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INACTIVATION OF HEART DIHYDROLIPOAMIDE DEHYDROGENASE BY COPPER FENTON SYSTEMS. EFFECT OF THIOL COMPOUNDS AND METAL CHELATORS

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Copper Fenton systems (Cu(II)/H_2O_2 and Cu(II)/Asc) inactivated the lipoamide reductase and enhanced the diaphorase activity of pig-heart lipoamide dehydrogenase (LADH). Cupric ions alone were less effective. As a result of Cu(II)/H_2O_2 treatment, the number of titrated thiols in LADH decreased from 6 to 1 per subunit. NADH and ADP (not NAD^+ or ATP) enhanced LADH inactivation by Cu(II). NADH also enhanced the effect of Cu(II)/H_2O_2. Dihydrolipoamide, dihydrolipoic acid, Captopril, acetylcysteine, EDTA, DETAPAC, histidine, bathocuproine, GSSG and trypanothione prevented LADH inactivation. 100 μM GSH, DL-dithiothreitol, N-(2-mercaptopropionylglicine) and penicillamine protected LADH against Cu(II)/Asc and Cu(II), whereas 1.0 mm GSH and DL-dithiothreitol also protected LADH against Cu(II)/H_2O_2. Allopurinol provided partial protection against Cu(II)/H_2O_2. Ethanol, mannitol, Na benzoate and superoxide dismutase failed to prevent LADH inactivation by Cu(II)/H_2O_2 or Cu(II). Catalase (native or denaturated) and bovine serum albumin protected LADH but that protection should be due to Cu binding. LADH inhibited deoxyribose oxidation and benzoate hydroxylation by Cu(II)/H_2O_2. It is concluded that site-specifically generated HO, radicals were responsible for LADH inactivation by Cu(II) Fenton systems. The latter effect is discussed in the context of ischemia-reoxygenation myocardial injury.

KEY WORDS: Lipoamide dehydrogenase, copper, hydrogen peroxide, ascorbate, thiol compounds, copper chelators, trypanothione.

ABBREVIATIONS: LADH, dihydrolipoamide dehydrogenase (NADH-lipoamide oxido-reductase, EC 1.6.4.3); Cu(II)/H_2O_2, Cu(II)-Fenton system; Cu(II)/Asc, Cu(II) ascorbate system; lipoamide, DL-6,8-thioctic acid amide; DCI, dichlorophenol-indophenol; Captopril, [1-(3-mercapto-2-methyl-1-oxopropyl-L-proline)]; GSH and GSSG, reduced and oxidized glutathione, respectively; penicillamine, 3-mercapto-D-valine; DETAPAC, diethylenetriaminepentacetic acid; DTNB, 5,5′- dithiobis(2-nitrobenzoic acid); TBA, thiobarbituric acid.

INTRODUCTION

Mammalian lipoamide dehydrogenase (LADH), a flavoprotein disulfide oxido-reductase, 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.[1] Two Cys and one His residue seem to be essential components of the catalytic site.[2] In most eukaryotic

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organisms, LADH is a matrix-located, mitochondrial enzyme and should therefore be accessible to "reactive oxygen species" generated in mitochondria.[3-6]

Cu(II)-Fenton systems[7] are able to oxidize proteins and inactivate enzymes.[8-10] Oxidative damage by Cu(II)/H_2O_2 and Cu(II)/Asc is attributed to production of "reactive oxygen species", namely, HO· radicals, H_2O_2 and/or hypervalent copper complexes.[11-15] Cu and Fe are mobilized following myocardial ischemia[16,17] and by virtue of their capacity to catalyze the production of HO· radicals, they play a causative role in ischemia-reoxygenation myocardial injury.[16,17] Myocardial injury affects mitochondrial enzymes[4,10] and, accordingly, it may affect mitochondrial LADH. This hypothesis fits in well with LADH inactivation by cupric salts[2] and by the Fe(II)-Fenton system.[18] Taking into account the foregoing information, it seemed of interest to investigate LADH inactivation by the Cu(II)-Fenton systems, as well as the effect of cardio-protective antioxidants on the Cu(II)-dependent LADH inactivation.

MATERIALS AND METHODS

Materials

$CuSO_4.5H_2O$, bovine serum albumin, Captopril, batocuproine, DL-histidine, L-cysteine, N-acetyl-L-cysteine, DL-penicillamine, N-(2-mercaptopropionylglycine), allopurinol and Na benzoate were purchased from Sigma Chemical Co., St. Louis, MO, USA. H_2O_2 was purchased from Carlo Erba, Milano. Fresh $CuSO_4$, Na ascorbate and H_2O_2 solutions were prepared immediately before use. Other reagents were as described previously.[18]

Enzyme Assay

LADH activity was measured by the rate of NADH oxidation using lipoamide as electron acceptor. Unless stated otherwise, the reaction medium contained 50 mM $KH_2PO_4 - K_2HPO_4$, pH 7.4, 0.2 mM NADH, 1.0 mM lipoamide and LADH as stated under Results. Spectrophotometric measurements were performed using a Perkin-Elmer 550UV/VIS spectrophotometer at 30°C. Other assays were as described previously.[18]

LADH Inactivation

Unless stated otherwise, LADH (1 μM) in 200 μl 50 mM $KH_2PO_4 - K_2HPO_4$, pH 7.4, 10 μM $CuSO_4$ and 3.0 mM H_2O_2 (or 0.5 mM ascorbate) with the additions or omissions indicated under Results was incubated in a test-tube for 5 min at 30°C. Aliquots were taken and residual LADH activity was measured as described above. The reaction was started by adding the substrates and the initial velocity of the LADH reaction was measured by the slope of the recorded tracings. Control samples without FS were incubated simultaneously. A similar procedure was followed for measuring LADH diaphorase activity.

Chemical Assays

Thiol groups in LADH were measured, under denaturing conditions, by the method of Ellman.[19] LADH was incubated with Cu(II)/H_2O_2, Cu(II) or H_2O_2 for 1 hour at

30°C, in the standard reaction medium. Samples were filtered on the Ultrafree-MC (NMWL 30.000), at 3000 g and 10°C for 20 min. The protein on the filter membrane was dissolved in 2.5 ml of 50 mM K-phosphate, pH 7.4 and the filtration step repeated twice. To the residual protein solution (0.4 ml), 2.5 ml of 6 M guanidine-HCl-3.0 mM EDTA – 50 mM K-phosphate, pH 7.4 and 0.1 ml of 10 mM DTNB in 50 mM K-phosphate, pH 7.4, were successively added. After 30 min incubation at 20°C, absorbance at 412 nm was measured. Other experimental conditions were as described.[18] Protein concentration was measured by the method of Lowry *et al.*[20] Measurement of benzoate hydroxylation was performed as described by Gutteridge,[21] using an Aminco-Bowman Spectrofluorometer at 310 nm (excitation) and 405 nm (emission).

Assay of TBA-reactive Products

Deoxyribose oxidation[22] was monitored by heating solutions (0.5 ml) for 10 min at 100°C with 0.5 ml TBA (1 g/100 ml 0.05 M NaOH) and 0.5 ml trichloroacetic acid (2.8 g/100 ml). Absorbance at 532 nm was read against a blank containing both reagents. Other experimental conditions were as already described.[18]

Expression of Results

(a) LADH activity is expressed in μmol NADH oxidized/min per mg protein (Table I). (b) Taking into account the limited variation in the activity of different LADH samples, in Figures 1–3, relative activity values are presented (100% for the control sample). (c) Protection (*P*) against inhibitors (Tables II–VII) was calculated from Equation 1 where *P*, *i* and *ip* are the protector relative activity, the inhibition of LADH activity by the

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

Cu(II)-Fenton system (or Cu(II)), and the inhibition by the Cu(II)-system (or Cu(II)) plus protector, respectively. Duplicate values deviated from the average by less than 5%. When more than two measurements were performed, the values presented are the average ± SD.

RESULTS

Incubation of LADH with the Cu(II) systems caused a significant diminution of lipoamide reductase activity. Results in Table I show that after 5 min incubation with LADH, Cu(II) plus H_2O_2 (or ascorbate) inactivated LADH by 87 and 83% respectively, whereas Cu(II) alone inactivated LADH by43%. After 30 s incubation under the same experimental conditions, the inactivation values for H_2O_2, Cu(II) and Cu(II)/H_2O_2 were 0, 20 and 37%, respectively (other experimental data omitted). The LADH dihydrolipoamide dehydrogenase activity was inhibited to a similar degree to the lipoamide reductase activity (experimental data omitted). At variance with these results LADH DCI-diaphorase activity increased significantly after treatment with Cu(II)/H_2O_2, the greatest increase (roughly 4-fold) being obtained with Cu(II)/H_2O_2 (Table 1). Titration of thiol groups in LADH under denaturing conditions showed that after treatment with Cu(II)/H_2O_2 only one thiol group per subunit reacted with DTNB whereas with Cu(II) or H_2O_2, 5 thiol groups were titrated.

TABLE I
Effect of Cu(II) systems on LADH activity and LADH thiol groups

Additions	LADH-activity		Thiol residues per LADH subunit
	Lipoamide reductase (A)	Diaphorase (B)	
None	110 ● 4.7	4.4 ± 0.4	6.0 ± 0.3
$Cu(II)/H_2O_2$	16 ± 8.9	17.9 ± 1.0	1.0 ± 0.1
Cu(II)	58 ± 15	11.2 ± 1.6	4.8
H_2O_2	107 ± 2.8	2.7 ± 0.1	4.9 ± 0.1
Cu(II)/Asc	19 ± 8.8	12.4 ± 0.7	-
Ascorbate	108 ± 1.0	6.4 ± 0.4	-

LADH inactivation. The reaction medium contained 1.0 μM LADH, 50 mM K-phosphate buffer, pH 7.4, 10 μM $CuSO_4$, 3.0 mM H_2O_2 and 0.5 mM ascorbate, as indicated above; total volume, 0.2 ml. After 5 min incubation at 30°C, activities were measured: A, μmol NADH/min per mg protein; B, μM DCI/min per mg protein. *Thiol oxidation.* The reaction medium contained 5.0 μM LADH, 50 mM K-phosphate buffer, pH 7.4, 5.0 μM $CuSO_4$ and 3.0 mM H_2O_2, as indicated above; total volume, 1.2 ml. After 1 h incubation at 30°C, LADH thiol groups were titrated as described under Materials and Methods. Values are the average ± S.E. of at least 3 measurements except for the thiol values in Cu(II)-treated LADH which are the average of duplicate measurements.

The corresponding value for the control sample was 6 out of 7 titratable thiol groups.[1]

Production of HO·, by $Cu(II)/H_2O_2$ in the presence of LADH was investigated by the deoxyribose and benzoate methods. The results obtained (Table II) indicate that (a) $Cu(II)/H_2O_2$ was an effective generator of HO· radicals; (b) Cu(II) or H_2O_2 alone were weak generators of HO· radicals and (c) LADH inhibited the

TABLE II
Effect of LADH on deoxyribose oxidation and benzoate hydroxylation

Assay	Additions	LADH	
		None	1.0 μM
		TBA reaction products (10^3 × A)	
A) Deoxyribose oxidation	Cu(II)	14	18
	H_2O_2	46	32 (30)
	$Cu(II)/H_2O_2$	142	59 (58)
		Fluorescence (units)	
B) Benzoate hydroxylation	Cu(II)	0	0
	H_2O_2	2	0.5
	$Cu(II)/H_2O_2$	29 ± 0.6	2.8 ± 0.6 (90)
	$Cu(II)/H_2O_2$	29 ± 0.6*	28 ± 1.3 (3.5)*

The reaction medium contained 50 mM K-phosphate, pH 7.4, 3.0 mM deoxyribose (exp. A) or 2.0 mM Na-benzoate (exp. B) and additions as indicated above. 10 μM Cu(II) and 3.0 mM H_2O_2. After incubation for 1 h at 30°C, TBA reaction products (exp. A) or fluorescent products (B), were measured. Other experimental conditions were as described under Materials and Methods. Exp. A: values include the blank absorbance (45). Exp. B: values are the average of duplicate measurements, except for $Cu(II)/H_2O_2$, which are the average ± SD of 3 measurements. * means that LADH was added to the reaction medium after incubation. In parenthesis, percentage inhibition of deoxyribose oxidation or benzoate hydroxylation.

TABLE III
Effect of NAD^+ NADH, ATP and ADP on LADH inactivation by Cu(II)/ H_2O_2 and Cu(II)

Experiment	Cu(II) (μM)	Addition	LADH inactivation (%)	
			Cu(II)/H_2O_2	Cu(II)
A	7.5	None	96	49
		NAD^+	96	28
		NADH	99	88
		ATP	80	50
		ADP	89	78
B	2.5	None	36	12
		NAD^+	32	8
		NADH	96	60

Experimental conditions were as described in Table I legend and under Materials and Methods. Additions were as follows: 1.0 mM NAD^+; 0.5 mM NADH, ATP or ADP and 1.0 μM LADH. In the absence of Cu(II), NAD^+, NADH, ATP and ADP did not affect LADH activity.

HO·-dependent oxidation reactions. The effect of LADH was greater when using the benzoate method. With this connection, it should be noted that LADH did not quench the fluorescence of benzoate hydroxylation reaction products (Table II).

LADH inactivation by Cu(II)/H_2O_2 and Cu(II) was modified by coenzymes and adenine nucleotides. Results in Table III indicate that (a) NAD^+ prevented LADH inactivation by Cu(II), but not by Cu(II)/H_2O_2; (b) NADH enhanced the effect of Cu(II)/H_2O_2; (c) ADP exerted a similar action on Cu(II) effect's; and (d) ATP prevented Cu(II)/H_2O_2 action to a limited degree. In order to observe NADH enhancement of oxidant action, in these experiment a relatively low concentration of Cu(II) was used.

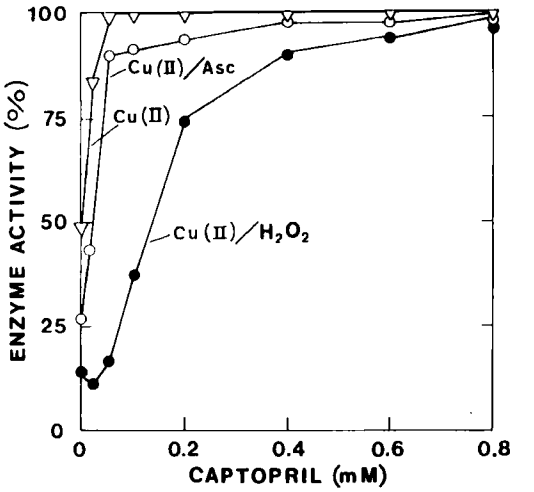

FIGURE 1 Effect of Captopril on LADH inactivation by Cu(II)/H_2O_2 (●), Cu(II)/Asc and Cu(II) (▽). Experimental conditions were as described under Materials and Methods. Captopril concentration was as indicated in the abscissa and Cu(II) systems were as indicated on the Figure. Control sample relative activity 100%.

Inactivation of LADH by all the Cu(II) systems assayed was counteracted by Captopril, a monothiol inhibitor of the angiotensin-converting enzyme.[23,24] Figure 1 shows that Captopril acted in a concentration-dependent manner, proving most effective against Cu(II) and least so against Cu(II)/H_2O_2. Figures 2 and 3 show the results of similar experiments using two different antioxidant thiols, namely, N-acetyl cysteine[25] and penicillamine.[26] N-acetylcysteine, prevented LADH inactivation by all the Cu(II) systems assayed, but protection against Cu(II)/H_2O_2 was only

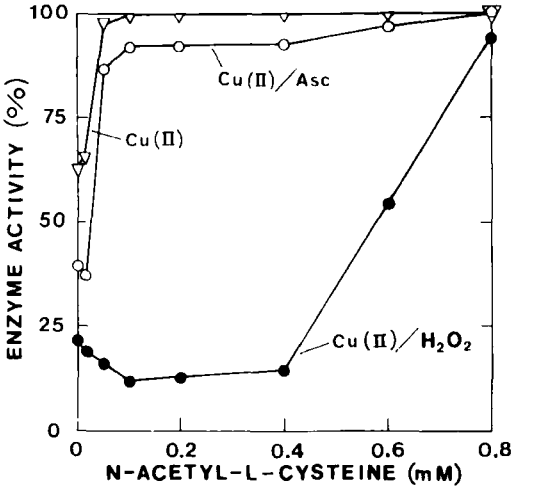

FIGURE 2 Effect of acetylcysteine on LADH inactivation by Cu(II)/H_2O_2 (Cu(II)-FS); (●), Cu(II)/Asc (●) and Cu(II) (▽). Conditions were as described in Figure 1 legend. Acetylcysteine concentration was as indicated on the abscissa.

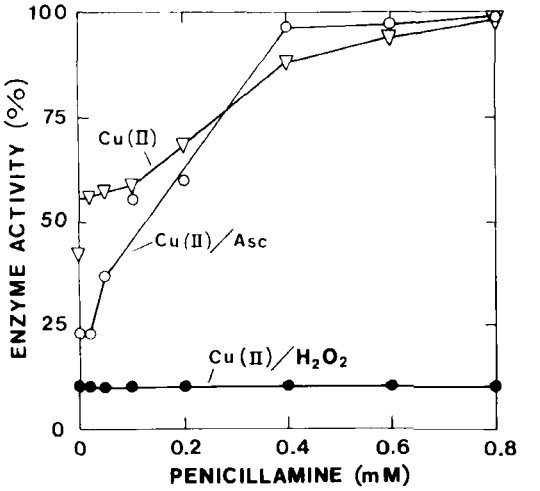

FIGURE 3 Effect of penicillamine on LADH inactivation by Cu(II)/H_2O_2 (●), Cu(II)/Asc (●) and Cu(II) (▽). Conditions were as described in Figure 1 legend. Penicillamine concentration was as indicated on the abscissa.

obtained at relatively high thiol concentrations. Penicillamine, was active against Cu(II)/Asc and Cu(II) but not against Cu(II)/H_2O_2 (Figure 3). Table IV summarizes the results obtained with other thiol compounds. It may be seen that (a) dihydrolipoamide and dihydrolipoic acid preserved LADH from inactivation by Cu(II)/H_2O_2 and Cu(II)/Asc, especially from the former; (b) GSH, DL-dithiothreitol, N-(2-mercaptopropionylglycine), cysteine and lipoamide were ineffective against Cu(II)/H_2O_2; (c) with Cu(II)/Asc, the thiol compounds were strong protectors of LADH except for cysteine and lipoamide. Similar results were observed when using Cu(II) alone as inhibitor of LADH (experimental data omitted).

Inactivation of LADH by Cu(II)/H_2O_2 and Cu(II)/Asc was affected by copper-chelators (Table V). Thus, at 100 μM concentration, EDTA, DETAPAC, histidine and bathocuproine counteracted Cu(II)/H_2O_2 to a significant degree. Similar results were obtained with Cu(II)/Asc and with this system, LADH inactivation was also

TABLE IV
Effect of thiol and related compounds on LADH inactivation by Cu(II) systems

Thiol compound (mM)	LADH protection (%)	
	Cu(II)/H_2O_2	Cu(II)/Asc
Dihydrolipoamide (0.05)	95	95
Dihydrolipoamide (0.10)	95	100
Dihydrolipoic acid (0.05)	39	99
Dihydrolipoic acid (0.10)	99	100
GSH (0.10)	14	100
DL-Dithiothreitol (0.10)	2	100
N-(2-mercaptopropionylglycine) (0.4)	1.4	96
Cysteine (0.10)	3	15
Lipoamide (1.0)	0	1

Experimental conditions were as described in Table I legend and under Materials and Methods. The reaction medium was supplemented with thiol compounds as indicated above. Inactivation (%) of LADH by Cu(II)/H_2O_2 and Cu(II)/Asc (*i* values): 98 and 86, respectively.

TABLE V
Effect of Cu(II) chelators on LADH inactivation by Cu(II) systems

Chelator (μM)	LADH protection (%)	
	Cu(II)/H_2O_2	Cu(II)/Asc
EDTA (10)	14	100
EDTA (100)	104	103
DETAPAC (10)	21	100
DETAPAC (100)	100	100
Histidine (10)	5	13
Histidine (100)	81	59
Bathocuproine (10)	71	60
Bathocuproine (100)	93	100
Allopurinol (100)	0	31
GSSG (100)	90	–
Trypanothione (100)	101	100

Experimental conditions were as described in Table I legend and under Materials and Methods. The reaction medium was supplemented with chelators as indicated above. Inactivation (%) of LADH by Cu(II)/H_2O_2 or Cu(II)/Asc (*i* values): 96 and 86, respectively.

prevented by allopurinol. On the other hand, at 10 μM concentration, the action of EDTA and DETAPAC against Cu(II)/H_2O_2 was partial and histidine was inactive. At variance with these results, 10 μM EDTA and DETAPAC provided full protection against the Cu(II)/Asc (Table IV) and Cu(II) alone (experimental data omitted). 100 μM GSSG and trypanothione (a spermidine-glutathione conjugate, oxidized form[27]) protected LADH against Cu(II)/H_2O_2 and the latter also protected against Cu(II)/Asc. Chelators prevented LADH inactivation by Cu(II) alone, at all concentrations assayed (experimental data omitted).

The action of thiol compounds and copper chelators on LADH modification allowed the latter's reversibility to be examined. The results in Table VI show that when thiol compounds or chelators were added to LADH before the oxidizing system, LADH activity was always conserved but when added after, the enzyme activity was not restored (Table VI).

Hydroxyl radical "scavengers" such as ethanol, mannitol, Na benzoate and superoxide dismutase did not modify LADH inactivation by Cu(II)/H_2O_2 (Table VII). LADH activity was preserved by bovine serum albumin and catalase, but native and denaturated catalase acted alike. Since catalase has a strong affinity for cupric ions,[28] catalase effect would result from Cu(II) binding, not from H_2O_2 decomposition. A similar mechanism may explain albumin effect in Table VII.

DISCUSSION

The results here described indicate that LADH inactivation by the Cu(II) Fenton systems depends on "reactive oxygen species", in all probability HO· radicals. Thus, (a) the greatest effect of the Cu(II)-systems occurred in the presence of both Cu(II) and H_2O_2 (or ascorbate) (Table I); (b) Cu(II)/H_2O_2 oxidized deoxyribose and hydroxylated benzoate, these effects being inhibited by LADH (Table II); (c) NADH enhanced LADH inactivation by Cu(II), in close agreement with HO·, production

TABLE VI
Irreversibility of LADH inactivation by Cu(II) systems

Addition (mM)	LADH protection (A) or reactivation (B)					
	Cu(II)/H_2O_2		Cu(II)/Asc		Cu(II)	
	A	B	A	B	A	B
Captopril (0.4)	88	1	103	0	100	−4
Dihydrolipoamide (0.5)	100	1	101	0	103	−25
GSH (1.0)	76	−2	97	0	100	−8
DL-Dithiothreitol (1.0)	95	2	100	−4	100	−4
EDTA (0.1)	104	2	100	0	104	0
DETAPAC (0.1)	100	−2	104	−1	105	−6

Expt. A: LADH was added to the reaction medium containing the Cu(II) system (or Cu(II)) and additions as indicated above. After 5 min incubation, substrates were added and LADH activity was measured. Expt. B: similar conditions except that protectors were added after 5 min incubation, immediately before the substrates. Other experimental conditions were as described under Materials and Methods and Tables IV and V.

TABLE VII
Effect of scavengers on LADH inactivation by Cu(II) systems

Scavenger	LADH inactivation (%)	
	$Cu(II)/H_2O_2$	Cu(II)/Asc
None	80	83
Ethanol (6.0 mM)	87 (−8)	-
Mannitol (300 mM)	87 (−8)	-
Na Benzoate (200 mM)	85 (−6)	-
Superoxide dismutase (80 μg/ml)	85 (−6)	-
Bovine serum albumin (50 μg/ml)	43 (46)	-
Catalase (40 μg/ml)	-	5 (94)
Catalase denatured (40 μg/ml; heated)	-	6 (93)

Experimental conditions were as described under Materials and Methods. The reaction medium was supplemented with scavengers as indicated above. Catalase inactivated (100%) by heating at 100°C for 10 min (denatured catalase). Catalase specific activity, 35 units/μg; superoxide dismutase especific activity, 5 units/μg. The figures in parenthesis indicate LADH protection (%).

by the Cu(II)-NADH system;[29] (d) Captopril (Figure 1), dihydrolipoamide and dihydrolipoic acid (Table IV), all of them effective radical scavengers,[11,23,24,30-32] protected LADH against the Cu(II) systems assayed; (e) copper chelators (Table V) prevented LADH inactivation by $Cu(II)/H_2O_2$ and Cu(II)/Asc system (Table V); (f) LADH modification by the $Cu(II)/H_2O_2$ system was a relatively slow, biphasic process, as indicated by the difference between the inactivation values after 0.5 and 5.0 min incubation (Table I and data in the text). Taken together with the absence of LADH protection by oxy-radical scavengers (Table VII), the summarized results are in close agreement with the site-specific nature of the copper-catalyzed inactivation of LADH.

The decrease of titratable thiols after LADH treatment with $Cu(II)/H_2O_2$ (Table I) supports thiol oxidation as a main cause of LADH inactivation. It is known[1] that a drop of 2 in the number of thiols titrated by cupric ions leads to (a) the loss of LADH lipoamide reductase activity and the increase in diaphorase activity.[1,2] An imidazole residue in LADH, in the vicinity of LADH active centre thiols,[2] may bind Cu(II), prior to its catalysis of thiol oxidation[1] since copper complexes catalyze the oxidation of thiol groups to their corresponding disulfides.[33] The irreversibility of LADH inactivation, by thiol compounds and copper-chelators (Table V), suggests that in addition to thiols, other amino acid residues (histidyl, prolyl and tyrosyl) should be affected by "reactive oxygen species", as expected from studies with other proteins.[8]

At variance with LADH inactivation by the Fe(II) Fenton system,[18] ATP prevented the effect of Cu(II) or $Cu(II)/H_2O_2$ only to a limited degree (Table III), whereas ADP and NADH enhanced the Cu(II) effect. It is known that copper salts oxidize NADH,[29] thus producing $HO\cdot$ radicals. The results in Table III suggest that depletion of NAD^+, ATP[34] and accumulation of NADH and/or ADP, as a result of myocardial hypoxia,[34] may facilitate the Fe(II) or Cu(II)-catalyzed inactivation of LADH.

Protection of LADH by thiol compounds deserves a special comment. Captopril and N-acetylcysteine (Figures 1 and 2, respectively) impeded LADH modification by all the Cu(II) systems assayed but N-acetylcysteine was effective against Cu(II)/

H_2O_2 only at relatively high concentrations. The effect of Captopril can be explained by scavenging HO· radicals and/or reductive chelation of Cu(II).[23,24] With this connection, it should be noted that Captopril, decreases the myocardial infarct size and prevents the corresponding electrocardiographic alterations in rats.[35] Dihydrolipoamide and dihydrolipoic acid protected LADH against Cu(II)/H_2O_2 and Cu(II)/Asc (Table IV) in close agreement with their antioxidant action *in vivo*, especially on heart mitochondria.[30-32] On the other hand, GSH (0.1 mM), DL-dithiothreitol (0.1 mM), N-(2-mercaptopropionylglycine) and penicillamine were inactive against Cu(II)/H_2O_2, despite their action against Cu(II)/Asc and Cu(II) alone (Table IV and Figure 3). Nevertheless, 1.0 mM GSHand DL-dithiothreitol preserved LADH from inactivation by Cu(II)/H_2O_2 (Table VI). GSH is present in myocardial mitochondria[36] where it may prevent LADH oxidation by endogenously generated "reactive oxygen species".[3-6] The antioxidant action of 1.0 mM GSH (Table VI) would result from scavenging of HO·, radicals and/or stabilization of copper in the cuprous form.[13,37] On the other hand, the relative inefficiency of 0.1 mM GSH against Cu(II)/H_2O_2 (Table IV) may be explained by (a) oxidation of GSH and production of HO· radicals[38] and/or (b) formation of Cu(III) as an intermediate during the reaction of Cu(I) with H_2O_2.[13]

EDTA, DETAPAC, histidine and bathocuproine are known to form complexes with Cu(II). At 100 μM concentration (about ten-fold Cu(II) concentration), these chelators counteracted effectively the action of Cu(II)/H_2O_2. Similar effects were observed with Cu(II)/Asc, allowance made for histidine (Table V), thus proving the essential role of Cu(II) in LADH damage. At 10 μM concentration, the chelators action against Cu(II)/H_2O_2 was partial, since non-chelated Cu(II) can produce HO· radicals and hence damage LADH. As regards histidine, it should be noted that, in addition to binding Cu(II), it scavenges singlet oxygen and HO· radicals.[39] Allopurinol did not impede LADH inactivation by Cu(II)/H_2O_2 but afforded partial protection against Cu(II)/Asc. Both histidine[39] and allopurinol[40] are cardioprotectors. The effect trypanothione (Table V), and its similar action against the iron Fenton system,[18] supports the hypothesis that trypanothione may protect trypanosomatids against "reactive oxygen species".

Taken together with LADH inactivation by the Fe(II) Fenton system,[18] the observations here described strongly point to LADH as a target for "reactive oxygen species" generated by myocardial ischemia-reperfusion.

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