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INACTIVATION OF RABBIT MUSCLE CREATINE KINASE BY HYDROGEN PEROXIDE

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The effects of xanthine + xanthine oxidase-generated reactive oxygen species (ROS) on rabbit muscle creatine kinase (CK) were studied. Xanthine (0.1 mM) + xanthine oxidase (30 mU/ml) inhibited activity of rabbit muscle CK (1.2 mU/ml). Catalase (100 U/ml), but not SOD (100 Uml), deferoxamine (100 μM) or mannitol (20 mM), protected CK from inactivation; suggesting that H_2O_2 was responsible for inactivation. These results were different from previously reported findings on bovine heart CK that superoxide radicals inactivate the enzyme. Thus, enzymes with homologous structures may have different reactivities to different ROS. H_2O_2-induced inactivation of rabbit muscle CK was accompanied by a decrease in its thiol group content, whereas no significant changes in the protein structure were detected by SDS-PAGE or carbonyl content. These results suggest that oxidation of -SH groups by H_2O_2 seems to be a major mechanism of activation of rabbit muscle CK by xanthine + xanthine oxidase. Such inactivation of CK by H_2O_2 may be important in ROS-induced pathology.

KEY WORDS: creatine kinase, hydrogen peroxide, oxidative damage, oxy-radicals, superoxide anion radical, xanthine oxidase.

INTRODUCTION

Electron reduction of molecular oxygen generates reactive oxygen species (ROS) including superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^\cdot$). These ROS are now implicated in the development of pathophysiological conditions including aging, cancer, heart disease, muscular dystrophy, and many others.[1-3] Thus, elucidating the mechansims of ROS-induced cellular damages is important. The dimeric enzyme creatine kinase (CK) [ATP: creatine *N*-phosphotransferase; EC 2.7.3.2] has been an important indicator in some of these pathological conditions.[4] Therefore, the effect of ROS on CK may be an important part of underlying mechanisms of ROS-induced cellular injury.

Inactivation of bovine heart CK ROS generated by xanthine oxidase plus its substrate xanthine has been reported.[5] It was found that O_2^- is capable of inhibiting CK through mechanisms which do not involve H_2O_2 or $HO^\cdot$. This is an important finding because the superoxide theory of oxygen toxicity proposed by McCord and Fridovich following their discovery of superoxide dismutase,[6] has been neglected after the realization of the subsequent generation of more reactive species.[7] Reconsideration

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of the superoxide theory of oxygen toxicity is supported by findings that several other enzymes have been reported to be inactivated by reactions with O_2^-.[5,8] This suggests that O_2^- possesses a specific reactivity to inactivate certain enzymes.

In the present study, we examined the effects of xanthine plus xanthine oxidase on CK from rabbit muscle. We found that this CK, unlike bovine heart CK, is not inactivated by O_2^-, but is inactivated by H_2O_2. We also found that the H_2O_2-induced inactivation of rabbit muscle CK is accompanied by a decrease in sulfhydryl (SH) content, but is not accompanied by protein fragmentation, aggregation, or carbonyl group formation. These results indicate that enzymes with homologous structures may possess different reactivities to ROS.

MATERIALS AND METHODS

Rabbit muscle CK (type I) and bovine heart CK (type III) were purchased from Sigma Chemical Company. 1.2 mg/ml CK was incubated in 50 mM potassium phosphate (pH 7.0) in the presence or absence of 0.1 mM xanthine (Sigma; X) and/or 30 mU/ml xanthine oxidase (EC 1.1.3.22, Grade III, from buttermilk, Boehringer Mannheim; XO) for 30 min at 37°C. 100 U/ml superoxide dismutase (EC 1.15.1.1., from bovine erythrocytes, Sigma; SOD), 100 U/ml catalase (EC 1.11.1.6, from bovine liver, Sigma; CAT), 20 mM D-mannitol or 100 μM deferoxamine mesylate (Sigma, DFA) were added before the initiation of O_2^- generation as scavengers for O_2^-, H_2O_2, $HO^{\cdot}$ and an inhibitor of the Fenton reaction, respectively. Activities of xanthine oxidase and SOD were tested by monitoring the reduction of ferricytochrome *c* (from horse heart, type VI, Sigma) by O_2^- at wavelength of 550 nm.[9]

CK activity was assayed using a colorimetric kit obtained from Sigma (#520) at a wavelength of 520 nm using a Perkin-Elmer Lambda 4B spectrophotometer. This assay determines the CK activity based on the following reactions:

$$\text{ADP} + \text{Phosphocreatine} \xrightarrow{\text{CK}} \text{ATP} + \text{Creatine}$$

$$\text{Creatine} + \alpha\text{-Naphthol} + \text{Diacetyl} \longrightarrow \text{Chromogen}$$

SH contents were measured as described by Suzuki *et al.*[10] using DTNB [5, 5′-dithiobis (2-nitrobenzoic acid)]. A molar extinction coefficient of $1.36 \times 10^4\,M^{-1}\,cm^{-1}$ at 412 nm was used for calculation.

Carbonyl content was measured as described by Levine *et al.*[11] using 2, 4-dinitrophenylhydrazine. Carbonyl content was calculated from the maximum absorbance (360–390 nm) using an extinction coefficient of $2.2 \times 10^4\,M^{-1}\,cm^{-1}$.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted using a vertical slab gel (1.5 mm thick; 10% T 2.7% C). Gels were stained with Coomassie Brilliant Blue R-250.

Significant differences between control groups and experimental groups were determined by the student's t-tests at $P < 0.05$.

RESULTS

As shown in Figure 1(A), the rabbit muscle CK (1.2 mg/ml) was inactivated by xanthine (0.1 mM) plus xanthine oxidase (30 mU/ml). This effect was completely

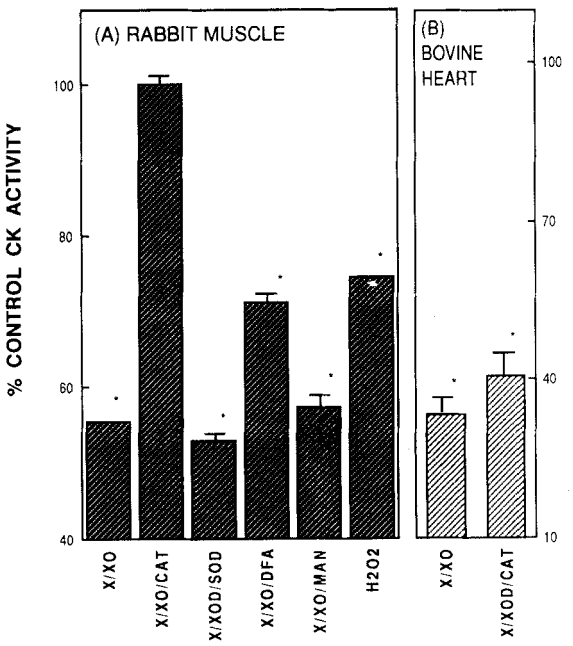

FIGURE 1 Effects of xanthine + xanthine oxidase on creatine kinase (CK) activity. (A) rabbit muscle creatine kinase; (B) bovine heart creatine kinase: [xanthine, X] = 0.1 mM; [xanthine oxidase, XO] = 30 mU/ml; [catalase; CAT] = 100 U/ml; [superoxide dismutase; SOD] = 100 U/ml; [deferoxamine; DFA] = 100 μM; [D-mannitol] = 20 mM; [hydrogen peroxide, H_2O_2] = 100 μM. Control contained XO for all X/XO experiments. Values ae means $\pm$ S.E. of 3 replicates. Value denoted with (*) represents significantly different value from control value at $P < 0.05$. The actual control values were (A) 2380 $\pm$ 87.5 and (B) 2836.4 $\pm$ 63.7 μmol $\cdot$ min^{-1} $\cdot$ l^{-1}

prevented by catalase (100 U/ml). SOD (100 U/ml) did not demonstrate any protective effect. This concentration of SOD completely inhibited the reduction of ferricytochrome *c* by xanthine plus xanthine oxidase at concentrations used in these experiments (data not shown), thus the preparation of SOD used herein had a reliable O_2^--eliminating activity. This combination of results suggests that H_2O_2, but not O_2^-, is involved in the inhibition of rabbit muscle CK. Deferoxamine (100 μM) partially protected against inhibition suggesting that $HO^\cdot$ may be involved. However, the observation that SOD had no protective effect argues against such an involvement of $HO^\cdot$ because SOD eliminates O_2^- which is needed for iron-catalyzed $HO^\cdot$ generation. The ineffectiveness of D-mannitol (20 mM) strengthens the notion that $HO^\cdot$ is not involved. Direct addition of 100 μM H_2O_2 significantly inactivated rabbit muscle CK activity. Thus, H_2O_2 seems to be the primary damaging ROS in the xanthine plus xanthine oxidase-induced inactivation of rabbit muscle CK. As shown in Figure 1(B), bovine heart CK (1.2 mg/ml) could be inactivated by xanthine (0.1 mM) plus xanthine oxidase (30 mU/ml). This effect was only partially prevented by catalase (100 U/ml) suggesting that O_2^- is capable of inactivating the enzyme. These results were consistent with the findings previously reported at pH 7.0.[5] Neither xanthine alone nor xanthine oxidase alone had any effects on the activity of either bovine heart CK or rabbit muscle CK. H_2O_2 (up to 200 μM) had no interfering effects on the CK assay system.

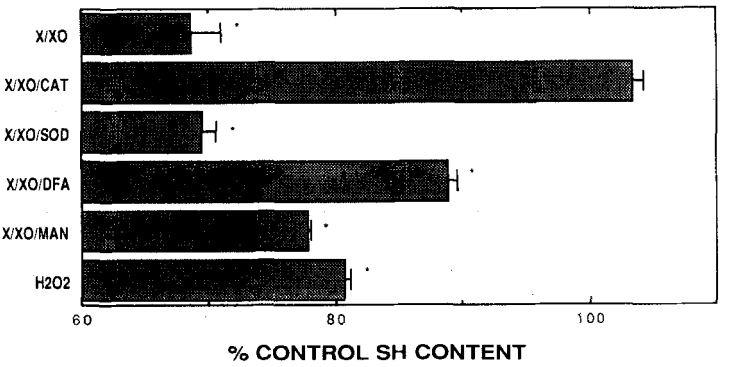

FIGURE 2 Effects of xanthine + xanthine oxidase on sulfhydryl (SH) content of rabbit muscle creatine kinase. [xanthine, X] = 0.1 mM; [xanthine oxidase, XO] = 30 mU/ml; [catalase; CAT] = 100 U/ml; [superoxide dismutase; SOD] = 100 U/ml; [deferoxamine; DFA] = 100 μM; [D-mannitol] = 20 mM; [hydrogen peroxide, H_2O_2] = 100 μM. Control contained XO for all X/XO experiments. Values are means $\pm$ S.E. of 3 replicates. Value denoted with (*) represents significantly different value from control value at $P < 0.05$. The actual control value was 42.67 $\pm$ 1.54 μM.

In order to elucidate the mechanisms by which ROS inactive rabbit muscle CK, we studied the effects of xanthine plus xanthine oxidase on sulfhydryl (SH) oxidation, carbonyl formation, and protein fragmentation and/or aggregation. The same concentrations of xanthine plus xanthine oxidase which inhibited the rabbit muscle CK also decreased the SH content of this enzyme (Figure 2). Exactly the same pattern was observed in the SH oxidation as the inactivation of the enzyme activity when the effects of reactive oxygen scavengers were tested. Catalase, but not SOD, deferoxamine or D-mannitol, completely protected the rabbit muscle CK from SH oxidation induced by xanthine plus xanthine oxidase. Deferoxamine was partially protective. These results strongly suggest that SH oxidation is involved in H_2O_2-induced inactivation of rabbit muscle CK.

Xanthine plus xanthine oxidase did not significantly affect the carbonyl content (Table I) or cause the fragmentation/aggregation as detected by SDS-PAGE (no changes in the 41 kDa band) of rabbit muscle CK. Thus, neither carbonyl formation nor fragmentation/aggregation seem to be involved in the mechanism of the inactivation of rabbit muscle CK by xanthine plus xanthine oxidase. These results suggest that the SH oxidation induced by H_2O_2 is the major mechanism of the inactivation of rabbit muscle CK by xanthine plus xanthine oxidase.

TABLE I
Effects of xanthine + xanthine oxidase on carbonyl content

	Carbonyl content (nmoles/mg protein)
CK + XO	0.52 $\pm$ 0.14 (n = 4)
CK + X/XO	0.57 $\pm$ 0.24 (n = 4)

Values are mean $\pm$ S.E.

DISCUSSION

The involvement of ROS in various pathological conditions and the alteration of CK in some of these conditions strongly suggest the importance of the relationship between ROS and CK. Thus, clarification of the mechanisms of the effects of ROS on CK is warranted.

The present study examined the mechanisms of ROS-induced damage to rabbit muscle CK using xanthine plus xanthine oxidase as a ROS generator. It was shown that the inactivation of rabbit muscle CK by xanthine plus xanthine oxidase is mediated by H_2O_2. Thus finding is different from a previous report that bovine heart CK is inactivated by O_2^-.[5] These results suggest that enzymes with homologous structures possess different reactivities to ROS.

Whether the inactivation of CK's by different ROS is due to a species difference or an organ difference is not clear. CK is a dimeric protein with two different types of monomers: M and B, approximately 40 kDa each.[12] The MM dimer is primarily found in skeletal muscle, the MB heterodimer in caridiac tissue, and a BB dimer in brain.[13] Thus, different isoforms of CK may possess different reactivities to ROS.

Our results strongly suggest that SH oxidation is a major mechanism of the xanthine plus xanthine oxidase-induced inactivation of rabbit muscle CK. The oxidation of the SH groups of CK by ROS is a very reasonable mechanism for the inactivation because a considerable number of reports point to the importance of SH groups in the activity of CK.[12] CK contains eight SH groups[14] and among them two are known to be titratable by DTNB in the absence of denaturants.[12] Our results are also consistent with a suggestion that ROS contribute to the *S*-thiolation of creatine kinase.[15]

ROS-induced biomolecular damage is generally thought to involve indiscriminate reactions mediated by $HO^{\cdot}$. More specific inactivation of enzymes by ROS, particularly O_2^- and H_2O_2, may be an important part of the underlying mechanisms of oxygen toxicity. These species are not indiscriminately reactive, thus their concentrations can more easily be conserved. If one biologically important molecule is susceptible to oxidative attack by O_2^- or H_2O_2, its inactivation may be enough to cause significant damage to the whole system. CK's may be one such susceptible physiological targets of ROS.

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