrofluorometer(Perkin-Elmer, Model 1000) at the excitation wavelength of 468 nm and the emission wavelength of 520 nm.

Measurement of myocardial xanthine oxidase activity

Myocardial contents of NAD-dependent dehydrogenase form(D-form), O_2-dependent oxidase form(O-form) and intermediate D/O-form of xanthine oxidase were determined separately by the method of Kaminski and Jezewska(1979). Heart removed from Langendorff apparatus was quickly forzen and powdered 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 1,000g and 4°C. The superantant was removed and recentrifuged for 60 min at 27,000g 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 20,000g. 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 contained 50 mM Tris-HCl buffer(pH 8.0), 60 μM xanthine and the enzyme preparation(0.3-0.5 mg protein/ml) with or withot 167.5 μM 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 to prevent the inhibition of the D-form enzyme(DellaCorte and Stirpe 1970). The molar absorption coefficient for NADH at 340 nm($E_{340}=6.22\times10^3$/M/Cm) and that for conversion of xanthine to uric acid at 290 nm(E_{290}, xanthine → urate $= 0.85 \times 10^4$/M/Cm) 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/Cm) was taken into account. Protein concentration in the enzyme preparation was determined by the method of Lowry *et al.* (1951).

Separation and determination of activities of forms D, D/O and O 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 absnce 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 uninhibted 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)].

Measurement of myocardial adenine nucleotides, hypoxanthine and xanthine

Heart was quickly forzen and powered in liquid nitrogen. The powdered frozen tissue was homogenized in 4 vol of 6% perchloric acid with a Polytron tissue disintegrator. The homgenate was centrifuged for 10 min at 3 ,000g with a refrigerated centrifuge. The resulting supernatant neutralized to pH 7.4 with 5 M K_2CO_3 was used for the assay of adenine nucleotides and their metabolites. ATP was assayed by UV-spectrophotometric method using hexokinase and glucose-6-phosphate dehydrgroenase(Trautschold *et al.* 1965). ADP and AMP were determined enzymatically by using myokinase, pyruvate kinse and lactic dehydrogenase with a UV-spectrophotometer(Jaworek and Welsch 1985). Hypoxanthine and xanthine were measured by enzymatic method using xantine oxidase(Jensen and Jorgensen 1985). The molar absorption coefficient for the enzymatic transforma-

Table 1. Effects of allopurinol and oxygen radical scavengers on coronary flow during reperfusion of ischemic myocardium in isolated Langendorff preparations of rat heart[1]

Conditions	No. of animal	Coronary flow(ml/min/g wet wt)[2]					
		Control	Reperfusion (min)				
			2	5	10	15	20
Untreated	6	15.5±1.3	8.3±0.4	7.3±0.5	7.5±0.6	7.7±0.6	7.9±0.5
SOD[3] (5000 U)	6	15.0±2.1	12.1±3.6*	11.5±2.5*	11.0±2.3*	11.0±2.1*	10.5±2.2*
Catalase[3] (12,500 U)	5	15.2±2.7	13.0±3.3*	12.5±2.3**	11.9±1.8**	11.0±1.5*	10.7±1.7*
Allopurinol[4]	7	15.3±0.9	12.7±1.8*	13.3±1.6**	13.6±1.5**	13.4±1.3**	13.1±1.1**

1. Perfusion pressure was maintained constantly at 100 cm H_2O.
2. Mean+S.E.
3. SOD and catalse were infused during reperfusion by using infusion pump at a rate of 0.5 ml/min.
4. Allopurinol(20 mg/kg) was administered orally two times at 24 hrs and 2 hrs before study.

* $p < 0.05$.

** $p < 0.01$.

tion of xanthine to uric acid at 290 nm($E_{290,X\rightarrow Urate} = 0.85 \times 10^4$/M/Cm) and that for the transformation of hypoxanthine to uric acid at 280 nm($E_{280,Hx\rightarrow Urate} = 0.7 \times 10^4$/M/Cm) were used for the calculation.

Administration of oxygen radical scavengers and allopurinol treatment

Oxygen radical scavengers were administered duirng reperfusion. Superoxide dismutase(SOD) which dismutates superoxide anion enzymatically, and catalase which degrades hydrogen peroxide were infused via aortic cannula by a infusion pump at a rate of 0.5 ml/min. Total amount of scavengers administered were 5,000 U for SOD and12,500 U for catalase. Rats were pretreated with allopurinol, a specific competitive inhibitor of xanthine oxidase. Allopurinol was administered orally two times at 24 hrs and 2 hrs before study at a dose of 20 mg/kg. In preliminary experiments, myocardial xanthine oxidase was confirmed to be compleletely inhibited by this selected dose of allopurinol.

RESULTS

Effects of oxygen radical scavengers and allopurinol on reperfusion injury

Coronary flow: Control hearts perfused continuously with oxygenated K-H solution maintained constant coronary flow(about 15 ml/min/g wet wt) throughout 90 min perfusion period. After 60 min of global ischemia, the coronary flow decreased to 50% of the control value upon reperfusion. This decrease in coronary flow was considerably prevented by SOD or catalse administered with the reperfusion. In addition to this, hearts isolated from rats pretreated with allopurinol also showed significantly improved coronary flow during the reperfusion period(Table 1). In control hearts, administration of SOD or catalse and allopurnol pretreatment did not affect the coronary flow.

Enzyme release: In control hearts, there was no significant release of CPK or LDH during 90 min of perfusion with oxygenated K-H solution. Sixty minutes of global ischemia followed by 20 min reperfusion caused massive enzyme release. This enzyme release was significantly prevented by oxygen radical scavengers as well as by allopurnol. The amount ot CPK released during 20 min period of reperfusion was 54.2 U/g wet wt. This release was reduced to 15 U/g wet wt by SOD or catalase. Allopurinol pretreatment also caused a significant reduction to below half of the untreated value (Fig. 1). In LDH release, total amount released during the reperfusion period was 40.2 U/g wet wt. It was also significantly suppressed by SOD and catalse as well as by allopurinol(Fig. 2). These oxygen radical scavengers or allopurinol neither induced the enzyme release in the control hearts nor affected the analytical methods for the enzyme assay.

Lipid peroxidation: Control perfusion with oxygenated K-H solution for 90 min did not induce any

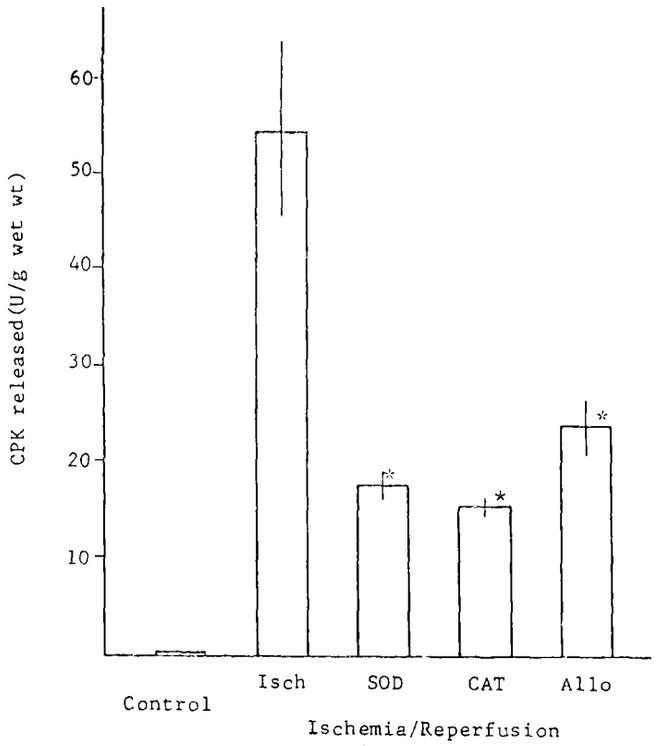

Fig. 1. Effects of allopurinol and oxygen radical scavengers on creatine phosphokinase release during reperfusion of ischemic myocardium of rat.
Isolated, Langendorff preparations of rat hearts were subjected to 60 min of global ischemia followed by 20 min of reperfusion with oxygenated Krebs-Henseleit solution. Superoxide dismutase (SOD, 5,000 U) and catalase(12,500 U) were infused to the hearts starting with reperfusion at a rate of 0.5 ml/min. Allopurinol (20 mg/kg) was administered orally two times at 24 hrs and 2 hrs before study. Control hearts were perfused for 90 min with oxygenated Krebs-Henseleit solution(37°C) at a constant perfusion pressure of 100 cm H_2O. Values are total amount of enzyme released into the coronary effluent during 20 min period of reperfusion(Mean±S.E., n=6). *$p < 0.01$

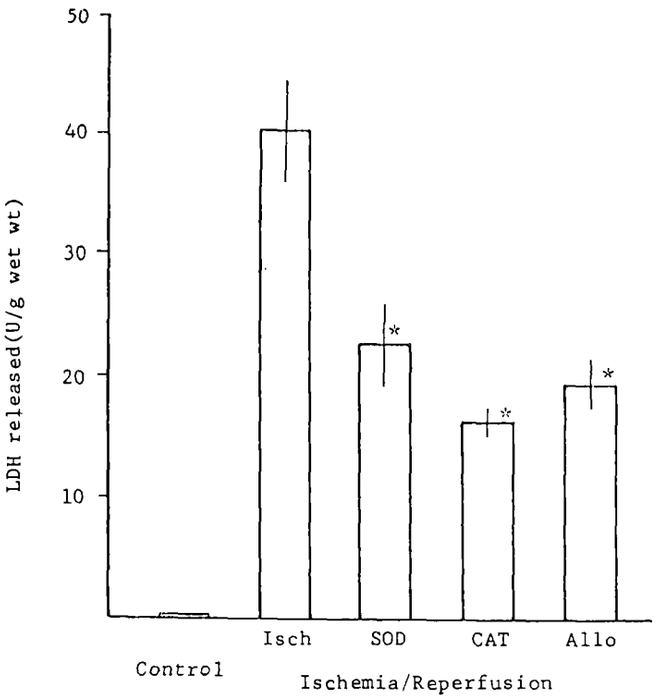

Fig. 2. Effects of allopurinol and oxygen radical scavengers on lactic dehydrogenase release during reperfusion of ischemic myocardium of rat.
Methods of perfusion and drug administration are the same as in Fig. 1. Values are total amount of enzyme released into the coronary effluent during 20 min of reperfusion period(Mean±S.E., n=6). *$p < 0.01$

significant release of MDA. In the heart subjected to 60 min of global ischemia, MDA release was massively increased upon reperfusion(49 nmoles/g wet wt/20 min). Administrations of SOD and catalse as well as allopurinol treatment significantly depressed the MDA release to one-third of that of the untreated ischemic-reperfused value(Fig. 3). The oxygen radical scavengers or allpurinol neither caused MDA release in control hearts nor affected the analytical method for MDA assay.

These results suggest that xanthine oxidase-linked oxygen radicals are probably produced during reperfusion and may play a significant role in the genesis of reperfusion injury of ischemic rat heart.

Oxygen radical production

We tried to obtain more direct evidence for the xanthine oxidase-linked oxygen radical production in in situ ischemic-reperfused heart. For the detection of superoxide anion, ferricytochrome C solution (100 μM, 0.5 ml/min) was infused to the heart in the presence or absence of SOD(10 U/ml). In the absence of SOD, there was sudden increase in ferricytochrome C reductin upon reperfusion. This increase was maintained throughout the reperfusion period. However, when ferricytochrome C was infused in the presence of SOD, the reduction was almost completely prevented. In addition to this, in the heart isolated from allopurinol treated rat, the ferricytochrome C reduction was also prevented to the same extent as with SOD(Fig. 4). H_2O_2 was directly measured in the coronary effluent by sensitive fluorometric method. As in Fig. 5, H_2O_2 was abruptly and markedly increased upon reperfusion. This increase in H_2O_2 was also signifcantly depressed in allopurinol treated heart. These results

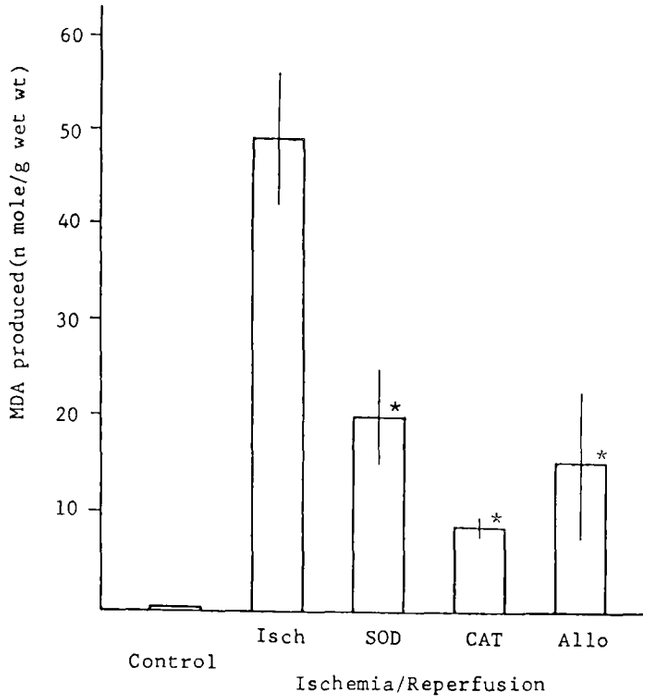

Fig. 3. Effects of allopurinol and oxygen radical scavengers on lipid peroxidation during reperfusion of ischemic myocardium in rat.
Lipid peroxidation of myocardial tissue was estimated from malondialdehyde(MDA) production assayed by thiobarbituric acid method. Perfusion conditions and methods of drug administration are the same as in Fig. 1. Values are total amount of MDA released into the coronary effluent during 20 min of reperfusion period(Mean± S.E., n=6). *p < 0.01

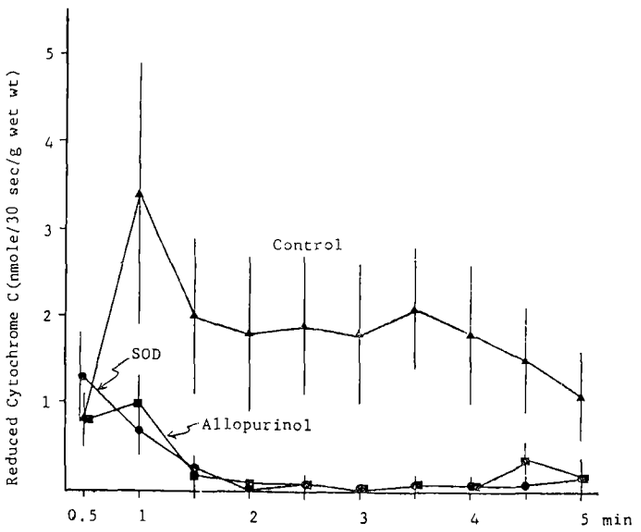

Fig. 4. Ferricytochrome C reduction during reperfusion of ischemic myocardium.
Langendorff preparations of isolated rat hearts were subjected to 60 min of ischemia followed by 20 min of reperfusion. During reperfusion, ferricytochrome C solution(100 μM) was infused to the heart through the aortic cannula at a rate of 0.5 ml/min. Concentrations of reduced ferrocytochrome C in the coronary effluents were determined by measuring the absorbance at 418 nm. For the estimation of SOD-inhibitable portion of ferricytochrome C reduction, SOD(10 U/ml) was mixed with ferricytochrome C. Allopurinol treatment was same as in the Fig. 1. Results are Mean±S.E.(n=6)

indicate that oxygen free radicals may be produced during the reperfusion of ischemic heart through the action of xanthine oxidase.

Xanthine oxidase actvity in sichemic myocardium

Total myocardial content of xanthine oxidase (5.3 μ/g.prot.) was not different between in normal control heart and in 60 min ischemic heart. However, compared with the control, the ischemic heart showed lower activities in D-form and D/O-from, but higher activity in O-form. The proportion of O-form to the total enzyme was about 16% in the ischemic heart, while that was only less than 1% in the normal control hearts(Fig. 6).

Adenine nucloetides, hypoxanthine and xanthine in ischemic myocardium

Total adenine nucleotide content was 16.5 umole/g dry wt in normal control heart. Proportions of ATP, ADP and AMP to the total adenine nucleotide were 85%, 11% and 4% respectively. After 60 min of global ischemia, total adenine nucleotide reduced to 40% of normal control vlaue. This reduction of total adenine nucleotide was paralled by decrease in ATP and ADP. Almost none of hypoxanthine and xanthine was found in control heart. However, in the ischemic heart these purine bases were increased significantly. In the heart isolated from allopurnol treated rat, 60 min of global ischemia caused similar decrease in levels of total adenine nucleotide, ATP and ADP. Only hypoxanthine, but not xanthine, was increased in this allopurinol treated heart. The level of hypoxanthine was equal to the sum of hypoxanthine plus xanthine found in the allopurinol non-treated heart(Table 2).

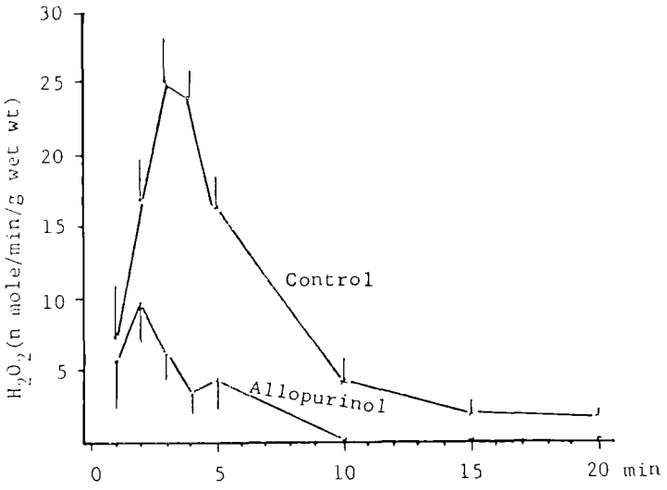

Fig. 5. H_2O_2 production during reperfusion of ischemic myocardium of isolated rat heart.
Isolated, Langendorff preparations of rat hearts were subjected to 60 min of global ischemia followed by 20 min of reperfusion with oxygenated Krebs-Henseleit solution-(Control). Allopurinol (20 mg/kg, oral) was pretreated to rats two times at 24 hrs and 2 hrs before study. H_2O_2 released into the coronary effluents during reperfusion period was measured by using fluorescent dichlorofluorescein and hose radish peroxidase. Results are Mean ± S.E. (n=6).

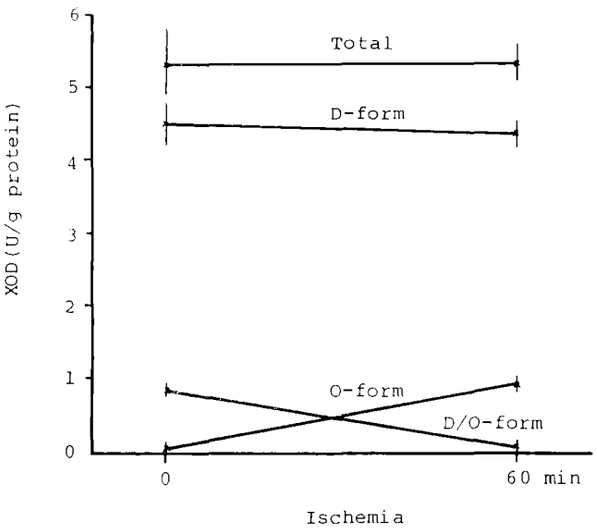

Fig. 6. Xanthine oxidase activity in ischemic heart of rat.
Isolated, Langendorff preparations of rat hearts were subjected to 60 min of global ischemia. NAD^+-dependent dehydrogenase form(D), O_2-dependent oxygen radical producing oxidase form(O) and intermediate D/O-form of the enzyme were determined separately by method of Kaminski & Jezewska(1979) as described in the Method and Material. Results are Mean±S.E.(n=6)

DISCUSSION

Since Dewall *et al.* (1971) has reported that allopurinol reduces arrhythmias in dog heart with regional ischemia, xanthine oxidase has been considered to play a certain role in myocardial ischemia and reperfusion injury of myocardium. Several other investigators have also shown that allopurinol supresses the development of arrhythimia in ischemic-reperfused myocardium(Manning *et al.* 1984), prevents Na,K-ATPase inhibition(Kim and Akera 1987) and creatine phosphokinase release(Myers *et al.* 1985) in sichemic-reperfused heart, and reduces the infarct isze in animal hearts(Chambers *et al.* 1985). In the present study we obtained similar result that allopurinol prevented the increased release of intracellular enzymes and MDA induced by reperfusion in ischemic rat heart. The effect of allopurinol was comparable to that of oxygen radical scavengers, SOD and catalase.

The mechanism of this cardioprotective effect of allopurinol, however, has been the subject of debate. During ischemia, tissue high energy phosphates continue to degrade to pruine bases, hypoxanthine and xanthine. This degradation process is finally terminated by xanthine oxidase-mediated conversion of hypoxanthine and xanthine to uric acid. In the early studies, it was postulated that allopurinol protected the heart by preventing the loss of pruine bases and then by salvaging the high energy phosphates in the ischemic myocardium (Dewall *et al.* 1971). However, in the current concept, hypoxanthine and xanthine are not expected to be saved by allopurinol in the ischemic tissue, because purine bases are so lipid soluble and diffusalbe to membranes that collateral blood flow can wash them out from the ischemic region(Chambers *et al.* 1985; Hearse *et al.* 1986). Our present result also supports this concept. After 60 min of ischemia, myocardial contents of hypoxanthine, xanthine and total adenine nucleotides were not higher in the allopurinol-treated heart than in the untreated one.

Recently, it has been suggested that cardioprotective effect of allopurinol in the ischemic-reperfused myocardium is likely to be due to its ability to inhibit O-form of xanthine oxidase and thereby prevent the production of reactive oxygen radicals(Hearse 1986). In 1982, McCord and Roy demonstrated in the intestine that a breif period of ischemia caused conversion of D-form of xanthine ox-

Table 2. Myocardial contents of adenine nucleotides, hypoxanthine and xanthine after 60 min of global ischemia in isolated rat heart[1]

Conditions	No. of animal	Adenine nucleotide (u mole/g dry wt)				Prufine base (u mole/g dry wt)	
		ATP	ADP	AMP	Total	Hypoxanthine	Xanthine
Control	6	13.97±1.25	1.84±0.16	0.64±0.08	16.45	N.D.[2]	N.D.[2]
Ischemia	6	4.35±0.82*	0.84±0.09*	1.26±0.07*	6.45	1.55±0.18*	0.79±0.20*
Ischemia[3] Allopurinol	5	3.19±0.39*	0.61±0.05*	1.06±0.10*	4.86	2.57±0.29*	N.D.[2]

1. Mean±S.E.
2. N.D.: Not detected.
3. Rat was pretreated with allopurinol(20 mg/kg, orally) two times at 24 hrs and 2 hrs before study.

* $p < 0.01$.

idase to O-form which produces superoxide anion by utilizing molecular oxygen as an electron acceptor. In addition to this, Chambers *et al.* (1985) also suggested the conversion of xanthine oxidase from D-form to O-form in the ischemic heart of dog. They reported that about 30% of total content of xanthine oxidase was in O-form in the ischemic myocardium, while only within 10% was in O-form in normal myocardium. In the present study using rat heart, we also similarly observed the increase in O-form of the enzyme in the ischemic condition. The proportion of O-form was about 16% in the 60 min ischemic heart, while almost none of O-form was detected in the normal control heart. Especially in our ischemic rat heart, the increase in O-form was paralleled by the decrease in D/O-form. From this, it is thought that in the ischemic rat heart, the conversion of xanthine oxidase may occur from D/O-form to O-form rather than from D-form to O-form. The D/O-form of xanthine oxidase is an intermediate form which can use both NAD and O_2 as electron acceptors. NAD and O_2 appear to compete for D/O-form at same site of the enzyme. The affinity of D/O-form is considerably greater for NAD than for O_2(Kaminiski and Jezewaka 1979). Considering this fact and an observation that a significant decrease in NAD is not noted until after 90 min or longer of ischemia(Jennings *et al.* 1981), D/O-form in our rat heart may probably act as NAD-dependent D-form even after the 60 min ischemia and during the reperfusion period.

When ferricytochrome C was infused in the presence of superoxide dismutase, the icreased reduction of ferricytochrome C during the reperfusion period was almost completely prevented. This is thought to provide evidence for the production of superoxide anion in this ischemic-reperfused rat heart. Additionally, hydrogen peroxide in the coronary effluent was also markedly elevated by the reperfusion. The present result that allopurinol has prevented these increases in superoxide anion and hydrogen peroxide strongly suggests xanthine oxidase-linked production of oxygen radicals in the ischemic-reperfused rat heart. Based on the O-form activity in the ischemic heart of dog(0.07 U/g tissue protein), Chambers *et al.* (1985) calculated the amount of superoxide anion as much as 5.6 nmole/min/g wet wt. This was quite similar to that estimated from the ferricytochrome C reduction in our present study(2-3.3 nmole/30 sec/g wet wt).

Other than xanthine oxidase, leucocyte, mitochondria and catecholamines are being included in the possible source of oxygen free radicals in the ischemic-reperfused heart. It has been demonstrated that polymorphonuclear leucocyte produces superoxide anion during phagocytosis(Salin and McCord 1974). Although the importance of leucocytes as a source of oxygen raidclas has been recognized in several pathologic conditions such as inflammation and ischemic tissue injuries(Fantone and Ward 1982; Jolly *et al.* 1984; Romson *et al.* 1983), it seems unlikely that leucocytes play a major role in the early ischemic-reperfusion injury of myocardium. It was reported that the leucocyte content of infarcted tissue was increased 18-fold in 24 hrs after the onset of ischemia(Romson *et al.* 1983). However, it barely doubles in the early ischemic region at 3 hrs after the onset of coronary occlusion(Hearse *et al.* 1986). Furthermore, in

case of the experimental ischemic-reperfusion injury using isolated heart, like in the present study, the involvement of leucocyte is excluded. Catecholamine is released from the sympathetic nerve endings in the ischemic region(Newman *et al.* 1971). Although this catecholamine has been known to produce oxygen radicals during its break-down by the enzyme monoamine oxidase(Rao *et al.* 1983; Singla *et al.* 1983), its role as a source of oxygen radicals in the ischemic-reperfusion injury of mycardium seems not to be significant. The actual amount of oxygen radical which can be produced by this catecholamine is yet to be determined but is thought to be small. In normal mitochondria, about 1% of the electron flow results in superoxide anion production at the NADH dehydrogenase step and near to the ubiquinone component(Bovaris *et al.* 1976; Bovaris and Chance 1973; Turner and Bovaris 1980). In normal conditions, this small amount of superoxide anion is eliminated by superoxide dismutase which is normally existed in cytosole as well as in mitochondria. During ischemia, mitochondrial function might well be altered so that more superoxide anion is produced. Currently, however, it has not been clarified that mitochondria act as a major source of oxygen radicals in ischemic-reperfused myocardium.

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= 국문초록 =

허혈심근 재관류손상에 있어서 Xanthine Oxidase의 역할

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허혈심근의 재관류손상에 있어서 유독성 산소라디칼의 관련과 그 산소라디칼의 출처 및 발생기전으로서의 xanthine oxidase의 역할을 규명코저 하였다.

Langendorff 관류, 흰쥐 적출심장의 대동맥 카뉼라를 차단하여 허혈상태(60분)를 만든 후 계속해서 산소가 포화된 Krebs-Henseleit용액(pH 7.4)으로 20분동안 재관류(관류압 100 cmH_2O, 37°C)하여 실험적인 재관류손상을 유도하였다. 재관류시 creatine phosphokinase(CPK), lactic dehydrogenase(LDH) 및 지질과산화산물의 하나인 malondialdehyde(MDA)의 유출을 세포손상의 지표로 삼아 xanthine oxidase의 억제약물인 allopurinol의 심근보호효과를 산소라디칼제거물질과 비교검토하고, 심근조직에서 산소라디칼의 생성을 직접 관찰하였으며, 한편으로는 허혈심근의 xanthine oxidase(D, O 및 D/O형) 함량과 이 효소의 기질인 hypoxanthine, xanthine 축적여부를 관찰하여 다음과 같은 성적을 얻었다.

1. CPK, LDH 및 MDA의 유리는 재관류와 더불어 급격히 증가하였으며, 산소라디칼제거물질인 superoxide dismutase(5,000 U), catalase(12,500 U)는 이들의 유리증가를 억제하였다.

2. Allopurinol의 전처치(20 mg/kg, 실험 24 hr과 2 hr전에 구강투여)는 이들 세포질효소 및 MDA의 유출증가를 산소라디칼 제거물질과 동등한 정도로 억제하였다.

3. 재관류와 더불어 SOD-억제성 ferricytochrome C 환원과 관상 관류액중의 H_2O_2농도가 증가하였다.

4. Allopurinol의 전처치는 재관류시 ferricytochrome C환원과 H_2O_2 농도증가를 억제하였다.

5. 허혈심근의 xanthine oxidase는 정상심근에 비하여 총함량에는 큰 차이가 없으나, D형 및 D/O형은 감소하는 반면 산소라디칼생성 O형은 증가하였다.

6. 허혈심근의 adenine nucleotide함량은 정상심근에 비하여 감소하나, 그 분해산물인 hypoxanthine 및 xanthine은 현저히 증가하였다.

이상의 결과에서 허혈심근의 재관류시에는 xanthine oxidase를 통한 유독성산소라디칼의 생성이 증가되며, 그 산소라디칼은 심근 손상 유발에 크게 관여할것으로 사료된다.