Mechanisms of Iron-Induced Oxidative Modifications of Creatine Kinase in Rat Brain *in vitro*. Possible Involvement of HNE

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Abstract. The model of oxidative stress induced by Fe/ascorbate in rat brain *in vitro* was used to compare the antioxidant capacity of known antioxidants. Creatine kinase (CK) was selected as a marker of protein injury in such studies. Of the antioxidant enzymes (catalase, superoxide dismutase), oxygen radical scavengers (mannitol, glutathione), and the chelator (EDTA) tested in this work and this system, only catalase and glutathione prevented the injury induced by oxidative stress, indicating that H_2O_2 and the glutathione peroxidase reaction were involved in the preventive effect. Additionally, the preventive effect of glutathione may be caused also by the fact that glutathione easily reacts with 4-hydroxynonenal (HNE), generated in rat brain homogenate, thus protecting CK from inactivation by this aldehyde. To find out whether and if at which concentrations CK may be oxidatively modified by HNE, pure CK was incubated in the presence of 10 and 64 μmol/l HNE for 30 min at 37 °C. The activity of CK incubated with HNE decreased significantly. Simultaneously, the protein carbonyls, determined by electrophoresis and immunoblotting increased at 10 μmol/l HNE or disappeared probably due to crosslinking of CK at 64 μmol/l HNE. The concentration of HNE in rat brain homogenates after oxidative stress was determined by HPLC and was in the range of 10–16 nmol/mg prot., corresponding to a concentration of 10–16 μmol/l HNE. This indicates that CK of rat brain homogenates oxidized by Fe/ascorbate may be impaired not only directly by oxygen radicals but also secondarily by HNE.

Key words: Brain oxidative stress — Creatine kinase — 4-hydroxynonenal — Antioxidants

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Introduction

Oxidative modification of proteins occurs in cells during oxidative stress in various pathological states including tissue injury provoked by ischemia-reperfusion and also during aging. These modifications can be primary or secondary. There are several primary mechanisms by which proteins can be oxidatively modified.

Most prominent mechanism of primary modification is probably that involving site-specific metal ion-catalyzed reactions (Stadtman 1990). Understanding of the mechanism of such oxidation is, however, based only on studies of a few enzymes, showing that His, Arg, Lys, Pro, Met, Cys residues are among the most common sites of oxidation by metal catalyzed oxidation (MCO) systems (Stadtman 1990, 1993). According to Levine et al. (1981) and Stadtman and Oliver (1991), the reduction of O_2 to H_2O_2 and Fe(III) to Fe(II) is provided by an electron donor system. It is believed that the Fe(II) binds to a metal binding site on the protein after which the protein-Fe(II) complex reacts with H_2O_2 to generate an oxygen species ($^{\bullet}OH$, ferryl compounds), which then reacts with the side chains of amino acid residues at the metal binding site *in situ*. This way free radical species directly attacks on amino acid converting it to a carbonyl group (Levine 1983; Amici et al. 1989; Davies et al. 1999). After oxidative modification, the protein becomes highly sensitive to proteolytic degradation, and in the case of an enzyme, it is converted to a catalytically inactive or less active and more thermolabile form (Stadtman and Oliver 1991).

Secondary modifications occur when proteins are modified by molecules originating through oxidation of other molecules, for example by 4-hydroxynonenal (HNE) produced by oxidation of omega-6-unsaturated fatty acids (Esterbauer et al. 1991). It was demonstrated that the histidine, cysteine and lysine residues of proteins are important targets for modification by HNE (Uchida and Stadtman 1992; Reinheckel et al. 1998; Eaton et al. 1999). HNE has a considerably longer half-life than free radical species and is capable of inhibiting the function of key enzymes (Uchida and Stadtman 1993; Siems et al. 1996), impair function of membrane transporters and promote neuronal death in variety of experimental models (Kruman et al. 1997; Mark et al. 1997).

We studied oxidative modification of creatine kinase (CK) in rat brain homogenates where oxidative stress was induced by *in vitro* addition of Fe/ascorbate. CK belongs to the enzymes sensitive to metal catalyzed oxidative modification (Fucci et al. 1983; Levine 1983; Aksenov et al. 1997). We analyzed the potential involvement of individual reactive oxygen species in oxidative modification of CK and tried to establish whether primary, secondary, or both mechanisms of modification were included in our model of oxidative stress.

Materials and Methods

Animals

Male normotensive rats of inbred Wistar strain (250–300 g) were used.

Agents and chemicals

Catalase, superoxide dismutase (SOD), mannitol were from Serva (Germany); histidine was from Reanal (Hungary). Protease inhibitors leupeptin, pepstatin, aprotinin and phenylmethylsulfonyl fluoride (PMSF), and creatine kinase were from Sigma (St. Louis, MO, USA). 4-hydroxynonenal was from Cayman Chemical (Ann Arbor, MI, USA).

Brain homogenate preparation

Animals were sacrificed by decapitation, the brains were removed and 10% homogenates were prepared by brain homogenization in icecold HEPES, pH 7.4, which contained (in mmol/l): 137 NaCl, 4.6 KCl, 1.1 KH_2PO_4, 0.6 $MgSO_4$ and protease inhibitors (in μg/ml): 0.5 leupeptin, 0.7 pepstatin, 0.5 aprotinin, and 40 phenylmethylsulfonyl fluoride (PMSF) to prevent proteins from degradation by most common proteases. A Janke Kunkel Ultra Turrax T25 homogenizer was used for homogenization. The brain homogenates were divided into small volumes and stored at −20°C until next day when CK activity was measured.

Oxidation of homogenates

Brain homogenates diluted in potassium phosphate buffer (50 mmol/l, pH 7.4) to the final concentration of 1 mg prot./ml were oxidized by $FeSO_4$ and ascorbic acid at final concentrations of 0.1 mmol/l and 0.5 mmol/l, respectively. The control sample contained homogenate only. Oxidation was started with ascorbic acid. The antioxidant enzymes or antioxidants were added to the homogenates prior to the oxidative system, to the final concentrations as indicated in Table 1. All samples were incubated at 37°C for 30 min. Oxidation was finished by addition of desferroxamine to the final concentration of 10 μmol/l. The samples were centrifuged at 10,000 × *g* and 5°C for 5 min, frozen and stored at −20°C until the following day when CK activity was determined. Each sample was investigated in four parallels.

CK activity determination

The Sigma diagnostics kit, procedure No. 661, was used for CK activity determination. The inorganic phosphate produced proportionally to CK activity was measured colorimetrically. In our experiments the rat brain supernatant (centrifuged at 10,000 × *g*) in the volume of 150 μl *per* sample was analyzed for CK activity.

Protein determination

Protein concentration was determined according to the method of Lowry et al. (1951). Bovine albumin (Serva, Germany) was used as a standard for protein determination.

Modification of pure CK by HNE

Pure CK was incubated for 30 min or 120 min in HEPES, pH 7.4, at 37°C either with HNE (at joint concentrations of 10 μmol/l or 64 μmol/l) or alone (at concentrations of 0.5 mg/ml or 1 mg/ml), see Table 2. A stock solution of CK was prepared

by dissolving 4 mg of pure CK in 200 μl of HEPES, pH 7.4. From this CK solution 3 μl or 6 μl were added to the final volume of 120 μl. Immediately after incubation, 2 μl of these samples were used to measure the CK activity as described above. Besides, a certain volume of some samples was used for electrophoretic identification of CK impairment.

Determination of CK protein carbonyls by Western blot analysis

Creatine kinase was incubated with or without HNE in final concentration of 10 or 64 μmol/l in HEPES, pH 7.4, at 37℃ for 30 min. The modified and control samples of creatine kinase were derivatized with dinitrophenylhydrazine (DNPH) for 18 min at room temperature and 1 μg of each protein was subjected to SDS-PAGE and immunoblot analysis using the Oxyblot Oxidized Protein Detection System (No. S7/150 KIT, Appligene, France), ECL Western blotting detection kit, together with a Hyperfilm MP (Amersham). Reactive carbonyl derivatives (RCD) in creatine kinase were evaluated by a BAS 2000 Bioimaging Analyser (Fuji Film Co., Tokyo, Japan).

Determination of HNE by HPLC

HNE determination was performed according to Kinter (1996). Rat brain homogenates (1 mg prot./ml) were incubated with Fe/ascorbate for 30 min at 37℃ as mentioned above. One ml of rat brain homogenate was taken immediately after Fe/ascorbate addition at time point zero and after 10, 20 and 30 min incubation. 0.5 ml DNPH (5 mmol/l) was added to 1 ml of each sample, vortexed and incubated for 1 h in dark. Subsequently, 250 μl and 500 μl of these samples underwent a three-fold extraction by 2 ml of hexane and the extracts were combined and evaporated to dryness under nitrogen at 40℃. The samples were stored at −60℃ until HPLC measurement. Each sample was vortexed with 140 μl acetonitrile to reconstitute the residue and 60 μl of water was added to adjust the solvent to 70% acetonitrile.

HPLC was performed on a 250 × 4 mm Nucleosil 120-5 C18 column (Watrex) using an isocratic mobile phase of acetonitrile/water (70 : 30, v/v) at a flow rate 1 ml/min with UV detection by a DeltaChrom UVD 200 at 355 nm.

A calibration curve in the range from 0–75 nmol of standard HNE was used for determination of HNE in rat brain homogenates.

Statistical evaluation

ANOVA and Bonferoni tests were used for statistical evaluation and expression of significances in experiments concerning the activity of CK and HNE level in rat brain homogenates.

Results

The effect of antioxidant enzymes, oxygen radical scavengers and a ferrous chelator on CK as a marker of protein injury was studied in oxidized rat brain homogenates. Decrease of CK activity was a measure of oxidative injury of this enzyme. The results are summarized in Tab. 1. The activity of CK from oxidized homogenates was

Table 1. Effect of antioxidants and antioxidative enzymes on activity of CK in rat brain homogenates

Antioxidant (*concentration*)	Activity of CK (U/mg prot.) Control	Oxidation	Antioxidant	Changes in CK activity *in percentage*
Catalase				
(49 000 U/ml)		($p < 0.01$)	($p < 0.05$)	
	169.9 ± 9.6	84.3 ± 5.5	128.3 ± 23.8	49
SOD				
(630 U/ml)		($p < 0.001$)	ns	
	174.3 ± 12.0	92.5 ± 2.0	66.2 ± 2.4	132
EDTA				
(200 μmo/l)		($p < 0.001$)	($p < 0.001$)	
	177.8 ± 2.5	92.5 ± 2.0	39.4 ± 1.0	162
Mannitol				
(30 μmol/l)		($p < 0.001$)	ns	
	209.1 ± 1.2	137.7 ± 0.4	139.8 ± 3.5	97
Mannitol				
(100 μmol/l)		($p < 0.001$)	($p < 0.05$)	
	209.1 ± 1.1	137.7 ± 0.4	120.6 ± 5.6	124
Glutathione				
(30 μmol/l)		($p < 0.001$)	($p < 0.001$)	
	213.4 ± 2.2	140.5 ± 2.0	181.0 ± 1.7	44
Glutathione				
(100 μmol/l)		($p < 0.001$)	($p < 0.001$)	
	213.4 ± 2.2	140.5 ± 2.0	192.9 ± 5.6	28

Rat brain homogenates (1 mg prot./ml) were oxidized by $FeSO_4$ (0.1 mmol/l)/ascorbic acid (0.5 mmol/l), 30 min at 37°C. Antioxidants or antioxidative enzymes were added prior to oxidation. CK activity of control and oxidized samples as well as oxidized samples and samples treated with antioxidants were compared by Bonferoni statistical test. The results are expressed as mean ± S.E.M. The significances are depicted between two compared values. The difference in the activity of CK in the control and oxidized sample was set as 100 percent decrease of CK activity. The percentage of decrease or increase of CK activity in samples with antioxidants was then calculated as difference between control samples and samples treated with antioxidants or antioxidant enzymes. ns – not statistically significant.

significantly lower in comparison with that from control samples in all experiments ($p < 0.001$ or $p < 0.01$). SOD, mannitol and EDTA did not protect CK from oxidative injury: SOD and mannitol (30 μmol/l) had no significant effect on CK activity when compared with oxidized samples; mannitol (100 μmol/l) even decreased the activity of this enzyme ($p < 0.05$). EDTA significantly profounded the decrease in CK activity induced by oxidative injury ($p < 0.001$). On the contrary, catalase and

Table 2. Effect of HNE on activity of pure CK

	Incubation: 30 min		Incubation: 120 min	
	CK U/mg prot.			
CK	142.6 ± 5.9		159.4 ± 14.2	
	($p < 0.01$)		($p < 0.001$)	
CK + HNE (10 μmol/l)	108.6 ± 6.2	(76.1%)	38.8 ± 0.3	(21%)
CK	204.8 ± 4.7		220.3 ± 5.0	
	($p < 0.001$)			
CK + HNE (64 μmol/l)	43.2 ± 2.9	(24.3%)	0	(0%)

CK activity in the absence of HNE was set as 100%. Results are expressed as mean ± S.E.M. Significances are depicted between two compared values.

glutathione had a protective effect on CK: the activity of CK significantly increased after treatment of homogenates with catalase ($p < 0.05$) and glutathione of both concentrations, 30 and 100 μmol/l ($p < 0.001$).

Results of effect HNE on pure CK are summarised in Tab. 2 and Fig. 1. Compared to the original activity of CK in absence of HNE, activity of CK incubated with HNE (10 μmol/l) decreased during 30 min to 76.1% and after 120 min to 21% (Tab. 2). This decrease was significant ($p < 0.01$, $p < 0.001$, respectively). Activity of CK incubated with HNE (64 μmol/l) decreased to 24.3% after 30 min incubation, which was a significant decrease ($p < 0.001$). After 120 min incubation no activity of CK was found in the sample with HNE. The same samples were evaluated by electrophoresis and immunoblotting. The signal corresponding to protein carbonyl groups at 10 μmol/l HNE was significantly more intense than that for the CK control, indicating increase of protein carbonyl groups. The signal completely disappeared at HNE concentration of 64 μmol/l, which may indicate fragmentation of enzyme (Fig. 1).

In order to determine the actual concentration of HNE generated in rat brain homogenates, the level of HNE during oxidation by Fe/ascorbate was measured by HPLC. A typical chromatogram and a calibration curve of HNE in rat brain homogenates are depicted in Fig. 2. The highest level of HNE (16.05 ± 0.62 nmol/mg prot. corresponding to μmol/l) was found immediately after addition of Fe/ascorbate (Tab. 3). After 10 minutes it dropped to about 10 nmol/mg prot. corresponding to 10 μmol/l concentration and remained approximately constant for the next 20 minutes.

Discussion

In the present work we studied the oxidative modification of CK as a model of protein oxidation in rat brain homogenates oxidized by Fe/ascorbate. We used this test to compare the antioxidant capacity of selected antioxidants.

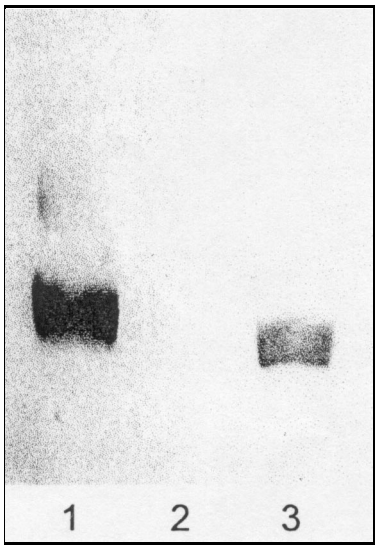

Figure 1. Western blot immunoassay for protein-bond carbonyl groups in pure creatine kinase (CK) after treatment with HNE. 1 – CK incubated with HNE (10 μmol/l) for 30 min at 37°C, 2 – CK incubated with HNE (64 μmol/l), 3 – CK in absence of HNE.

Table 3. HNE concentration in rat brain homogenates in the presence of Fe/ascorbate

Time of incubation (min):	0	10	20	30
HNE (nmol/mg prot. corresponding to μmol/l)	16.05 ± 0.62	10.03 ± 0.25	10.06 ± 0.98	12.17 ± 0.31

All values are expressed as mean ± S.E.M.

We expected that in rat brain homogenates CK would be modified both primary by oxygen radicals, similarly as pure CK is modified by the metal catalyzed oxidation (MCO) system (Fucci et al. 1983) and also secondary by impairment caused by reaction of the end products of lipid peroxidation (4-hydroxynonenal) with cysteine, histidine or lysine.

The site-specific nature of the MCO is indicated by the fact that inactivation of enzymes is relatively insensitive to inhibition by free radical scavengers (OH radical scavengers) as e.g., mannitol (Stadtman and Oliver 1991). These authors also excluded the involvement of superoxide anion in this reaction as SOD did not inhibit the metal catalyzed oxidative modification. Neither mannitol nor SOD prevented decrease of CK activity in rat brain homogenates oxidized by Fe/ascorbate in our experiments.

Metal catalyzed oxidation of amino acids can be either stimulated or inhibited by metal ion chelators (Stadtman 1993). In our experiments, impairment of CK in rat brain homogenates was elevated by EDTA, which means that CK has the ability to bind the EDTA-metal chelate complex, promoting a site specific modification of amino acid residues at the EDTA-binding site. These results were in agreement

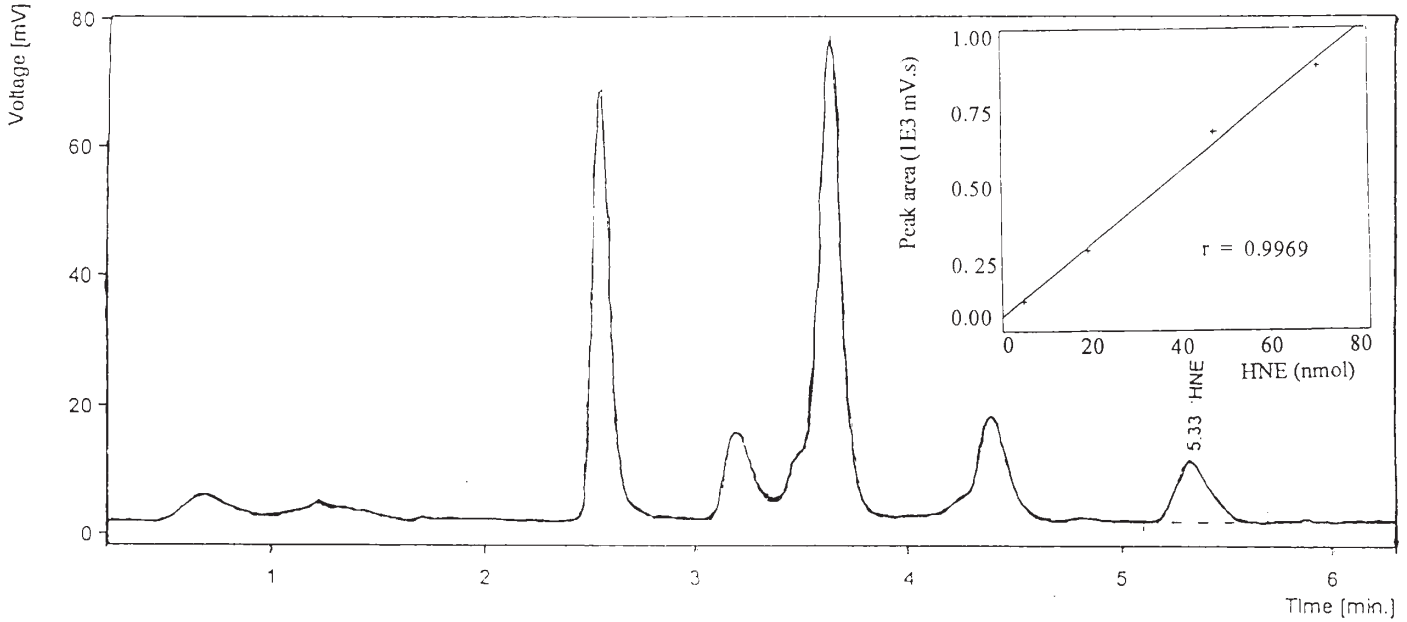

Figure 2. Typical chromatogram of HNE in rat brain homogenate incubated with Fe/ascorbate. HLPC column (250 × 4 mm Nucleosil 120-5 C18 Watrex), mobil phase 70% acetonitril, flow 1 ml/min, sample injection volume 20 μl. The calibration curve of standard HNE is given at top right.

with the results on pure CK where CK inactivation was stimulated by EDTA and other chelating agents.

Involvement of H_2O_2 in this oxidative reaction of rat brain homogenates is indicated by the fact that the oxidative modification of CK in our experiments was inhibited by catalase, which is in agreement with the results of Fucci et al. (1983) on pure CK.

Glutathione strongly prevented decrease in CK activity in our experiments (Tab. 1). This could be due both to the rapid consumption of GSH *via* glutathione peroxidase reaction and to the high reactivity of HNE generated from lipids during oxidation with sulfhydryl groups. Glutathione easily reacts with HNE (Esterbauer et al. 1991). Enzymes inactivated by HNE can be reactivated by glutathione excess (Schauenstein et al. 1971). Yet GSH is also a singlet oxygen quencher and this property could be involved in preventing oxidative modification of CK. Moreover, GSH is an OH radical scavenger, but it is known that OH radicals are not involved in metal catalyzed oxidative modification. This was confirmed also in our experiments where mannitol, a known OH radical scavenger, did not influence CK activity.

In the experiment with pure CK we found that the activity of CK was decreased by HNE in the concentrations used (10 and 64 μmol/l) (Tab. 2), while simultaneously the protein carbonyls significantly increased after 30 min incubation with HNE (10 μmol/l). HNE at 64 μmol/l induced complete disappearence of the carbonyl band, which may indicate fragmentation of the enzyme. The actual concentration of HNE in rat brain homogenates during the time course of oxidation, determined by HPLC was in the range of 10–16 nmol/mg prot., corresponding to 10–16 μmol/l (Tab. 3). Thus we may suppose that the concentrations of HNE found in rat brain in our experiments were high enough to impair CK, as even 10 μmol/l HNE was able to induce significant ($p < 0.01$) impairment of pure CK during 30 min oxidation (Tab. 2).

We conclude that CK of rat brain homogenates oxidized by Fe/ascorbate may have been impaired not only directly by metal catalyzed oxidation but also by HNE which level in rat brain homogenates was high enough to cause CK inactivation. This work is a contribution to the mechanism of oxidative injury in rat brain *in vitro* where oxidative stress was induced by Fe/ascorbate. Antioxidants and antioxidative enzymes with known specific effect to individual reactive oxygen species were used to analyze which of them were involved in this oxidative injury. Accordingly also the mechanisms of new antioxidants will be estimated.

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