Rapid and irreversible inhibition of creatine kinase by peroxynitrite

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Abstract We examined the ability of peroxynitrite and other •NO-derived oxidants to inhibit creatine kinase (CK). Peroxynitrite potently inhibited CK activity and depleted protein thiols. The rate constant for this reaction was 8.85×10^5 M^{-1} s^{-1}. Glutathione did not reactivate CK activity nor did it regenerate protein thiol content. In contrast, glutathione reactivated CK, and regenerated protein thiols, after inhibition by either •NO or oxidized glutathione (GSSG). Peroxynitrite did not irreversibly inhibit CK after it had been treated with GSSG to block protein thiols. We conclude that thiol oxidation is a critical event leading to inactivation of CK by peroxynitrite.

Key words: Peroxynitrite; Nitric oxide; Superoxide; Creatine kinase

1. Introduction

Creatine kinase (CK; ATP:creatine *N*-phosphoryltransferase, EC 2.7.3.2) catalyzes the reversible transfer of the phosphoryl group from phosphocreatine (PCr) to ADP, to form ATP (Eq. 1). The rate of turnover of the CK reaction exceeds the rate

$$\mathrm{PCr} + \mathrm{ADP} \rightleftharpoons \mathrm{Creatine} + \mathrm{ATP} \quad (1)$$

of ATP synthesis in the cell by an order of magnitude [1]. This explains the ability of excitable tissues, such as cardiac, skeletal muscle, neurons and retina, to cope with changing energy consumption rates during periods of increased performance [2]. Proposed functions of the cellular CK systems include temporal energy buffering, regulation of oxidative phosphorylation and the transport of chemical potential, in the form of PCr, between sites of ATP production and energy utilization [2,3]. Inhibition of myocardial CK compromises contractile function and end-diastolic pressure at higher workloads [4–6]. Depression of CK activity in the heart has been implicated in the pathogenesis of cardiomyopathies and heart failure [7,8].

The active site of CK contains a cysteinyl residue that is essential for substrate binding. Substitution of this cysteine with a serine results in a 500-fold decrease in enzyme activity [9]. Consequently, CK is susceptible to inactivation by oxidative reactions [10,11]. In addition, post-translational modification of thiol groups by transnitrosation from *S*-nitrosothiols inhibits its catalytic activity [12,13]. Peroxynitrite, formed in the reaction between superoxide and •NO, is a potent oxidant of GSH forming GSSG [14], and will oxidize BSA thiols to unknown products via the formation of a protein thiyl radical [14,15]. In addition, it oxidizes the Zn finger thiols of alcohol dehydrogenase with a rate constant of $2.6–5.2\times10^5$ M^{-1} s^{-1} [16]. It has been also shown that peroxynitrite is formed in muscle tissues during sepsis, autoimmune, inflammatory conditions and ischemia-reperfusion injury [17–21]. In this study, we investigated the effect of •NO, superoxide and peroxynitrite on the activity of CK from muscle tissue. We show that peroxynitrite is a rapid and irreversible inhibitor of CK activity. The combination of •NO and superoxide inhibited CK more potently than either of these agents alone.

2. Materials and methods

2.1. Reagents

Xanthine oxidase (XO), superoxide dismutase (SOD), imidazole, and CK from rabbit muscle were purchased from Boehringer Mannheim (Indianapolis, IN). 1,3-Propanediamine-*N*-[4-[1-(3-aminopropyl)-2-hydroxy-2-nitrozohydrazino[butyl](spermine NONOate, SNN) and SIN-1 hydrochloride were obtained from Cayman Chemical (Ann Arbor, MI). All other reagents were obtained from Sigma (St. Louis, MO).

2.2. Assay of CK activity

The activity of CK was measured as described by Forster et al. [22]. ssay medium contained (in mM): imidazole, 100; glucose, 20; $MgSO_4$, 10; ADP, 1; NAD^+, 0.7; PCr, 35. The reaction was followed by detecting the formation of NADH at 340 nm after addition of hexokinase (1 U/ml) and glucose 6-phosphate dehydrogenase (1 U/ml) [22]. *N*-Acetylcysteine was omitted from the assay medium to preserve the status of CK thiol groups. Compounds used to inactivate CK did not interfere with the activities of hexokinase and glucose 6-phosphate dehydrogenase used in the assay.

2.3. Synthesis of peroxynitrite

Peroxynitrite was prepared as described previously [23]. Briefly, an ice cold solution of sodium nitrite (0.6 M) was rapidly mixed with cold acidified hydrogen peroxide (0.6 M in 0.8 M HCl) and quenched with sodium hydroxide (1.4 M). Excess hydrogen peroxide was removed with manganese dioxide. All reactions were performed on ice. The concentration of peroxynitrite was determined using UV-visible spectroscopy at $\lambda = 302$ nm ($\varepsilon = 1670$ M^{-1} cm^{-1}). The stock solution of peroxynitrite was diluted in sodium hydroxide (100 mM) before use.

2.4. Generation of superoxide and •NO

Two systems were employed to simultaneously generate •NO and superoxide. •NO was formed upon decomposition of SNN in imidazole buffer (100 mM, pH 6.9). Superoxide radical was generated in a system containing xanthine (10 μM) and XO (2 mU/ml). SIN-1, a sydnonimine, spontaneously decays in aerobic buffer to generate both nitric oxide and superoxide [24] and has been used as a model for the continuous formation of peroxynitrite [25]. SIN-1 was decomposed to peroxynitrite in aerobic imidazole buffer (100 mM, pH 6.9).

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Abbreviations: CK, creatine kinase; •NO, nitric oxide; GSH, glutathione, reduced form; GSSG, glutathione, oxidized form; SNN, spermine NONOate; SIN, 1,3-morpholino-sydnonimine; XO, xanthine oxidase; SOD, superoxide dismutase; PCr, phosphocreatine; GSNO, *S*-nitrosoglutathione; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); BSA, bovine serum albumin; iNOS, inducible •NO synthase

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2.5. Chromatographic separation of CK

After incubation with inhibitors or GSH, CK was reisolated in imidazole buffer (100 mM, pH 6.9) on a Sephadex G-25 column. After separation protein was concentrated using Centricon-50 ultrafiltration devices (Millipore, Bedford, MA).

2.6. Assays of protein thiols and protein concentration

Protein thiols were measured using 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) at $\lambda = 412$ nm ($\varepsilon = 11\,000$ M^{-1} cm^{-1}). Protein was determined using the Bradford assay [26].

3. Results

3.1. Effect of peroxynitrite on CK activity

As shown in Fig. 1A, peroxynitrite caused a concentration dependent inhibition of CK (4 μg/ml or 48 nM) activity with an IC_{50} (concentration required to inhibit 50% of enzyme activity) of 2.5 μM. At this concentration of peroxynitrite and CK, approximately 50 mol of peroxynitrite are required to inactivate 1 mol of enzyme. Decomposed peroxynitrite (i.e. allowed to decompose in imidazole buffer, 100 mM, pH 6.9 for 10 min) did not inhibit CK activity (not shown). A kinetic analysis of the data shown in Fig. 1A was performed using the approach taken by Padmaja et al. [27]. If it is assumed that a 1:1 stoichiometric ratio exists between peroxynitrite and enzyme

$$CK_{Active} + ONOO^- \xrightarrow{k_1} CK_{Inactive} + Products \quad (2)$$

$$ONOO^- \xrightarrow{k_2} Products \quad (3)$$

$$[ONOO^-] = Z(A_0 - A_i) + \left(\frac{k_2}{k_1}\right)\ln\left(\frac{A_0}{A_i}\right) \quad (4)$$

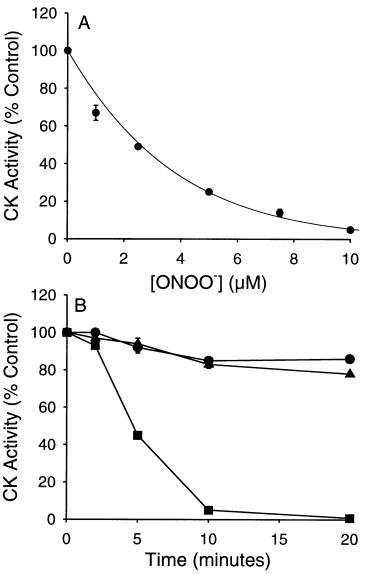

Fig. 1. Effect of peroxynitrite on CK activity. A: Peroxynitrite (0–10 μM in 100 mM NaOH) was added to CK (4 μg/ml in 100 mM imidazole, pH 6.9, containing 100 μM DTPA) and vortex-mixed immediately. The pH of the mixture was not significantly altered by addition of peroxynitrite. CK activity (●) was determined within 10 min. The solid line represents a fit of Eq. 4 to the data where $k_1 = (8.85 \pm 1.1) \times 10^5$ M^{-1} s^{-1}. B: CK (4 μg/ml) in 100 mM imidazole, pH 6.9, containing 100 μM DTPA) was incubated with SNN (1 μM) (●), XO (2 mU/ml) and xanthine (10 μM) (▲), or a combination of all three agents (■), for 20 min. Aliquots were removed at the indicated times and assayed for CK activity. Data represent mean ± S.E.M. ($n = 3$).

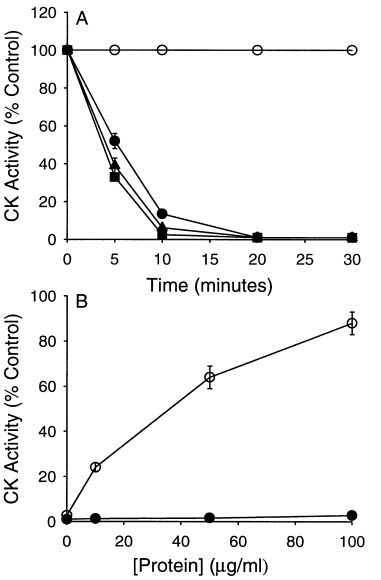

Fig. 2. Effect of SIN-1 on CK activity. A: Effect of SIN-1 and SIN-1C on CK activity. CK was incubated in 100 mM imidazole buffer (pH 6.9) containing 100 μM DTPA at 37°C with 100 μM SIN-1 or with the product of SIN-1 decomposition, SIN-1C; (○, SIN-1C; ●, SIN-1; ▲, SIN-1 in the presence of 1 mM of $NaHCO_3$; ■, SIN-1 in the presence of 25 mM $NaHCO_3$). B: Effect of SOD on SIN-1 induced inhibition of CK activity. CK was incubated with SIN-1 (100 μM) and the indicated concentrations of SOD (◯) or BSA (●) for 30 min at 37°C in imidazole buffer (100 mM, pH 6.9) containing 100 μM DTPA. SOD activity was 5000 U/mg protein. Data represent mean ± S.E.M. ($n = 3$).

inactivation (Eq. 2), and that the only other route of peroxynitrite decomposition is spontaneous decay (Eq. 3), then competition analysis gives Eq. 4, where A_0 is enzyme activity before addition of peroxynitrite, A_I is enzyme activity after peroxynitrite, Z is a factor to convert enzyme activity to concentration of active enzyme. The value of k_2 is 2.84 s^{-1} at pH 6.9 (calculated from $k_2 = 0.9$ s^{-1} at pH 7.4 and $pK = 6.8$ for peroxynitrite). Fig. 1A shows a fit of Eq. 4 to the experimental data giving $k_1 = (8.85 \pm 1.10) \times 10^5$ M^{-1} s^{-1} and $Z = 6.9$ nM. The inhibitory activity of peroxynitrite was not dependent on the buffer system as the irreversible inhibition of CK was also observed using phosphate buffer (100 mM, pH 6.9, containing 100 μM DTPA).

Incubation of CK in buffer containing the •NO donor, SNN (1 μM) or components of the superoxide generating system (10 μM xanthine + 2 mU/ml XO) caused little inactivation of CK (Fig. 1B). However, in combination, these agents caused a time dependent inhibition of enzyme activity, leading to complete inactivation within 20 min (Fig. 1B).

SIN-1, in the presence of oxygen, decomposes to form equal amounts of •NO and superoxide which combine to form peroxynitrite [24,25]. SIN-1 (100 μM) completely inhibited enzyme activity after 20 min (Fig. 2A). In the presence of bicarbonate (1 and 25 mM), the inhibition of CK was slightly enhanced (Fig. 2A). The decomposition product of SIN-1, SIN-1C, had no effect on CK activity (Fig. 2A). SOD was used to scavenge superoxide and thus prevent the formation of peroxynitrite during SIN-1 decomposition. SOD protected CK from SIN-1-dependent inhibition in a concentration dependent manner (Fig. 2B). This protection was specific for

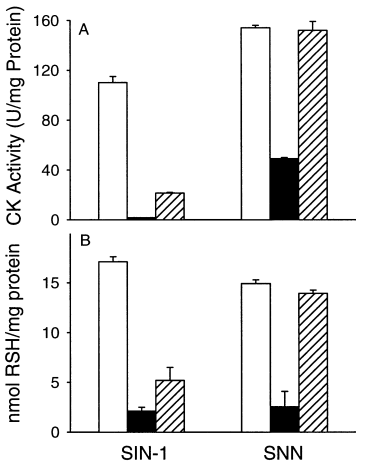

Fig. 3. Effect of GSH on protein thiols and activity of CK inhibited with peroxynitrite donor SIN-1 or •NO donor SNN. CK was incubated at room temperature for 30 min with SIN-1 (10 mM) or for 120 min with SNN (10 mM) in imidazole (100 mM) buffer containing 100 μM DTPA. After incubation CK was separated from SIN-1 and incubated with 10 mM GSH, and then concentrated and separated from GSH for protein thiols and activity assays. Control, white bar; after SIN-1 treatment, black bar; after SIN-1 followed by GSH treatment, hatched bar. Data represent mean ± S.E.M. ($n = 3$).

SOD, as bovine serum albumin (BSA) did not affect SIN-1-induced inhibition of CK.

3.2. Recovery of CK activity and protein thiols

Reduced glutathione (GSH) is a predominant cellular thiol present in cytosol at high concentrations (1–10 mM) that acts to maintain intracellular thiols in a reduced state. It follows that any inhibition of enzyme activity that occurs as a result of thiol oxidation may be subject to repair by GSH. We examined if GSH was able to reactivate CK after inactivation by the simultaneous generation of •NO and superoxide. CK, inactivated to 0.8% of initial activity, was incubated for 30 min with 10 mM GSH. After this treatment, enzyme activity was recovered to only 5% of its initial value (not shown). Thus, the inactivation of CK, from the simultaneous generation of •NO and superoxide could not be reversed by GSH. Similar results were obtained using dithiothreitol as a thiol reducing agent (not shown).

CK previously inactivated by SIN-1, was incubated with GSH (10 mM) for 30 min. GSH treatment recovered CK activity to only 20% of the initial value (Fig. 3A). In contrast, the activity of CK inhibited with SNN was completely recovered upon addition of 10 mM GSH (Fig. 3A). SIN-1 dramatically decreased the amount of DTNB detectable thiol groups (Fig. 3B). After addition of 10 mM GSH, only 20% of thiols were regenerated indicating that SIN-1 chemically modifies CK thiols predominantly to a form that is not reducible by GSH. SNN also reduced the amount of thiols, however, addition of GSH completely regenerated protein thiols to initial values (Fig. 3B).

Peroxynitrite is a fairly non-specific oxidant and several other amino acid residues, such as tyrosine and methionine, can be modified by reaction with peroxynitrite. In order to determine if protein thiol modification is the mechanism for CK inactivation, CK thiol groups were shielded by reversible thiolation with oxidized glutathione (GSSG). Incubation of CK with GSSG decreased both CK activity and the protein associated thiol concentration (Table 1). This preparation of CK was exposed to SIN-1 (10 mM for 60 min), after which it was incubated with GSH (10 mM for 60 min). GSH regenerated thiols after SIN-1 treatment and enzyme activity was completely recovered (Table 1). This result indicates that the oxidation of protein thiols is the major mechanisms of SIN-1 dependent enzyme inactivation and that additional modification of other amino acid residues does not affect the activity of CK.

4. Discussion

The study shows that CK is extremely sensitive to both authentic peroxynitrite and the simultaneous generation of •NO and superoxide. The rate constant for the reaction between peroxynitrite and the active-site thiol of CK was determined to be $(8.85 \pm 1.1) \times 10^5$ M^{-1} s^{-1}. This rate constant is similar to that determined for heme-containing peroxidases of $3.3–64 \times 10^5$ M^{-1} s^{-1} [28] and for the Zn finger thiols of alcohol dehydrogenase ($2.6–5.2 \times 10^5$ M^{-1} s^{-1}) [16]. Interestingly, the rate constant is significantly larger than that for the selenol-containing glutathione peroxidase (4.5×10^4 M^{-1} s^{-1}) [27] and for the thiol group of bovine serum albumin ($2.6–2.8 \times 10^3$ M^{-1} s^{-1}) [14]. Peroxynitrite is a substantially more potent agent than either superoxide, •NO (Fig. 1B), or hydrogen peroxide (unpublished observations).

Peroxynitrite is able to oxidize thiol groups [14,15], and to nitrate tyrosine residues [29]. The active site of CK contains an essential cysteine residue [9], and the MgADP binding site contains tyrosine residues [30]. These could be the targets for peroxynitrite and modification/oxidation of these residues may be responsible for enzyme inhibition. This study suggests that peroxynitrite induced CK inhibition is due to the oxidation of protein thiol groups. CK activity correlates well with DTNB detectable thiol concentration, and it has previously been established that only the active site cysteine of CK can be detected in non-denaturing conditions [31]. Inhibition of CK activity by peroxynitrite and SNN is associated with a decreased amount of protein thiols, and the degree of the enzyme reactivation by GSH is paralleled by the regeneration

Table 1
Effect of GSH on protein thiols and activity of CK exposed to SIN-1, after shielding protein thiol groups with GSSG

	Activity (U/mg protein)	Protein thiols (nmol/mg protein)
CK	142 ± 8	18.2 ± 0.5
CK+GSSG	23 ± 0.8	2.0 ± 0.6
CK+GSSG+SIN-1	6 ± 0.4	1.3 ± 0.2
CK+GSSG+SIN-1+GSH	135 ± 5	14.5 ± 0.2

CK was incubated with 5 mM GSSG for 60 min in imidazole buffer (100 mM, pH 6.9 containing 100 μM DTPA). After separation, CK was treated with SIN-1 (10 mM) for 60 min. Isolated protein was reactivated with GSH (10 mM) for 60 min. Data represent mean ± S.E.M. (n=3).

of thiol groups (Fig. 3). In addition, SIN-1 did not cause irreversible inhibition of CK when the enzyme thiol groups had been shielded by reversible thiolation with GSSG (Table 1).

CK previously inactivated with peroxynitrite could not be reactivated by thiol reducing agents nor could the active site thiol be repaired. This suggests that neither *S*-nitrosation nor sulfenic acid formation is the mechanism of enzyme inactivation. The nitrosating agent, *S*-nitrosoglutathione (GSNO), is also known to potently inhibit CK, but its inhibitory effect is reversed by the excess of GSH [12,13] (unpublished results). High concentrations of •NO also inactivate the enzyme (Fig. 3), but, as in the case of GSNO, the inhibition was completely reversed by GSH. These results implicate peroxynitrite as the most likely and potent oxidant to cause irreversible inactivation of CK in vivo. Peroxynitrite is known to combine rapidly with carbon dioxide, present at relatively high concentrations inside the cells, to form $ONOOCO_2^-$ [32]. Therefore it is important to examine the effect of bicarbonate on peroxynitrite dependent reactions. Inactivation of CK by SIN-1, in the presence of bicarbonate was slightly accelerated. Interestingly, bicarbonate has been demonstrated to inhibit peroxynitrite dependent thiol oxidation [32] and partially to inhibit the inactivation of glutathione peroxidase [27]. This suggests that the active site of CK does not behave like a low molecular weight thiol and that neighboring residues appear to influence the reactivity of peroxynitrite with the active site thiol. The influence of close neighbor residues on peroxynitrite chemistry has previously been demonstrated, as peroxynitrite dependent tyrosine nitration is facilitated by the presence of glutamate [33].

Exogenous peroxynitrite has been reported to impair cardiac contractile function without reduction in energy substrate utilization and oxygen consumption [34]. The decreased cardiac efficiency is likely to be due to uncoupling between production of ATP and its utilization by myofibrils. This could be due to the inhibition of CK, which provides the coupling between the energy producing and energy utilizing processes within the cell. Inhibition of myocardial CK in isolated rat heart results in reduced contractile reserve indicating that CK ensures mechanical function during periods of increased performance [5,6]. Peroxynitrite formation in cardiac tissue as a result of pathological processes may consequently lead to impaired contractile function. For example, the reduced myocardial contractility and deteriorated hemodynamics associated with experimental autoimmune myocarditis, which is improved by iNOS inhibitors, may be due to the formation of peroxynitrite [35,36].

In conclusion, the present data indicate that peroxynitrite is a rapid and irreversible inactivator of CK. In contrast, •NO and superoxide alone are much less potent inhibitors of CK and their effects can be reversed by GSH.

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