Free Rad. Res., Vol. 30, pp. 105–117
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Inactivation of Myocardial Dihydrolipoamide Dehydrogenase by Myeloperoxidase Systems: Effect of Halides, Nitrite and Thiol Compounds

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Accepted by Prof. V. Darley-Usmar

(Received 21 May 1998; In revised form 19 August 1998)

Dihydrolipoamide dehydrogenase (LADH) lipoamide reductase activity decreased whereas enzyme diaphorase activity increased after LADH treatment with myeloperoxidase (MPO) dependent systems (MPO/H_2O_2/halide, MPO/NADH/halide and MPO/H_2O_2/nitrite systems. LADH inactivation was a function of the composition of the inactivating system and the incubation time. Chloride, iodide, bromide, and the thiocyanate anions were effective complements of the MPO/H_2O_2 system. NaOCl inactivated LADH, thus supporting hypochlorous acid (HOCl) as putative agent of the MPO/H_2O_2/NaCl system. NaOCl and the MPO/H_2O_2/NaCl system oxidized LADH thiols and NaOCl also oxidized LADH methionine and tyrosine residues. LADH inactivation by the MPO/ NADH/ halide systems was prevented by catalase and enhanced by superoxide dismutase, in close agreement with H_2O_2 production by the LADH/NADH system. Similar effects were obtained with lactoperoxidase and horseradish peroxidase suplemented systems. L-cysteine, N-acetylcysteine, penicillamine, N-(2-mercaptopropionylglycine), Captopril and taurine protected LADH against MPO systems and NaOCl. The effect of the MPO/H_2O_2/$NaNO_2$ system was prevented by MPO inhibitors (sodium azide, isoniazid, salicylhydroxamic acid) and also by L-cysteine, L-methionine, L-tryptophan, L-tyrosine, L-histidine and reduced glutathione. The summarized observations support the hypothesis that peroxidase-generated "reactive species" oxidize essential thiol groups at LADH catalytic site.

Keywords: Dihydrolipoamide dehydrogenase, myeloperoxidase, peroxidase, halides, hypochlorous acid, nitrite, thiol compounds

Abbreviations and chemical terms: LADH, dihydrolipoamide dehydrogenase (NADH-lipoamide oxido-reductase, E.C. 1.6.4.3); MPO, myeloperoxidase; HRP, horseradish peroxidase; LPO, lactoperoxidase; HOCl (OCl^-); hypochlorous acid (anion); DCI, dichlorophenol-indophenol; SOD, superoxide dismutase; DTNB, 5,5′-dithio-*bis*(2-nitrobenzoic acid); DETAPAC, diethylenetriamine pentaacetic acid; K-phosphate, KH_2PO_4-K_2HPO_4; GSH and GSSG, reduced and oxidized glutathione, respectively; Cys, L-cysteine; NAC, N-acetylcysteine; MPG, N-(2-mercaptopropionylglycine); PAM, penicillamine (3-mercapto-D-valine); CPT, Captopril [1-(3-mercapto-2-methyl-1-oxopropyl-L-proline)]; Isomiazid, 4-pyridine carboxylic acid hydrazide

INTRODUCTION

Mammalian lipoamide dehydrogenase (LADH), a mitochondrial enzyme, is a flavoprotein which

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reversibly catalyses the oxidation of dihydrolipoamide by NAD^+, to yield lipoamide and NADH (Reaction (1)). The enzyme is a component of α-oxoacid dehydrogenase complexes, such as the

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

pyruvate dehydrogenase complex, the α-oxoglutarate dehydrogenase complex and the branched chain α-oxoacid dehydrogenase complex.[1] Myocardial LADH is inactivated by oxygen radicals generated by Fenton systems ($Fe(II)/H_2O_2$ and $Cu(II)/H_2O_2$).[2–4] Catecholamines enhance LADH inactivation by $Cu(II)/H_2O_2$ whereas thiol compounds preserve LADH from oxidative damage.[4]

Myeloperoxidase (MPO) plays a fundamental role in oxidant production by neutrophils.[5] This heme enzyme uses H_2O_2 and chloride ions to catalyze the production of hypochlorous acid,

$$H_2O_2 + Cl^- + H^+ \longrightarrow HOCl + H_2O \quad (2)$$

(Reaction (2)) which under physiological conditions is the major strong oxidant generated by neutrophils in appreciable amounts.[5] Among the multiple effects of MPO and/or HOCl stand the inhibition of respiratory dehydrogenases,[6] the modification of structural proteins[7] and damage to cardiomyocytes.[8] HOCl exerts diverse effects on enzymes and proteins. Thus, it activates collagenase,[9] but inactivates glutathione peroxidase, SOD, catalase[10,11] and *Escherichia coli* F1-ATPase.[12] The kinetics of enzyme inactivation by HOCl depends on the enzyme structure: it is very fast with glutathione peroxidase, fast with catalase and relatively slow with SOD.[10]

Neutrophils are major effector cells in tissue damage occurring in many pathological conditions, including inflammation and myocardial ischemia-reperfusion injury.[13–15] Accordingly, MPO can contribute to neutrophil-dependent oxidative damage in infiltrated zones. On these grounds, it seemed of interest to investigate (a) myocardial LADH modification by MPO generated "reactive species", using physiologically accessible MPO substrates, namely halides and nitrite, and (b) the role of thiol compounds as LADH protectors against the reactive species generated by the MPO-dependent systems.

MATERIALS AND METHODS

Enzyme Preparations

Porcine heart LADH (Type III), human leukocytes MPO, HRP, LPO, SOD, microperoxidase-11 and catalase were obtained from Sigma Chemical Co., (St. Louis, MO, USA). Catalase was denatured at 100°C for 10 min. The specific activity of LADH preparations ranged from 100 to 130 units/mg. Enzyme suspensions were diluted ten-fold with 50 mM K-phosphate, pH 7.4 and kept at 4°C for subsequent use. MPO specific activity ranged from 50 to 100 units/mg, as determined using the guaiacol/H_2O_2 assay system.[16] HRP and LPO were used as supplied.

Reagents

NaOCl was obtained from Carlo Erba, Milano, as a 7% (w/v) solution and sample concentration was determined spectrophotometrically at 292 nm ($\varepsilon = 0.35\ mM^{-1}\ cm^{-1}$). H_2O_2 was obtained from E. Merck, Damstadt, Germany and sample concentration was determined spectrophotometrically at 240 nm ($\varepsilon = 0.04\ mM^{-1}\ cm^{-1}$). Analytical grade NaCl, KI, NaBr, KSCN and $NaNO_2$ were from J.T. Baker, Phillipsburg, NJ, U.S.A. Ultrafree MC (NM N 30000 filters, DTNB, NAC, PAM, MPG, CPT, DCI, DETAPAC, taurine, isoniazide, sodium azide, salicylhydroxamic acid and guanidine-HCl were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sodium peroxynitrite (100 mM) was kindly supplied by Dr. H. Rubbo, Uruguay. The solution concentration was determined spectrophotometrically, at 302 nm ($\varepsilon = 1670\ M^{-1}\ cm^{-1}$). Other reagents were as described previously.[2–4]

LADH Inactivation

LADH (1 μM) in 200 μl 50 mM K-phosphate, pH 7.4 was incubated for different periods with MPO, H_2O_2 and the corresponding additions, at 30°C, as indicated under Results. Aliquots (10 or 20 μl) of inactivation mixture were added to the assay medium and residual LADH activity was measured as described below. 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 peroxidase or other additions were incubated simultaneously.

LADH Assays

LADH activity was measured by the rate of NADH oxidation using lipoamide as electron acceptor. Unless stated otherwise, the standard reaction medium contained 50 mM K-phosphate, pH 7.4, 0.2 mM NADH, 1.0 mM lipoamide and 3.3 or 6.6 nM LADH. Diaphorase activity was measured by the rate of DCI reduction using NADH as electron donor.[3] The corresponding reaction medium contained 50 mM K-phosphate, pH 7.4, 0.2 mM NADH, 20 nM LADH and 40 μM DCI, total volume, 1.0 ml. DCI reduction was measured spectrophotometrically at 600 nm ($\varepsilon = 19\,mM^{-1}\,cm^{-1}$). Spectrophotometric measurements were performed using a Perkin-Elmer 550 UV/VIS spectrophotometer at 30°C. Other assays were as described previously.[2–4]

Chemical Assays

Production of H_2O_2 by the LADH/NADH system was measured by the microperoxidase method,[17] previously standardized with H_2O_2 solutions of known concentration.

Amino acid analysis of LADH samples was performed using a Beckman 119 CL Analyser, according to standard procedures. The sample (1.0 mg LADH) was incubated for 30 min at 30°C with NaOCl; the oxidant/enzyme ratio being 140 nmol NaOCl per nmol of LADH. After incubation, oxidized protein was filtered through the MC (NMWL 30000) membrane, at 3000*g* and 10°C, for 20 min. The protein was dissolved in 2.0 ml of 50 mM K-phosphate, pH 7.4, and the filtration procedure was repeated twice. To the residual LADH, 50 mM K-phosphate, pH 7.4 was added up to 0.4 ml of total volume. Samples were then hydrolysed with 6 N HCl, at 110°C, for 24 h, and the amino acid analysis was performed. The method used excluded tryptophan and cysteine residues.

Thiol groups in LADH were titrated, under denaturing conditions, by the method of Ellman.[18] Briefly, LADH samples (12.5 μM in 50 mM K-phosphate, pH 7.4) were supplemented with additions as indicated in Table I. Total volume of inactivation mixture and incubation time were as indicated under Results. Immediately after incubation, 1.0 mM L-methionine was added, to scavenge any remaining NaOCl (or chloramines).[7] Samples were then thoroughly mixed with 0.75 ml of a 6.0 M guanidine-HCl, 3.0 mM EDTA and 50 mM K-phosphate, pH 7.4. Subsequently, 18 μl of a solution containing 10 mM DTNB and 50 mM K-phosphate, pH 7.4 was added (final DTNB concentration, 0.18 mM). After 15 min incubation at 30°C, absorbance was measured spectrophotometrically at 412 nm. Thiol concentration was calculated using $\varepsilon = 13.600\,M^{-1}cm^{-1}$.

Expression of Results

LADH specific activity is expressed in μmol NADH oxidized/min per mg protein. Taking into account the limited variation of LADH specific activity, relative activity values are presented as the percentage value of the control sample activity (100%). LADH inactivation by pro-oxidant systems $I(\%)$ was calculated from the equation $I(\%) = 100(A_c - A_{ox}/A_c)$ where A_c and A_{ox} are the control sample activity and the oxidized (inactivated) sample activity, respectively. Protection (P) by thiol compounds against pro-oxidant systems

or NaOCl, was calculated from the equation $P(\%) = 100(i(\%) - ip(\%)/i(\%))$ where P, i and ip are the protector relative activity, the inhibition of LADH activity by the pro-oxidant system and the inhibition of LADH activity by the latter system plus protector (p), respectively. When the activity of LADH samples was measured at different incubation times, values represent the mean of duplicate measurements. These values deviated from the experimental values by less than 5%. When more than two measurements were performed, the values presented are means ± SD. Statistical analysis was performed using Student's t-test for paired values. Figures show results representing at least two duplicate measurements.

RESULTS

LADH Inactivation by MPO/H_2O_2/ Halide Systems

Figure 1 shows typical results illustrating the effect of the MPO/H_2O_2/NaCl system on LADH activity. It is to be seen that incubation of LADH with the MPO system produced a time-dependent enzyme inactivation which reached its maximum value after 60 min incubation. LADH inactivation depended on H_2O_2 concentration since it was greater with 100 μM than with 50 μM H_2O_2. Similar treatments of LADH with H_2O_2/NaCl, MPO/H_2O_2(Figure 1), MPO/NaCl or MPO alone (data not shown), failed to modify LADH activity to a significant degree. LADH inactivation by the MPO/H_2O_2/NaCl system was pH dependent since it was greater at pH 6.0 (a possible pH in phagosomes[5]) than at pH 7.4 (Figure 2). The pH variation failed to affect LADH activity, in the absence of the MPO/H_2O_2/NaCl system. In contrast to lipoamide reductase inactivation, the MPO/H_2O_2/NaCl system produced a progressive increase in LADH diaphorase activity (LADH-catalysed electron transfer from NADH to DCI) (Figure 3).

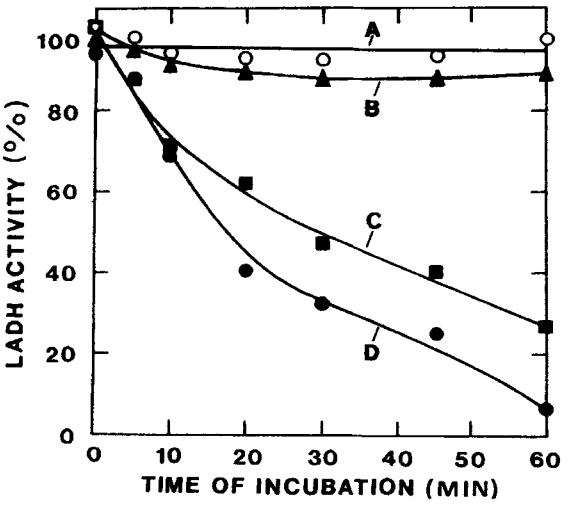

FIGURE 1 Time-course of LADH inactivation by the MPO/H_2O_2/NaCl system. Reagent concentrations were: 1.0 μM LADH (A–D); 0.5 U/ml MPO (B–D); 100 μM H_2O_2 (A,B,D), 50 μM H_2O_2 (C), 100 mM NaCl (A,C,D); 50 mM K-phosphate, pH 7.4 (A–D). After incubation for the time indicated on the abscissa, LADH activity was measured. Other experimental conditions were as described under Materials and Methods. Values represent the means of duplicate measurements.

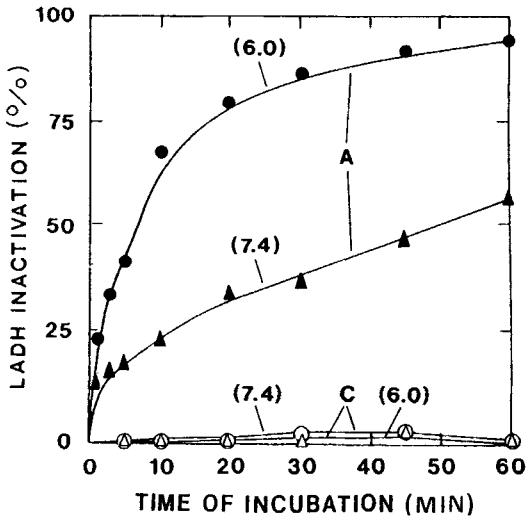

FIGURE 2 (A): Effect of pH on LADH inactivación by the MPO/H_2O_2/system. Experimental conditions were as in Figure 1 legend except pH, which is indicated by the numbers in parentheses. (C): Control assay in which LADH was preincubated as above except the MPO/H_2O_2/NaCl system (omitted). After incubation, LADH activity was measured at pH 7.4.

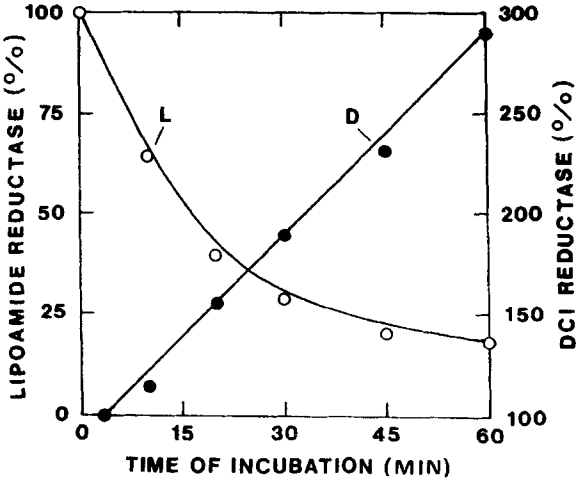

FIGURE 3 Diaphorase (D) activation and lipoamide reductase (L), inactivation by the MPO/H_2O_2/NaCl system. Experimental conditions were as described in Figure 1 legend and under Materials and Methods. Activity values are presented as percentage of the control sample relative activity (100%).

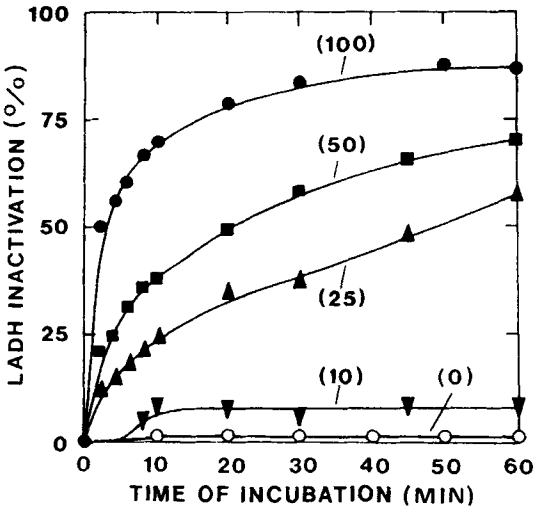

FIGURE 4 Time-course of LADH inactivation by NaOCl. Reagent concentrations were: 1.0 μM LADH, 50 mM K-phosphate, pH 7.4 and NaOCl (μM) as indicated by the numbers in parenthesis. Other experimental conditions were as described in Figure 1 legend.

The results in Figure 1 indicate that HOCl generated by chloride oxidation should be responsible for LADH inactivation and, accordingly, the effect of NaOCl was assayed. The results in Figure 4 show that, as expected, NaOCl inactivated LADH in a concentration-dependent manner, significant effects being observed with a NaOCl concentration as low as 10 μM. The inactivation kinetics was biphasic, with an initial fast phase, lasting for about 10 min followed by a relatively slow phase lasting to the end of incubation. Moreover, NaOCl effect did not depend on transition metals since 200 μM DETAPAC increased (instead of decreasing) LADH inactivation by 25 μM NaOCl, from 53% (control sample) to 64% (DETAPAC sample) whereas 100 μM or lower concentrations of DETAPAC did not modify the effect of NaOCl, under experimental conditions as in Figure 4 (data not shown).

Titration of LADH thiols after enzyme treatment with NaOCl or the MPO/H_2O_2/NaCl system yielded the results summarized in Table I. These results indicate that after LADH treatment with 0.2 or 0.4 mM NaOCl, 2 or 3 SH groups per subunit, respectively, were oxidized. With the MPO/H_2O_2/NaCl system, only one SH group per subunit was oxidized. As regards the relatively limited number of thiols titrated, it seems worth recalling that thiols in native LADH are remarkably unreactive.[1] L-methionine prevented SH oxidation to a significant degree, thus confirming the pro-oxidant activity of NaOCl. Thiol oxidation by NaOCl or the MPO/H_2O_2, NaCl system correlated with the loss of LADH activity (Table I). Amino acid analysis of NaOCl-inactivated LADH showed a decrease in Met and Tyr but no significant modification of Ala, Arg, Asp, Glu, Gly, His, Ile, Leu, Lys, Phe, Ser, Thr and Val, content since the ratio of modified LADH versus native LADH amino acid residues was invariably 1.0 ± 0.1. On the other hand, calculation of the number of Met and Tyr residues in NaOCl-treated LADH, yielded a decrease of 6 Met and 2 Tyr residues, per mole of LADH subunit, as compared with the amino acid composition of native LADH. Met and Tyr modification correlated with a 87% decrease in

TABLE I Effect of NaOCl and the MPO/H_2O_2/NaCl system on LADH thiol groups

Experiment	Additions (mM)	Thiol groups (SH/LADH subunit)	LADH inactivation (%)
A	None	5.8 ± 0.15 (4)	0
	NaOCl (0.2)	3.8 ± 0.14 (5)*	76
	NaOCl (0.2) + MET	5.4 ± 0.21 (4)**	0
	NaOCl (0.4)	2.4 ± 0.36 (3)*	82
	NaOCl (0.4) + MET	5.1 ± 0.09 (3)***	0
B	None	6.2 ± 0.20 (4)	0
	MPO/NaCl	6.0 ± 0.05 (5)****	0
	MPO/H_2O_2/NaCl	4.9 ± 0.24 (5)*	70
	MPO/H_2O_2/NaCl + MET	5.8 ± 0.34 (3)****	5

Reagent concentrations were 12.5 μM LADH, 50 mM K-phosphate, pH 7.4, 3.75 U/ml MPO, 0.2 mM H_2O_2 100 mM NaCl, 5.0 mM L-methionine (MET), and NaOCl as stated above; total volume, 0.4 ml. After 30 (A) or 60 (B) min incubation at 30°C, LADH activity was measured. The enzyme was then denaturated with guanidine and thiol groups were titrated. Other experimental conditions were as described under Materials and Methods. Values represent means ± SD; in parentheses, number of determinations.
* $p < 0.0001$; ** $p < 0.01$; *** $p < 0.0004$; **** $p > 0.05$.

TABLE II Inactivation of LADH by the MPO/H_2O_2/halide systems

Halide	LADH inactivation (%)	
	Incubation: 5 min	Incubation: 30 min
KI (2.5 μM)	92	96
KI (10 μM)	94	98
NaBr (20 μM)	43	97
NaBr (50 μM)	74	97
KSCN (25 μM)	50	73
KSCN (100 μM)	66	91
NaCl (50 mM)	55	94
NaCl (100 mM)	56	95

Reagent concentrations were 1.0 μM LADH, 0.5 U/ml MPO, 0.1 mM H_2O_2 50 mM K-phosphate, pH 6.0. Halides were added as indicated above. Other experimental conditions were as indicated under Materials and Methods. Values represent means of duplicate measurements.

TABLE III Effect of MPO/H_2O_2/halide systems on LADH diaphorase and lipoamide reductase activities

Pro-oxidant	LADH activity	
	Diaphorase (μmol DCI/min)/(mg LADH))	Lipoamide reductase (%)
None	3.45 ± 0.24 (100)	100
MPO/H_2O_2/NaCl	6.62 ± 0.29 (192)*	42
MPO/H_2O_2/NaBr	10.68 ± 0.22 (310)	3
MPO/H_2O_2/KI	10.38 ± 0.77 (301)*	2
MPO/H_2O_2/KSCN	4.64 ± 0.13 (135)*	25

Reagent concentrations were 1.0 μM LADH, 0.5 U/ml MPO, 0.1 mM H_2O_2 100 mM NaCl, 0.1 mM KI, 0.5 mM NaBr and 0.5 mM KSCN. Time of incubation, 30 min. Other experimental conditions were as described in Figures 1and 5 legends and under Materials and Methods. Diaphorase values represent means ± SD ($n \geq 3$). In parentheses is relative activity.
* $p < 0.001$, as compared with the control sample. Lipoamide reductase values represent means of duplicate measurements.

LADH lipoamide reductase activity in the oxidized sample.

In order to correlate LADH inactivation with hypohalous acid production by the MPO/H_2O_2/halide systems, iodide, bromide and thiocyanate-supplemented MPO/H_2O_2 systems were assayed. The results obtained indicate that these latter systems inactivated LADH at much lower concentration than the MPO/ H_2O_2/NaCl system (Table II). Halides were more effective at pH 6.0 (Table II) than at pH 7.4 (experimental data omitted) in close agreement with the results with NaCl (Figure 2). Under the same experimental conditions, the halide supplemented systems, especially MPO/H_2O_2/KI and MPO/H_2O_2/NaBr increased LADH diaphorase activity, despite their effect on LADH lipoamide reductase activity (Table III).

LADH inactivation by NaOCl was prevented by thiol compounds, such as GSH, L-cysteine, NAC, PAM, MPG and CPT. Similar protection was obtained against the MPO/H_2O_2/NaCl system (Table IV) and also against the more effective MPO/H_2O_2/KI system (experimental data omitted). LADH protection depended on the thiol compound structure and the incubation time, as shown in Figure 5 with PAM and MPG. These

TABLE IV Protection of LADH by thiol compounds against inactivation by NaOCl and the MPO/H_2O_2/NaCl system

Thiol compound (μM)	LADH protection (%)	
	Oxidant: NaOCl	Oxidant: MPO/H_2O_2/NaCl
GSH (50)	69	42
GSH (100)	95	92
L-Cysteine (100)	89	84
NAC (50)	27	36
NAC(100)	75	90
PAM (100)	98	100
MPG (100)	99	100
CPT (50)	—	70
CPT (100)	100	97

Reagent concentrations were 1.0 μM LADH, 100 μM NaOCl, 0.5 U/ml MPO, 0.1 mM H_2O_2, 100 mM NaCl (MPO/H_2O_2/NaCl system) and 50 mM K-phosphate, pH 7.4. Thiol compounds were added as indicated above. Time of incubation, 30 min (consistent values obtained at shorter incubation times, omitted). Other conditions were as described under Materials and Methods. LADH inactivation values in the absence of thiol were 76% and 58% with NaOCl and the MPO/H_2O_2/NaCl system, respectively. Values represent means of duplicate measurements.

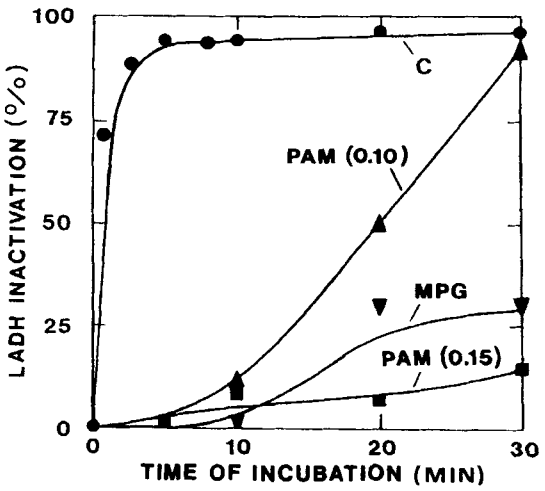

FIGURE 5 Effect of MPG and PAM on LADH inactivation by the MPO/H_2O_2/KI system. Reagent concentrations were: 1.0 μM LADH, 0.5 U/ml MPO and 0.1 mM H_2O_2, 0.1 mM KI and 50 mM K-phosphate, pH 7.4; MPG (0.15 mM) and PAM (0.10 or 0.15 mM) were added as indicated in the figure. Other conditions were as described under Materials and Methods. C, control sample (thiol compound omitted).

thiol compounds were highly effective after 5 or 10 min incubation but less (MPG) or not at all effective (0.10 mM PAM) after 60 minutes incubation. GSSG was less effective than GSH since 200 μM GSSG provided 50% protection after 30 min incubation against 92% protection provided by 100 μM GSH (Table IV). Taurine was also a protector of LADH against the MPO/H_2O_2/NaCl system or NaOCl but a relatively high concentration (1.0 mM) was required to obtain 38% and 48% protection against the standard MPO/H_2O_2/NaCl system or NaOCl, under experimental conditions as described in Table IV (30 min incubated samples).

TABLE V LADH inactivation by MPO/NADH/halide systems

NAD(P)H	Halide (mM)	LADH inactivation (%)	
		+MPO	−MPO
NADH	KI (0.1)	95	12
	KI (0.1) + Catalase	4	—
	KI (0.1) + Catalase (D)	96	12
	NaBr (1.0)	79	0
	KSCN (1.0)	40	0
	NaCl (150)	30	0
NADPH	KI (0.1)	6.0	40
	NaCl (100)	8.0	20

Reagent concentrations were 1.0 μM LADH, 0.5 U/ml MPO, 0.1 mM NAD(P)H, 50 mM K-phosphate, pH 7.4; halides were added as indicated above; catalase, 150 U/ml (5 μg protein/ml); denatured catalase (D), 5 μg protein/ml). Time of incubation, 30 min (consistent values obtained at shorter incubation times, omitted). Other experimental conditions were as described under Materials and Methods. Values represent means of duplicate measurements.

LADH Inactivation by MPO/NADH/Halide Systems

Table V shows that LADH was inactivated by MPO systems in which H_2O_2 was replaced by NADH (not by NADPH). LADH inactivation was prevented by catalase, as illustrated by the MPO/NADH/KI system. Other peroxidases, namely, HRP and LPO, also catalyzed LADH inactivation by the NADH halide systems but were less effective than MPO, despite the relatively lower concentration at which MPO was used (Table VI). Differences between peroxidase effects were remarkable after short-term incubation (Table VI). Figure 6 shows the time-course of LADH inactivation by the MPO/NADH/halide

TABLE VI LADH inactivation by peroxidase/NADH/KI systems

Peroxidase (U/ml)	LADH inactivation (%)	
	Incubation: 5 min	Incubation: 30 min
MPO (0.5)	85 ± 1.7	96 ± 1.7
LPO (1.0)	39 ± 0.7*	55 ± 2.4*
HRP (2.5)	10 ± 0.1*	95 ± 1.0

Reagent concentrations were 1.0 μM LADH, 0.1 mM NADH and 50 mM K-phosphate, pH 7.4. Values represent means ± SD ($n = 3$). * $p < 0.0001$.

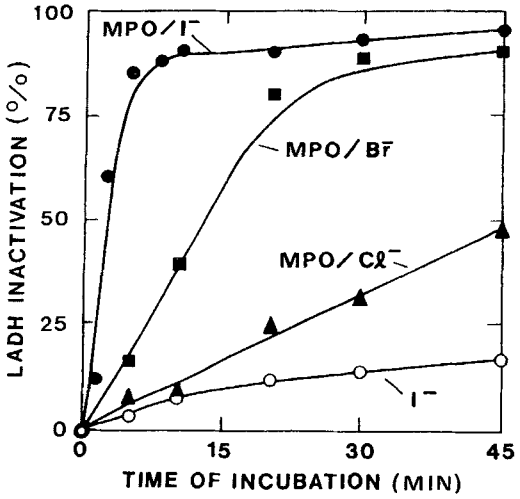

FIGURE 6 Time-course of LADH inactivation by MPO/NADH/halide systems. Reagent concentrations were: 1.0 μM LADH, 0.5 U/ml MPO, 0.1 mM NADH and 50 mM K-phosphate, pH 7.4; 0.1 mM KI (I^-), 1.0 mM NaBr (Br^-) or 150 mM NaCl (Cl^-) were added as indicated in the figure. Other conditions were as described under Materials and Methods.

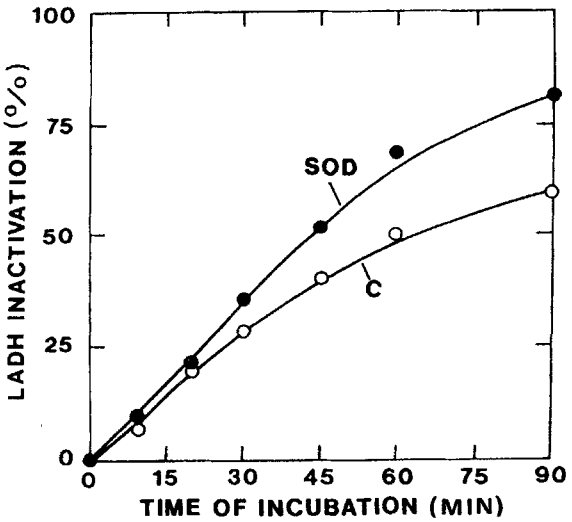

FIGURE 7 Effect of SOD on LADH inactivation by the MPO/NADH/NaCl system. Reagent concentrations were: 1.0 μM LADH, 0.1 mM NADH, 100 mM NaCl and 50 mM K-phosphate, pH 7.4; 100 U/ml SOD was added as indicated in the figure. C, control sample (SOD omitted).

TABLE VII H_2O_2 production by the MPO/NADH/KI system

MPO	H_2O_2 production	
	Experiment A (μmol/min)/(mg LADH)	Experiment B (μmol/min)/(mg LADH)
Omitted	0.079 ± 0.006	0.63 ± 0.12
Added	0.085 ± 0.012	0.42 ± 0.10*

Experiment A: The reaction medium contained 0.2 μM LADH, 0.25 U/ml MPO, 0.1 mM NADH, 3.3 μM microperoxidase and 50 mM K-phosphate, pH 7.4; total volume, 1.0 ml. The reaction was started by adding NADH and the rate of the H_2O_2–microperoxidase complex productions was measured. Experiment B: The reaction medium contained 1.0 μM LADH, 0.5 U/ml MPO, 0.1 mM NADH, 0.1 mM KI and 50 mM K-phosphate, pH 7.4; total volume, 0.2 ml, After 30 min incubation at 30°C, 50 μl of LADH inactivation medium were added to 3.3 μM microperoxidase in 50 mM K-phosphate, pH 7.4, total volume, 1.0 ml. H_2O_2 accumulated in the LADH inactivation medium was measured. Other conditions were as described under Materials and Methods. Values represent the means ± SD ($n = 3$). * $p < 0.08$.

systems. Iodide, bromide and chloride effectiveness decreased in the given order notwithstanding their different concentrations. Addition of MPO was essential for obtaining the halide effect.

Under adequate experimental conditions LADH is an effective generator of superoxide.[19] Superoxide dismutation yields H_2O_2, thus explaining the effect of SOD in Figure 7. In order to confirm H_2O_2 production by the LADH/NADH system, two different experiments (A and B) were performed (Table VII). In experiment A, the reaction medium in the spectrophotometer cell, contained the MPO/NADH/KI system and microperoxidase, in order to detect H_2O_2 production. Under these conditions, the initial rate of H_2O_2 production (2–3 min incubation) was measured. In experiment B,

LADH was incubated for 30 min with the MPO/NADH/KI system (0.2 ml inactivation sample) for 30 min, as described in Table VII. Fifty-µl samples were then added to the microperoxidase-containing medium in the spectrophotometer cell, and H_2O_2 accumulation was measured. The results obtained in both experiments demonstrated H_2O_2 production. Interestingly enough, calculation of possible H_2O_2 concentrations for the inactivation media yielded values compatible with an effective activity of the MPO-dependent systems, namely 47 ± 3.1 and 51 ± 7.2 µM H_2O_2 (experiment A) and 63 ± 12 and 42 ± 10 µM H_2O_2 (experiment B). MPO produced a small decrease in H_2O_2 accumulation (experiment B) as expected from the operation of MPO-catalyzed reactions, but the difference was at the limit of significance.

LADH Inactivation by the MPO/H_2O_2/Nitrite System

LADH was inactivated by the MPO/H_2O_2/$NaNO_2$ system, the latter effect depending on both H_2O_2 and $NaNO_2$ concentrations (Figures 8 and 9) respectively. $NaNO_2$ effect correlated with the production of a saturable MPO-nitrite complex,[20] as suggested by the limited effect resulting from the increase in nitrite concentration from 0.25 to 0.50 mM (Figure 9). Inactivation of LADH lipoamide reductase activity (65%) was accompained by the increase of diaphorase activity (190%). The MPO/H_2O_2/ $NaNO_2$ system activity was greater at pH 6.0 than at pH 7.4, as shown by comparing Figures 8 and 9. At variance with these results, 100 µM Na-peroxynitrite, at pH 7.4 failed to modify LADH activity (experimental data omitted). MPO inhibitors (sodium azide,[7,21] isoniazid[22] and salicylhydroxamic acid[23]), prevented LADH inactivation by the MPO/ H_2O_2/$NaNO_2$ system (Table VIII), thus confirming the essential role of MPO in the nitrite-dependent LADH modification. Thiol compounds (GSH and cysteine) and several amino acids (L-methionine, L-tryptophan, L-tyrosine and L-histidine) prevented the effect of the MPO/H_2O_2/$NaNO_2$ system (Table IX).

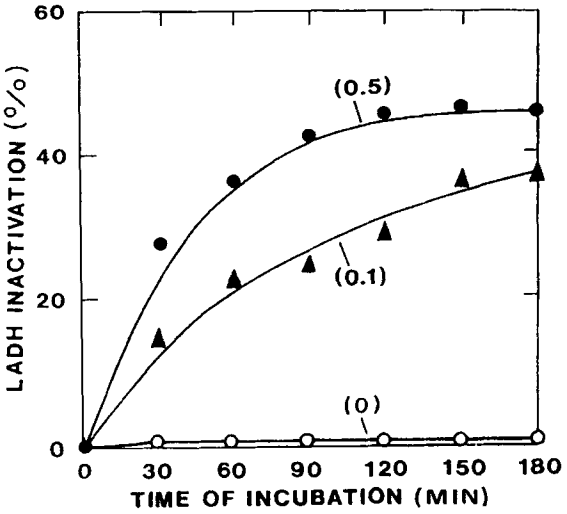

FIGURE 8 Time-course of LADH inactivation by the MPO/H_2O_2/$NaNO_2$ system. Reagent concentrations were: 0.5 µM LADH, 0.5 U/ml MPO, 50 mM K-phosphate, pH 7.4 and 0.5 mM $NaNO_2$; H_2O_2 (mM) was added as indicated by the figures in parentheses. Other conditions were as described under Materials and Methods.

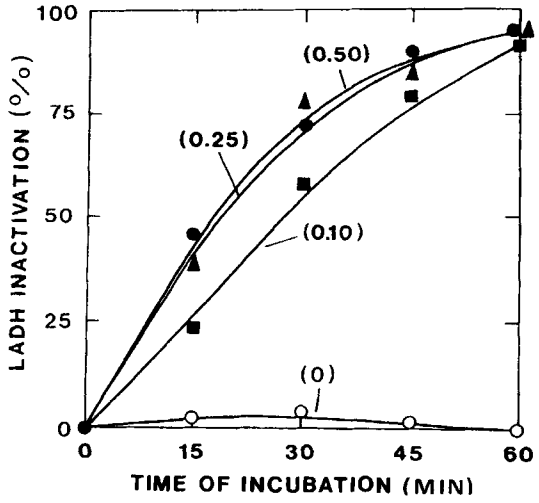

FIGURE 9 Time-course of LADH inactivation by the MPO/H_2O_2/$NaNO_2$ system. Reagent concentrations were: 0.5 µM LADH, 0.5 U/ml MPO, 0.25 mM H_2O_2 and 50 mM K-phosphate, pH 6.0; $NaNO_2$ (mM) was added as indicated by the figures in parentheses. Other experimental conditions were as described under Materials and Methods.

Under the same experimental conditions 0.25 mM PAM (100%), NAC (93%), MPG (100%) and CPT (93%) protected LADH, as indicated by the numbers in parenthesis.

TABLE VIII Effect of MPO inhibitors on LADH inactivation by the MPO/H_2O_2/$NaNO_2$ system

MPO inhibitor (mM)	LADH inactivation (%)	
	Incubation: 30 min	Incubation: 60 min
None	57.1 ± 5.2	89.4 ± 2.6
NaN_3 (0.1)	7.8 ± 1.3*	13.4 ± 0.8*
NaN_3 (0.2)	3.1 ± 0*	2.1 ± 1.5*
Isoniazid (0.1)	19.7 ± 2.5**	29.6 ± 2.0*
Isoniazid (0.2)	7.5 ± 2.5*	10.3 ± 3.9*
Salicylhydroxamic acid (0.2)	32.3 ± 2.6***	50.3 ± 1.9*
Salicylhydroxamic acid (0.4)	7.2 ± 2.4*	14.5 ± 4.0*

Reagent concentrations were 1.0 μM LADH, 0.5 U/ml MPO, 0.25 mM H_2O_2, 0.25 mM $NaNO_2$, 50 mM K-phosphate, pH 6.0 and MPO inhibitors as described above. Other experimental conditions were as described under Materials and Methods. The H_2O_2/$NaNO_2$ and MPO/H_2O_2 systems modified LADH activity in a non-significant degree (< 5.0%). Values represent means ± SD ($n = 3$). * $p < 0.0001$; ** $p < 0.0004$; *** $p < 0.0002$.

TABLE IX Effect of amino acids and GSH on LADH inactivation by the MPO/H_2O_2/$NaNO_2$ system

Amino acid (0.5 mM)	LADH protection (%)
L-Cysteine	100
L-Methionine	100
L-Tryptophan[a,b]	71
L-Tyrosine	41
L-Histidine	22
GSH[b]	91

Reagent concentrations were 1.0 μM LADH, 0.75 U/ml MPO, 0.25 mM H_2O_2, 0.25 mM $NaNO_2$ 50 mM K-phosphate, pH 6.0; amino acid were added as stated above. Time of incubation, 15 min. LADH inactivation by the MPO/H_2O_2/$NaNO_2$ system, 71%. Other conditions were as described under Materials and Methods. Values represent means of duplicate measurements. [a] 0.4 mM; [b] MPO 0.5 U/ml.

DISCUSSION

The findings described here indicate that MPO-dependent systems inactivate LADH, as a result of the modification of LADH specific amino acid residues by MPO-generated pro-oxidant species. Among those species stands HOCl which was produced by the MPO/H_2O_2/NaCl system, at NaCl concentration compatible with those present in biological media (100–140 mM).[24] MPO catalyzed LADH inactivation, using either exogenous H_2O_2 (most of the experiments) or H_2O_2 generated by the LADH-NADH system (Tables V–VII). HOCl would be the main agent of the MPO/H_2O_2/NaCl (or MPO/NADH/NaCl) systems since (a) in the absence of NaCl, the effect of the MPO system was negligible (Figure 1); (b) NaOCl significantly inactivated LADH (Figure 4 and Table I); (c) L-methionine, an HOCl scavenger,[7] prevented LADH inactivation by both the MPO/H_2O_2/NaCl system and NaOCl (Table I); (d) both NaOCl and the MPO/H_2O_2/NaCl system oxidized LADH thiol group, NaOCl being the most effective (Table I) and (e) thiol compounds prevented LADH inactivation by either the MPO/H_2O_2/NaCl system or NaOCl (Table IV). The effect of pH on the activity of the MPO/H_2O_2/halide and the MPO/H_2O_2/$NaNO_2$ systems (Figures 2, 8 and 9) may imply different mechanisms such as MPO greater activity at pH 6.0;[5] the greater activity of protonated hypohalous acids, or both.

LADH modification by NaOCl and the MPO/H_2O_2/halide systems was expressed by two opposite effects, namely, (a) inactivation of lipoamide reductase activity and (b) enhancement of electron transfer from NADH to the artificial electron acceptor DCI, termed diaphorase activity (Figure 3 and Table III). It is known[25] that LADH lipoamide reductase activity depends on one-electron transfer through the distal nascent thiol (Cys-45) to the lipoamide disulfide, whereas diaphorase activity depends on electron transfer from the flavin to the artificial electron acceptor.[25] The effect of the MPO/H_2O_2/NaCl system and NaOCl on LADH activity fits in well with previously reported effects of the Cu(II)-Fenton system[3] and Cu(II).[1] Such effects were explained as a consequence of LADH nascent thiol oxidation, since Cu(II)-oxidized LADH had an extra disulfide linkage, in addition to the active site disulfide.[1] Titration of LADH thiols after treatment with NaOCl or the MPO/H_2O_2/NaCl system (Table I) supports thiol oxidation as suggested by the increase in diaphorase activity. Cys-45 and/or Cys-50 (the active site thiols)[25] should be suitable targets for oxidants. The oxidation of LADH thiols by NaOCl fits in well with

NaOCl action on GSH and protein thiols.[26–28] NaOCl also oxidized LADH Tyr and Met residues, as shown by amino acid analysis of NaOCl-treated LADH. Apparently, HOCl was selective on LADH amino acid residues, as compared with its effect on other proteins.[29] Tyrosine chlorination[30,31] or oxidation[32] by MPO-dependent systems has been consistently reported.[30,31] Enhancement of LADH-diaphorase activity after NaOCl oxidation was, however, at variance with Tyr-118 chlorination (by HOCl) or nitration (by nitrite-dependent systems) since this residue binds NADH adenine to LADH active site.[25] Accordingly, LADH, Tyr-19, Tyr-350 or other Tyr residues might be more suitable targets for modification by oxidative attack.

It is known that MPO oxidizes halides (Cl^-, Br^-, I^-) and the pseudohalide SCN^-, producing the corresponding hypohalous acids.[24] LADH modification by the MPO/H_2O_2/halide systems, correlated at least qualitatively with the rate of halides oxidation by peroxidases (Table II). Peroxidases catalyse one-electron oxidation of iodide and two-electron oxidation of other halides.[33] MPO Compound I reacts with iodide to form the generating intermediate which catalyes iodide transfer to GSH and tyrosine.[33] The results in Table II and Figure 6 are in this regard illustrative since the MPO/H_2O_2/KI system was the most effective inactivator of LADH, at halide concentrations far lower than those of the other halides.

LADH was inactivated by the MPO/NADH/KI system in which NADH-generated H_2O_2 replaced exogenous H_2O_2 (Tables V–VII) Catalase completely prevented LADH inactivation by the latter system whereas HRP and LPO were able to replace MPO (Table VI), thus confirming the role of H_2O_2 in LADH inactivation by the MPO-NADH/halide systems. The NADH/LADH system produces superoxide radicals[19] which may modulate MPO activity[5] and dismutate producing H_2O_2. The resulting reactive oxygen species would contribute to LADH inactivation, as suggested by the effect of catalase and SOD (Table V and Figure 7).

Nitrite (Na) was also an effective supplement of MPO/H_2O_2 systems (Figures 8 and 9). Oxidation of nitrite by hemoprotein peroxidase enzymes and H_2O_2 is a well known reaction.[20,35,36] Peroxidase inhibitors prevented LADH inactivation (Table VIII), thus confirming the essential role played by both MPO and H_2O_2 in MPO/H_2O_2/$NaNO_2$ system. Peroxidase Compound I reacts with nitrite in a one-electron transfer process, forming Compound II. This reaction requires that its product should be a free radical, like $NO_2^{\bullet}$ (see Refs. [20,34,35] Protection of LADH by several free amino acids (Table IX) suggests (a) similar effects of these aminoacids in stressed tissues and (b) the modification of the corresponding residues in LADH, as result of attack by the nitrite system. The MPO/H_2O_2/$NaNO_2$ toxicity on LADH fits in well with the antimicrobial activity of nitrite[36,37] and the LPO catalyzed oxidation of mitoxanthrone by NO_2 (see Ref. [35]).

Thiol compounds protected LADH against NaOCl and MPO/H_2O_2/halide systems (Table IV and Figure 4). The thiol compounds assayed included GSH, CPT, NAC, MPG and PAM, some of them with specific medical uses. Enzyme protection by these thiols, as illustrated by LADH, might contribute to their anti-inflammatory action. The action of thiol compounds against NaOCl (Table IV) may be explained by Reactions (3)–(5) in which one molecule of thiol (e.g. GSH) inactivates three molecules of HOCl.[26–28]

$$GSH + HOCl \longrightarrow GSCl + H_2O \quad (3)$$

$$GSCl + 2HOCl \longrightarrow GSO_2Cl + 2Cl^- + 2H^+ \quad (4)$$

$$GSCl + GSH \longrightarrow GSSG + Cl^- + H^+ \quad (5)$$

Moreover, the sulfonyl chloride derivative may produce a sulfonamide and other non-toxic products. A fourth molecule of HOCl can be neutralized by reacting with GSH amino group, to form the chloramine derivative,[38] a reaction that would explain the antioxidant effects of taurine, as described under Results. At variance with the

above-described protection of LADH by thiol compounds, thiol oxidation by MPO and HRP can produce "reactive oxygen species"[39,40] which may contribute to LADH inactivation. This complex set of reactions would explain the peculiar effect of 0.1 mM PAM in Figure 5, which protected LADH solely during the first 10 min of incubation whereas with 0.15 mM PAM a more sustained protection was obtained. It remains to be established whether thiol oxidation by MPO occurs with all the thiol compounds assayed.

The relevance of LADH inactivation as a cause of oxidative damage in isolated cells and tissues is worth a consideration. In most eukaryotic cells. LADH is a mitochondrial enzyme whose accessibility for extracellular cytotoxic agents is limited by plasma and mitochondrial membranes. Nevertheless, stimulated neutrophils discharge HOCl and other diffusible reactive species (the "oxidative burst"). HOCl reacts with intracellular primary and secondary amines producing chloramines.[38] These latter molecules retain the oxidizing capability of HOCl and promote cytotoxic effects distant from their source. Moreover, MPO and its reaction products increase membrane permeability[41] thus faciliting the access of damaging agents to the intracellular macromolecules. In this connection, it should be noted that, in bacteria, inhibition of DNA macromolecule synthesis appears to be an indicator of oxidative damage by MPO systems.[41] Whether similar effects may affect LADH remains to be established.

Acknowledgements

We are grateful to Dr. H. Rubbo, Faculty of Medicine, Montevideo, Uruguay for the supply of Na-peroxinitrite. This work was aided by grants from the University of Buenos Aires and the Roemmers Foundation. J.G.C. is a Research Fellow of CEDIQUIFA (Argentina). M.G. Gutiérrez and M.A.E. Veron lent able technical assistance.

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