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Catecholamines Enhance Dihydrolipoamide Dehydrogenase Inactivation by the Copper Fenton System. Enzyme Protection by Copper Chelators

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Catecholamines (CAs: epinephrine, norepinephrine, dopamine, L-DOPA, 6-hydroxydopamine) and *o*-diphenols (DOPAC and catechol) enhanced dihydrolipoamide dehydrogenase (LADH) inactivation by Cu(II)/H_2O_2 (Cu-Fenton system). The inhibition of LADH activity correlated with Cu(II), H_2O_2 and CA concentrations. Similar inhibitions were obtained with the assayed CAs and *o*-diphenols. CAs enhanced $HO^{\bullet}$ radical production by Cu(II)/H_2O_2, as demonstrated by benzoate hydroxylation and deoxyribose oxidation; LADH counteracted the pro-oxidant effect of CAs by scavenging hydroxyl radicals. Captopril, dihydrolipoamide, dihydrolipoic acid, DL-dithiothreitol, GSSG, trypanothione and histidine effectively preserved LADH from oxidative damage, whereas N-acetylcysteine, N-(2-mercaptopropionylglycine) and lipoamide were less effective protectors. Catalase (though neither bovine serum albumin nor superoxide dismutase) protected LADH against the Cu(II)/H_2O_2/CAs systems. Denatured catalase protected less than the native enzyme, its action possibly depending on Cu-binding. LADH increased and Captopril inhibited epinephrine oxidation by Cu(II)/H_2O_2 and Cu(II). The summarized evidence supports the following steps for LADH inactivation: (1) reduction of LADH linked-Cu(II) to Cu(I) by CAs; (2) production of $HO^{\bullet}$ from H_2O_2 by LADH-linked Cu(I) (Haber-Weiss reaction) and (3) oxidation of aminoacid residues at the enzyme active site by site-specifically generated $HO^{\bullet}$ radicals. Hydrogen peroxide formation from CAs autoxidation may contribute to LADH inactivation.

Key words: lipoamide dehydrogenase, copper, hydrogen peroxide, hydroxyl radicals, oxidative damage, catecholamines, thiol compounds, copper chelators, Captopril

Abbreviations: LADH, dihydrolipoamide dehydrogenase (NADH-lipoamide oxido-reductase, E.C. 1.6.4.3); lipoamide, DL-6,8-thioctic acid amide; Cu(II)/H_2O_2 or Cu-FS, Cu(II)-Fenton system; CA, catecholamine; epinephrine, 1-(3,4-dihydroxyphenyl)-2-methylaminoethanol; norepinephrine, 1-(3,4-dihydroxyphenyl)-2-aminoethanol; dopamine, 3,4-dihydroxyphenylethylamine; 6-OHDA, 6-hydroxydopamine; L-DOPA, 3-(3,4-dihydroxyphenyl)-alanine; DOPAC, 3,4-dihydroxyphenylacetic acid; Captopril, [1-(3-mercapto-2-methyl-1-oxopropyl-L-proline)]; NAC, N-acetylcysteine; MPG, N-(2-mercaptopropionylglycine); GSH and GSSG, reduced and oxidized glutathione, respectively; penicillamine, 3-mercapto-D-valine; SOD, superoxide dismutase; TBA, thiobarbituric acid.

INTRODUCTION

Mammalian lipoamide dehydrogenase (henceforth LADH), a mitochondrial enzyme, is a

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flavoprotein which reversibly catalyzes the oxidation of dihydrolipoamide by NAD^+, to yield lipoamide and NADH (Reaction 1). The enzyme is a common component of α-oxoacid dehydrogenase complexes, such as the pyruvate dehydrogenase complex, the α-oxoglutarate dehydrogenase complex and the branched chain α-oxoacid dehydrogenase complex. Previous studies[1,2] have demonstrated that myocardial LADH is inactivated by $Fe(II)/H_2O_2$ and $Cu(II)/H_2O_2$ (Cu-FS) whereas thiol compounds, such as Captopril, dihydrolipoamide and dihydrolipoic acid, prevent LADH inactivation.

$$\text{Dihydrolipoamide}+NAD^+ \rightarrow \text{Lipoamide}+NADH+H^+ \quad (1)$$

Catecholamines and L-DOPA, due to spontaneous or copper-catalyzed oxidations, can generate toxic products such as hydrogen peroxide, oxygen radicals (e.g. $HO^\bullet$) and semiquinones, thus exerting cytotoxic effects, especially on the nervous system.[3–15] These effects include mitochondrial damage, namely, the inhibition of NADH-dehydrogenase by dopamine and the inhibition of complexes I and IV by L-DOPA.[12–14] Copper-catalyzed oxidation of CAs has also been proposed as a source of oxygen radicals, leading to myocardial damage.[15] In close agreement with that hypothesis, Cu(II) ions[16] and norepinephrine[17] are released by the ischemic myocardium.

Taking into account the foregoing information, it seemed of interest to investigate (a) the action of CAs and related *o*-diphenols on LADH inactivation by the Cu-FS; (b) the role of oxygen radicals in the possible action of CAs, and (c) the effect of oxygen radical scavengers as protectors of LADH against the combined action of CAs and the Cu-FS. The following CAs and catechols were assayed: epinephrine, norepinephrine, L-DOPA, dopamine, 6-OHDA, DOPAC and catechol.

MATERIALS AND METHODS

Materials

Epinephrine, norepinephrine (arterenol), dopamine, 6-OHDA, L-DOPA, DOPAC and catechol and adrenochrome, were purchased from Sigma Chemical Co., St. Louis, MO, USA. Other reagents were as described previously.[1,2]

Enzyme assay

LADH activity was measured by the rate of NADH oxidation using lipoamide as electron acceptor. Unless stated otherwise, the assay medium contained 50 mM KH_2PO_4-K_2HPO_4 (K-phosphate), pH 7.4, 0.2 mM NADH, 1.0 mM lipoamide, and LADH, pre-treated as indicated below. Final volume, 3.0 ml. The reaction was started by adding the substrates and the initial velocity of LADH reaction was measured by the slope of recorded tracings. Control samples without Cu-FS or CAs were incubated simultaneously. Spectrophotometric measurements were performed using a Perkin Elmer 550 UV/VIS spectrophotometer, at 30°C. Other assays were as described previously.[1,2]

LADH inactivation

Unless stated otherwise, LADH (1.0 μM) in 200 μl of 50 mM KH_2PO_4-K_2HPO_4, pH 7.4, with the additions indicated under Results (the 'inactivation medium'), was incubated in a test-tube for 5 min at 30°C. Ten μl duplicate samples of the 'inactivation medium' were taken, added to the enzyme assay medium and residual LADH activity was measured as described above.

Assay of benzoate hydroxylation and deoxyribose oxidation

Benzoate hydroxylation[18] was monitored by incubating solutions (2.0 ml) containing 50 mM KH_2PO_4-K_2HPO_4, pH 7.4, 2.0 mM Na benzoate

and the additions indicated under Results, at 20–22°C, for 1 hour. After incubation, fluorescence was measured using an Aminco-Bowman Spectrofluorometer, set at 310 nm (excitation) and 405 nm (emission). Deoxyribose oxidation[22] was monitored by the production of TBA-reactive products, as described previously.[2]

CA oxidation

Epinephrine oxidation was measured spectrophotometrically by adrenochrome production at 310 nm, using as standards 0.1 and 0.025 mM recently prepared adrenochrome solutions.

Expression of results

LADH specific activity is expressed in μmol NADH oxidized/min per mg protein. Taking into account the limited variation in the activity of different LADH samples, relative activity values are also presented (100% for the control sample). Protection (P) against inhibitors was calculated from Equation 1 where P, i and ip are the protector relative activity, the inhibition of LADH activity by Cu(II)/H_2O_2, Cu(II)/H_2O_2/CA, Cu(II) or Cu(II)/CA and the inhibition by the same systems plus protector, respectively. Duplicate values deviated from the average by less than 5%. When more than two measurements were performed, the values presented are average ± S.D. Protection values lower than 10% were considered non- significant, because of the propagation of experimental error to the calculated P values. Statistical analysis was performed using Student's t-test for paired values.

$$P\,(\%) = 100\,(i\,(\%) - ip\,(\%))/i\,(\%) \qquad (1)$$

RESULTS

Effects of CAs on LADH inactivation by Cu(II)/H_2O_2 (Cu-FS)

Table 1 shows the effect of CAs and related catechols on LADH inactivation by the Cu-FS. It is to be seen that all the CAs assayed enhanced the inhibitory action of the Cu-FS ($P<0.001$) but, interestingly enough, significant effects were also observed with catechol and DOPAC, two o-diphenols lacking the amino group. H_2O_2 was necessary in order to observe the effect of CAs, as illustrated in Table 2 using 10 μM Cu(II) and epinephrine as pro-oxidant. Table 3 shows the effect of Cu(II) concentration on LADH inactivation by

TABLE 1 Effect of CAs and catechols on LADH inactivation by Cu(II)/H_2O_2.

Addition (0.4 mM)	LADH activity (μmol NADH/min) mg protein	LADH inactivation (%)
None	83 ± 17 (18)	43
Epinephrine	29 ± 12 (5)	80*
Norepinephrine	32 ± 8 (4)	79*
Dopamine	18 ± 10 (4)	88*
L-DOPA	32 ± 3 (4)	78*
6-OHDA	19 ± 9 (7)	87*
DOPAC	18 ± 4 (5)	88*
Catechol	23 ± 7 (6)	85*
(Control sample)	149 ± 10 (18)	—

The inactivation medium contained 5.0 μM Cu(II), 3.0 mM H_2O_2, 1.0 μM LADH, 50 mM K-phosphate, pH 7.4 and the addition indicated above. The control sample contained neither Cu(II)/H_2O_2 nor addition. Values represent the average ± S.D. of the number of measurements indicated in parenthesis. Other conditions as described under Materials and Methods.

*$P<0.001$ as compared with LADH inactivation by the Cu-FS (43%).

TABLE 2 Effect of H_2O_2 concentration on LADH inactivation by Cu(II)/H_2O_2/epinephrine.

H_2O_2 (μM)	Epinephrine concentration (μM)		
	0	10	100
None	30	30	31
1.0	66	83	89
3.0	83	94	97

The inactivation medium contained 10 μM Cu(II), 1 μM LADH, 50 mM K-phosphate, pH 7.4, H_2O_2 and epinephrine as indicated above. Other experimental conditions were as indicated under Materials and Methods. Values represent LADH inactivation (%).

TABLE 3 Effect of Cu(II), epinephrine and dopamine on LADH inactivation by the Cu(II)/H_2O_2/CA systems.

Cu(II) (μM)	Catecholamine (0.4 mM)	LADH inactivation (%) by Cu(II)/H_2O_2	LADH inactivation (%) by Cu(II)
2.5	None	14	3
	Epinephrine	29	1.0
	Dopamine	39	−1.0
5.0	None	43	18
	Epinephrine	80	9
	Dopamine	88	10
10	None	85	47
	Epinephrine	98	34
	Dopamine	99	36

The inactivation medium contained 3.0 mM H_2O_2, 1.0 μM LADH, 50 mM K-phosphate buffer, pH 7.4, Cu(II) and CA as indicated above. Values for samples containing only LADH and CA were (average ± SD; in parenthesis, number of measurements): epinephrine 0 ± 0 (3), and dopamine, 0.2 ± 0.3 (5).

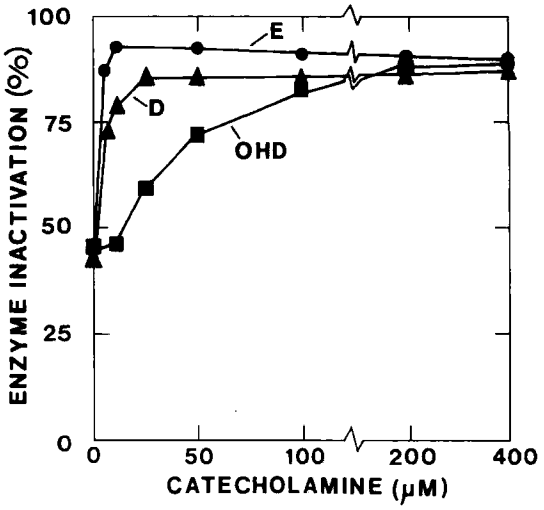

FIGURE 1 Effect of CA concentration on LADH inactivation by Cu(II)/H_2O_2/CA systems. The inactivation medium contained 5.0 μM Cu(II), 3.0 mM H_2O_2), 1.0 μM LADH, 50 mM K-phosphate, pH 7.4 and the CA at the concentration indicated on the abscissa. E, epinephrine; D, dopamine; OHD, 6-OHDA. Total volume 0.2 ml. After 5 minutes incubation at 30°C, lipoamide reductase activity was measured as described under Materials and Methods.

the Cu-FS. In the absence of Cu(II), enzyme activity was not affected, but with 2.5 and 5.0 μM Cu(II), LADH activity decreased as a function of Cu(II) concentration. With 10 μM Cu(II), LADH inactivation reached an apparent maximum, which decreased in the presence of epinephrine and dopamine. With 2.5 μM Cu(II), the CAs systems and the $HO^{\bullet}$-producing, NADH-system[19] added their actions (Table 4). Under the given experimental conditions, a direct effect of Cu(II) on LADH in the assay medium could be disregarded because of the thirty-fold dilution of the 'inactivation medium' by the assay medium.

Figure 1 shows the effect of increasing concentrations of epinephrine, dopamine and 6-OHDA on LADH inactivation by the Cu-FS. In the 10–50 μM range, epinephrine was the most effective, followed by dopamine, but at 400 μM concentration, maximum enzyme inhibition was obtained and, therefore, differences between CA activities were negligible.

Figure 2 shows the time course of LADH inactivation by Cu(II)/H_2O_2/6-OHDA. It is to be seen

TABLE 4 Effect of NADH on LADH inactivation by Cu(II)/H_2O_2/CA systems.

Cu(II) (μM)	NADH (mM)	LADH inactivation (%) by Cu(II)/H_2O_2/epinephrine	Cu(II)/H_2O_2/dopamine	Cu(II)/H_2O_2
2.5	0	24 ± 4.8	30 ± 4.0	14 ± 7.0
	0.15	69 ± 2.4	65 ± 9.6	74 ± 7.7
5.0	0	86 ± 7.0	80 ± 3.0	43 ± 6.1
	0.15	88 ± 9.2	87 ± 6.0	96 ± 2.2

The inactivation medium contained 3.0 mM H_2O_2, 1.0 μM LADH, 50 mM K-phosphate, pH 7.4 and additions (Cu(II), NADH and CA (0.1 mM)) as indicated above. Other experimental conditions were as described under Materials and Methods. Values represent the average ± S.D. of 3 measurements.

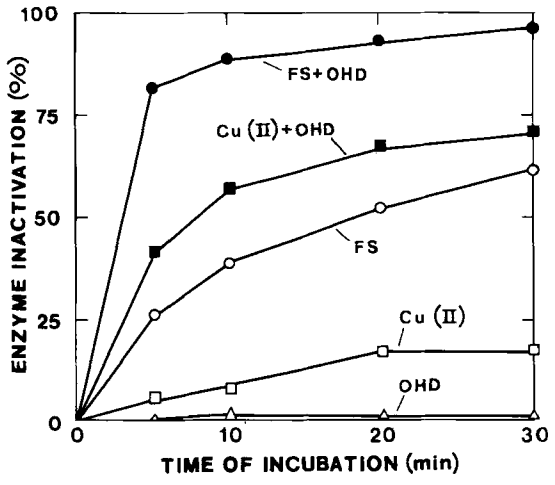

FIGURE 2 Time-course of LADH inactivation by Cu(II)/H_2O_2/6-OHDA. Reagents concentration was: 4.0 μM Cu(II), 3.0 mM H_2O_2, 0.4 mM 6-OHDA, 1.0 μM LADH and 50 mM K-phosphate, pH 7.4. The inactivation medium contained reagents as indicated on the figure (FS, Cu(II)/H_2O_2; OHD, 6-OHDA). Incubation time was as indicated on the abscissa. Other experimental conditions were as described under Materials and Methods. Values represent the average of duplicate measurements.

that with the complete system, inactivation was relatively fast and, after 5 min incubation, LADH activity decreased about 80%. When H_2O_2 was omitted, the inactivation process was slower than with the complete system, but faster than with the Cu-FS or Cu(II) alone. In the absence of Cu(II), 6-OHDA failed to affect LADH to a significant degree. Similar effects were obtained when 0.4 mM epinephrine, L-DOPA, dopamine, 6-OHDA, DOPAC, or catechol were added to the Cu-FS. In every case, the loss of activity was higher than 82%, the maximum effects corresponding to dopamine, 6-OHDA and DOPAC that produced 88% inhibition. After 30 min incubation of LADH with the same pro-oxidant systems, enzyme inactivation was always higher than 95%.

Effect of CAs on HO• production

HO• radicals play an essential role in LADH inactivation by the Cu-FS (Reference 2) and, accordingly, production of HO• radicals by the Cu(II)/H_2O_2/CA systems was examined. The results obtained with the benzoate hydroxylation

TABLE 5 Effect of CAs and LADH on benzoate hydroxylation and deoxyribose oxidation by Cu(II)/H_2O_2 and Cu(II)

Experiment	Addition (mM)	LADH: None		LADH: 1.0 μM	
		Cu(II)/H_2O_2	Cu(II)	Cu(II)/H_2O_2	Cu(II)
		Fluorescence (units)			
A	Epinephrine (0.1)	93 ± 2.0	2.5	30 ± 2.5 (68)	1.0
	Dopamine (0.1)	77 ± 1.2	0.9	25 ± 2.0 (70)	2.2
	6-OHDA (0.1)	97 ± 2.9	17	18 ± 1.7 (81)	2.5
	L-DOPA (0.1)	75 ± 0.7	24	38 ± 2.1 (49)	5.5
	DOPAC (0.1)	98 ± 8.0	1.0	30 ± 2.2 (69)	1.0
	None	24 ± 4.1	0	4.2 ± 1.4 (82)	0
		TBA reaction products (10^3 × A)			
B	Epinephrine (0.4)	451	17	64 (86)	11
	Dopamine (0.4)	299	28	148 (50)	3
	6-OHDA (0.4)	927	159	323 (65)	65
	DOPAC (0.4)	741	18	83 (88)	6
	None	194	6	63 (69)	4

The reaction mixture contained 2.0 mM Na-benzoate (exp. A), 3.0 mM deoxyribose (exp. B), 5.0 μM Cu(II), 3.0 mM H_2O_2, 50 mM K-phosphate, pH 7.4, and addition as indicated above. After incubation for 1 hour at 30°C, fluorescent products (exp. A) or TBA reaction products (exp. B) were measured. Other experimental conditions were as described under Materials and Methods. Values are the average ± S.D. of 3 measurements (exp. A) or the average of duplicate measurements (exp. B). In parenthesis, inhibition of HO· production by LADH.

method (Table 5) indicate that (a) at 0.1 mM concentration, the assayed CAs and DOPAC significantly enhanced. $HO^{\bullet}$ production; (b) omission of H_2O_2 almost nullified the effect of epinephrine, dopamine and DOPAC, but not that of L-DOPA and 6-OHDA; (c) LADH inhibited $HO^{\bullet}$ production by the Cu(II)/H_2O_2/CA and Cu(II)/CA systems, the inhibition depending in some degree on CA structure. Similar results were obtained with the deoxyribose method, and 0.4 mM CAs (Table 5). Investigation of superoxide anion production by Cu(II)/H_2O_2/epinephrine, using cytochrome *c* (16 μM) as detector, yielded negative results since addition of SOD (40 units/ml) failed to modify the rate of cytochrome *c* reduction to a significant degree ($P>0.05$; other experimental conditions omitted).

Protection of LADH against Cu(II)/H_2O_2/CA systems

Inactivation of LADH by Cu(II)/H_2O_2/CA systems was prevented by Captopril, a monothiol antihypertensive agent.[20] Figure 3 illustrates a typical experiment using epinephrine as pro-oxidant. Control assays were performed using NAC and MPG, two thiol antioxidants.[21,22] The results presented indicate that Captopril was a relatively efficient protector, acting in a concentration dependent manner in the 0 to 0.4 mM range. Moreover, at 0.4 mM and greater concentrations, Captopril almost entirely prevented LADH inactivation. On the other hand, at the 0 to 0.4 mM concentration range, NAC and MPG preserved LADH activity to a limited or negligible degree, respectively, but at greater concentrations, NAC significantly protected the enzyme. Figure 4 shows the results of a similar experiment using 6-OHDA as pro-oxidant. Captopril effectively protected LADH; NAC and MPG also preserved LADH activity, though less than Captopril. Table 6 summarizes the action of a series of monothiols, namely, Captopril, NAC, MPG, penicillamine and L-cysteine, against L-DOPA, dopamine, DOPAC and catechol systems. In order to compare the efficiency of the antioxidants assayed, they were used at 0.4 mM concentration, except Captopril that was also assayed at 0.1 mM

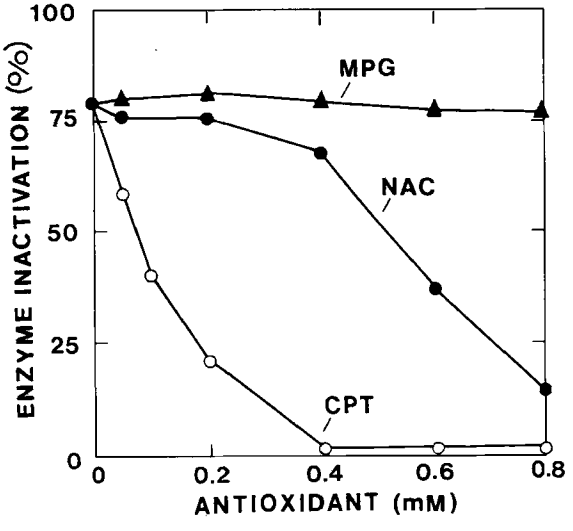

FIGURE 3 Effect of Captopril (CPT), NAC and MPG on LADH inactivation by Cu(II)/H_2O_2/epinephrine. The inactivation medium contained, 5.0 μM Cu(II), 3.0 mM H_2O_2, 0.4 mM epinephrine, 1.0 μM LADH, and the antioxidant indicated on the figure, at the concentration stated on the abscissa. Other experimental conditions were as described under Materials and Methods.

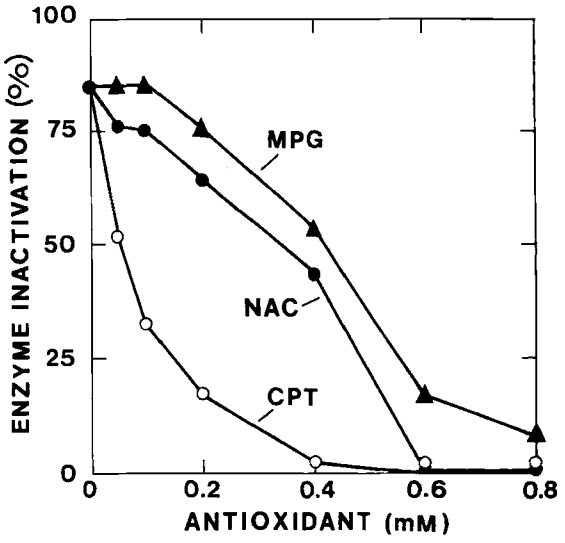

FIGURE 4 Effect of Captopril (CPT), NAC and MPG on LADH inactivation by Cu(II)/H_2O_2/6-OHDA. Experimental conditions were as in Figure 3 legend, except for CA which was 0.4 mM 6-OHDA.

TABLE 6 Protection of LADH by thiol compounds against inactivation by Cu(II)/H_2O_2/CA systems.

Thiol compound (mM)	LADH protection (%) against systems containing			
	L-DOPA	Dopamine	DOPAC	Catechol
Captopril (0.4)	80 ± 2.0	81 ± 1.7	84 ± 0.9	90 ± 1.9
Captopril (0.1)	64	80	53	53
NAC (0.4)	62	90	73	45
MPG (0.4)	12	80	10	3
Penicillamine (0.4)	45	37	19	9
L-cysteine (0.4)	28	15	18	18

Experimental conditions were as described in Figures 3 and 4 legends and under Materials and Methods. The inactivation medium was supplemented with Cu(II)/H_2O_2 (Cu-FS), 0.4 mM CA (or catechol) and thiol compound as indicated above. In the absence of thiol compound, LADH inactivation (%) was (average ± S.D.; in parenthesis, number of determinations): 43 ± 10 (18) (Cu-FS only); 78 ± 2 (4) (Cu-FS + L-DOPA); 88 ± 7 (5) (Cu-FS + dopamine); 88 ± 3 (6) (Cu-FS + DOPAC) and 85 ± 5 (6) (Cu-FS + catechol). In every case, $P<0.001$, as compared with the Cu-FS value. Protection values were calculated from Equation 1 and represent the average of duplicate measurements except with 0.4 mM Captopril (average ± S.D. of 3 measurements).

concentration. The results presented indicate that (a) 0.4 mM Captopril protected LADH over 90% against all pro-oxidants; (b) NAC protected over 60% against L-DOPA, dopamine and DOPAC systems, the greatest effect being obtained with dopamine; (c) MPG was effective only against dopamine; (d) penicillamine provided a relatively low protection (< 50%) against all the systems assayed, including the epinephrine and 6-OHDA systems, which are omitted from Table 6.

Table 7 shows the action of several dithiol compounds against the epinephrine system. It may be seen that two potent antioxidants, namely dihydrolipoamide and dihydrolipoic acid,[23,24]

TABLE 7 Protection of LADH by dithiol compounds against inactivation by Cu(II)/H_2O_2/epinephrine.

Dithiol or disulfide compound	Concentration (mM)	LADH	
		Inactivation (%)	Protection (%)
Dihydrolipoamide	0.05	14	82
	0.10	1	99
Lipoamide	0.10	58	26
Dihydrolipoic acid	0.05	32	59
	0.10	5	94
Lipoic acid	0.10	74	5
Dithiothreitol	0.05	22	72
	0.10	11	86
None	—	78 ± 4 (8)	—

Experimental conditions were as described in Table 6 legend, except the CA, which was 0.4 mM epinephrine and the thiol compound, which was as indicated above. Unless stated otherwise, or disulfide values represent the average of duplicate measurements. LADH inactivation by the Cu-FS alone (CA omitted) was: 39 ± 7.0 (14).

preserved LADH from inactivation and similar results were obtained with dithiothreitol. Interestingly enough, the dithiol structure was essential for LADH protection, as indicated by the lesser or insignificant effects of lipoamide and lipoic acid, respectively (Table 7). Other compounds assayed were trypanothione (at 100 and 200 μM concentrations), GSSG (at 200 μM) and L-histidine (at 100 μM). These compounds prevented LADH inactivation by 72, 95, 95 and 86% respectively. GSH (200 μM) protected 74%, which is less than GSSG, that protected 95%.

The effect of catalase and SOD on LADH inactivation by the Cu(II)/H_2O_2/CA systems was investigated as described in Table 8. Native and heat-denatured catalase preserved LADH activity, particularly the former. At the same concentration (20 μg/ml) serum albumin was ineffective. LADH protection by native catalase is easily understood considering the essential role of H_2O_2 in LADH inactivation (Table 3), whereas the effect of the denatured enzyme may be explained by Cu(II) binding.[25] Ethanol (6.0 mM), Na-benzoate (0.2 M) and mannitol (0.3 M) failed to prevent LADH inactivation (experimental data omitted). 1.0 mM ATP, ADP or NAD^+ did not modify LADH inactivation by the Cu(II)/H_2O_2/epinephrine (or 6-OHDA) systems (experimental data omitted), at variance with effects observed with Fe(II)/H_2O_2 (Reference 1).

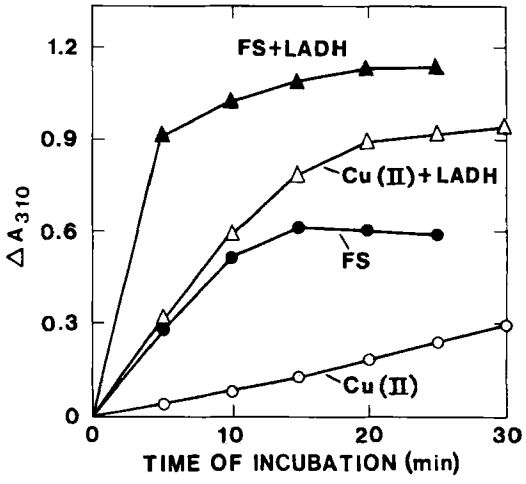

FIGURE 5 Oxidation of epinephrine by Cu(II)/H_2O_2. Reagents concentration was: 25 μM (Cu(II), 3.0 mM H_2O_2, 0.1 mM epinephrine, 1.0 μM LADH, and 50 mM K-phosphates, pH 7.4. Additions were as indicated on the figure. FS, Cu(II)/H_2O_2. A reference sample for each condition contained the same reagents, except the Cu-FS or Cu(II). Oxidation of epinephrine was measured by the absorbance difference between the assay and the reference sample (ΔA_{310}). Other experimental conditions were as described under Materials and Methods. Values represent the average of duplicate measurements.

Effect of LADH on CAs oxidation

Incubation of CAs with Cu(II)/H_2O_2 produced their oxidation to the corresponding chromogens. Figure 5 shows typical results using epinephrine as oxidizable substrate. It is to be seen that: a) adrenochrome production with Cu(II)/H_2O_2 was

TABLE 8 Effect of scavengers on LADH inactivation by Cu(II)/H_2O_2/CA systems.

Scavenger (μM)	LADH protection (%) against systems containing		
	Epinephrine	Dopamine	None
Catalase (20 μg/ml)	100	100	85
Catalase denatured (20 μg/ml)	49	44	57
Bovine serum albumin (20 μg/ml)	1.2	11	6.4
SOD (80 μg/ml)	−2.4	8.0	−4.2

Experimental conditions were as described in Figure 1 legend and under Materials and Methods. The inactivation medium contained 5 μM Cu(II), 3.0 mM H_2O_2, 0.4 mM CA 50 mM K-phosphate, pH 7.4 and scavenger as indicated above. Catalase inactivated 100% by heating at 100°C for 10 min (denatured catalase). Native catalase specific activity, 35 units/μg; SOD specific activity, 5 units/μg.

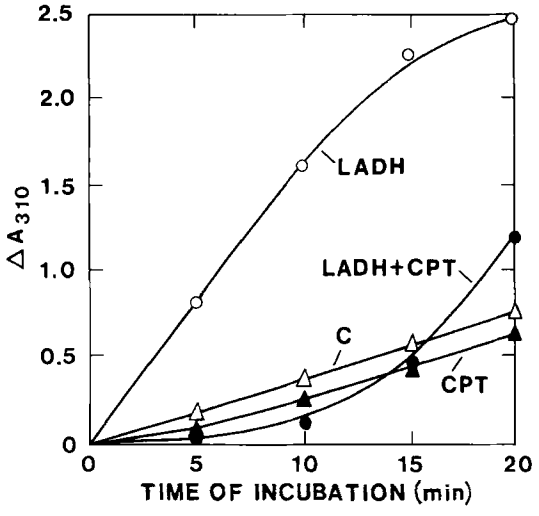

FIGURE 6 Effect of Captopril and LADH on epinephrine oxidation by Cu(II)/H_2O_2. The reaction medium contained 25 μM Cu(II), 3.0 mM H_2O_2, 0.4 mM epinephrine, 50 mM K-phosphate, pH 7.4 and additions as indicated on the figure. CPT, 0.4 mM Captopril; LADH, 1.0 μM; C, control sample, without Captopril and LADH. Other experimental conditions here as described in Figure 5 legend.

greater than with Cu(II) alone; b) addition of LADH significantly increased the rate of chromogen production; c) a similar effect was observed with Cu(II) as sole catalyst of epinephrine oxidation. From data in Figure 5, it can be calculated that adrenochrome production after 5 min incubation was (μM): 92 (Cu-FS + LADH); 33 (Cu(II) + LADH); 29 (Cu-FS); and 4.0 μM (Cu(II)). These values indicate that, after the incubation period used for LADH inactivation and under the best conditions for epinephrine oxidation, adrenochrome production reached 92 (%) of the assayed epinephrine. The effect of LADH depended on the enzyme structure since bovine serum albumin, at the same concentration, scarcely increased the rate of adrenochrome production (experimental data omitted). Moreover, thermal denaturation significantly decreased the pro-oxidant activity of LADH (experimental data omitted). Finally, Captopril (0.4 mM) inhibited significantly epinephrine oxidation by the Cu-FS (Figure 6).

DISCUSSION

The observations described here indicate that CAs contributed to LADH inactivation (Table 1) by increasing the production of $HO^{\bullet}$ by the Cu-FS, an essential complement for CA action. This assumption is borne out by the following evidence: (a) Cu(II) and H_2O_2 requirement for LADH inactivation (Tables 2 and 3); (b) the effect of CA concentration on the extent of LADH inactivation (Figure 1); (c) the summation of the effects of Cu(II)/H_2O_2/NADH and Cu(II)/H_2O_2/CA systems (Table 4), considering that the former was an $HO^{\bullet}$ producer[19]; (d) the enhanced production of $HO^{\bullet}$ by the CA-supplemented Cu-FS (Table 5); (e) LADH protection by Captopril (Figures 3 and 4; Table 6) and dithiols, including dihydrolipoamide and dehydrolipoic acid (Table 7); (f) LADH protection by Cu chelators, such as histidine,[26] GSSG and trypanothione (cited in text); (g) LADH protection by catalase, not by SOD (Table 8 and text); (h) the scavenging of $HO^{\bullet}$ radicals by LADH (Table 5), in close agreement with the loss of LADH activity. The kinetics of LADH inactivation by Cu(II)/H_2O_2/CA systems was relatively fast, as illustrated with 6-OHDA in Figure 2. On the other hand, participation of superoxide anion in LADH inactivation was contradicted by the inactivity of SOD as LADH protector (Table 8).

Important differences regarding the relative abilities of the various CAs to oxidize are worth noting. Thus, epinephrine failed to autoxidize to generate $HO^{\bullet}$ radicals, whereas 6-OHDA and L-DOPA did so (Table 5). As a result of this difference, epinephrine did not support LADH inactivation in the absence of added peroxide (Table 2), whereas 6-OHDA did so (Figure 2). This chemistry was seen again in a comparison of Figures 3 and 4, which show the effect of antioxidants on epinephrine- and 6-OHDA-stimulated LADH inactivation, respectively. Thus, MPG prevented inactivation by epinephrine (Figure 3) but not by 6-OHDA (Figure 4), a difference which reflects the comparative ease

with which the latter produced oxygen radicals, via autoxidation.[29]

Analysis of the structure-activity relationship in Tables 1 and 5 indicates that the CA *o*-diphenol group played an essential role in both LADH inactivation and $HO^{\bullet}$ production. In fact, catechol, and DOPAC were as effective as several CAs on (a) LADH inactivation (Table 1) and (b) $HO^{\bullet}$ radical production (Table 5). Taking into account previous reports on catechol reactions[27,28] it seems reasonable to postulate the following reaction sequence for CAs-dependent LADH inactivation. Firstly, CAs *o*-diphenol moiety would reduce Cu(II), by a one-electron transfer reaction, yielding Cu(I) and the semiquinone radical (Reaction 2: QH_2 and $QH^{\bullet}$, *o*-quinol and semiquinone, respectively). Secondly, Cu(I) would react with H_2O_2, according to the Haber-Weiss reaction, yielding the $HO^{\bullet}$ radical and Cu(II) (Reaction 3). Thirdly, oxygen reduction by the semiquinone from some CAs (e.g. 6-OHDA) (Reaction 4) might be an important contributory factor to the observed results, specially in the absence of H_2O_2, since O_2^- disproportionation would yield H_2O_2. Finally, site-specifically generated $HO^{\bullet}$ radicals would oxidize neighbouring aminoacid residues at the LADH active site, especially thiyl and histidyl.[2] The site-specificity of the postulated reaction mechanism is borne out by the lack of action of ethanol, mannitol and benzoate as protectors of LADH (cited in the text), whereas the production of the Cu-LADH complex, which would be involved in the catalytic activity of Cu ions, is supported by the effect of LADH on (a) the Cu-catalyzed oxidation of epinephrine (Figures 5 and 6) and (b) the inhibition of the same reaction by Captopril, a Cu-chelator (Figure 6). LADH capability to bind Cu ions,[30] as well as that of Cu(II)-protein or peptide complexes for catalysing the Haber-Weiss reaction, has been extensively investigated.[31,32] Cu(II) binding by LADH would depend on the protein structure, as indicated by the lesser action of denatured LADH as cited in the text.

$$QH_2 + Cu(II) \rightarrow QH^{\bullet} + Cu(I) + H^+ \quad (2)$$

$$Cu(I) + H_2O_2 \rightarrow Cu(II) + HO^{\bullet} + HO^- \quad (3)$$

$$QH^{\bullet} + O_2 \rightarrow Q + O_2^{\bullet} + H^+ \quad (4)$$

Disproportionation of CAs semiquinone produces the corresponding *o*-quinone (Q in Reaction 5), and the parent *o*-quinol. The *o*-quinones may react with thiol-proteins, thus producing enzyme inactivation.[33] However, under the experimental conditions used for LADH inactivation, adrenochrome production reached a maximum concentration of about 90 μM (Figure 6) at which it did not inhibit LADH activity to a significant degree (< 4%). In close agreement with other quinone-dependent systems,[33] *o*-quinone intermediates would be of limited importance, if any, as LADH inhibitors.

$$QH^{\bullet} + QH^{\bullet} \rightarrow Q + QH_2 \quad (5)$$

Protection of LADH by Captopril, may be explained considering that Captopril reduces Cu(II) and then forms a stable chelate with Cu(I) (Reference 34), which is unable to attack H_2O_2 to form the $HO^{\bullet}$ radical and to catalyse epinephrine oxidation, as indicated by results in Figure 3. Furthermore, Captopril may also scavenge 'reactive oxygen species'.[35] In connection with the high activity of Captopril as LADH protector (Figures 3, 4 and Table 6), it seems pertinent to recall Captopril cardio-protective action *in vivo*, as shown by the decrease in the myocardial infarct size and the prevention of electrocardiographic alterations in rats.[36] Taken together with LADH inactivation by Fe(II)/H_2O_2 and the Cu-FS,[1,2] the observations here described confirm LADH as a possible target for 'reactive oxygen species' generated as a result of myocardial ischemia-reoxygenation.[37] A similar damage may be assumed to occur for neuronal LADH, under conditions of brain ischemia-reoxygenation.

NAC was weaker than Captopril as LADH-

protector and even lesser protections were also achieved with MPG and penicillamine (Figures 3, 4 and Table 6). Since Captopril, NAC, MPG and penicillamine are all thiol compounds, a similar action against oxy-radical producing systems might be expected for all these compounds. However, the differences observed (Figures 3, 4; Table 6) indicate that, as reported previously,[38] other groups in the specific molecules may contribute to the antioxidant action.

Oxidative attack on CAs leads to a chain of reactions, some of whose products are in turn reactive. Thus, dopamine oxidation yields H_2O_2, norepinephrine, 6-OHDA and DOPAC. Dopamine, 6-OHDA and DOPAC are generated in neurons[9,11] and therefore the effects on LADH reported here might contribute to neuronal damage, especially in disorders such as Parkinson's disease.[39]

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