Biochemical Pharmacology, Vol. 54, pp. 669–675, 1997.

ISSN 0006-2952/97/$17.00 + 0.00
PII S0006-2952(97)00218-9

Inactivation of Intracellular Proteolysis and Cathepsin B Enzyme Activity by Dehydroascorbic Acid and Reactivation by Dithiothreitol in Perfused Rat Heart

*Thomas D. Lockwood**

DEPARTMENT OF PHARMACOLOGY AND TOXICOLOGY, SCHOOL OF MEDICINE, WRIGHT STATE UNIVERSITY, DAYTON, OH 45435 U.S.A.

ABSTRACT. The selective inhibition of some subcomponents of intracellular protein degradation was characterized under exposure to the cyclic multiketone thiol oxidizing agents dehydroascorbic acid (DHA) and alloxan. Proteins of the isolated perfused rat heart were labeled *in vitro* with a 10-min infusion of [^{3}H] leucine, and subsequent release of radiolabeled amino acid from cell proteins was measured. As determined previously, four subcomponents of total proteolysis can be distinguished; the first three subcomponents are reversibly inhibited by the thiol-reactive agent diamide: (a) The rapid turnover proteins comprise most of the [^{3}H] leucine release from 20 min to 3 hr after labeling. (b) Following 3 hr of degradation, the lysosomal (insulin-responsive) subcomponent comprises 35–40% of [^{3}H] leucine release. (c) A third nonlysosomal (adrenergic-responsive) subcomponent comprises 35%. (d) A fourth nonlysosomal subcomponent consisting of 25% of [^{3}H] leucine release is uninhibited by diamide. Infusion of supraphysiologic DHA (1 mM) or alloxan (1.5 mM) promptly mimicked the proteolytic inhibitory action of diamide on the first three subcomponents, but did not inhibit the diamide-resistant subcomponent. Infusion of a physiologic extracellular DHA concentration of 5 μM caused little or no change in proteolysis. The proteolytic inhibitory action of DHA (1 mM) could be reversed by concurrent infusion of dithiothreitol (DTT, 1.5 mM) simultaneously with continued DHA. DHA (1 mM) caused direct inhibition of the purified sulfhydryl proteinase cathepsin B (EC 3.4.22.1), which was reversible by subsequent excess DTT (5 mM). Results indicate that a nontoxic endogenous multiketone thiol oxidant can reversibly inhibit some proteolytic processes in viable tissue; however, approximately 25% of the observed proteolysis is uninhibited. Reversible inactivation of sulfhydryl proteinases, including cathepsin B, is among the possible multiple mechanisms of this DHA action. BIOCHEM PHARMACOL **54**;6:669–675, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. protein degradation; cathepsin B; dehydroascorbic acid; alloxan; dithiothreitol

In the isolated perfused rat heart, three distinct proteolytic processes are reversibly inhibited by the nontoxic thiol probe diamide; however, a fourth process is simultaneously independent of diamide action [1]. *N*-Ethylmaleimide or the direct sulfhydryl proteinase inhibitor L-*trans*-epoxysuccinyl-leucyl (4-guanidino) butane (E-64) inhibited all or most of these diamide-sensitive proteolytic pathways, respectively. It was speculated that maximal function of some cell proteolytic pathways might somehow require reduced thiols, whereas other cell proteolysis proceeds independent of such a reducing requirement.

The activity of sulfhydryl enzymes can be limited by the balance between factors promoting sulfhydryl inactivation versus reactivation [2]. Reduced sulfhydryl proteinases such as cathepsin B tend to lose activity spontaneously in the presence of oxygen, various ions, and other agents. Maximal *in vitro* assay requires chelator, such as EDTA, and thiol reductant, such as DTT† [3, 4]. Several cell reductase systems can reduce oxidized protein thiols [5–8]; however, the reversible oxidative inactivation and reductive reactivation of protein degradation or sulfhydryl proteinases have not been demonstrated under intracellular conditions.

Various experimental agents can inactivate sulfhydryl enzymes by several mechanisms [9]. Agents such as *N*-ethylmaleimide irreversibly form covalent adducts with sulfhydryl groups. Diamide catalyzes reversible formation of hetero- or homodisulfides, including reactions between protein sulfhydryls and low weight sulfhydryls such as glutathione, i.e. thiolation reactions [10, 11]. A variety of other oxidizing agents can catalyze sulfhydryl oxygenation to SO, SO_2, or SO_3 [8]. Some agents might form reversible associations with sulfhydryl groups, as suggested with ring multiketones such as alloxan [12, 13]. However, the *in vivo* relevance of any of these experimental reactions to the

* Correspondence. Tel. 937-775-2168; FAX 937-775-7221.
Received 13 December 1996; accepted 1 April 1997.

† *Abbreviations:* AA, ascorbic acid; DHA, dehydroascorbic acid; and DTT, dithiothreitol.

intracellular inactivation–reactivation of sulfhydryl proteinases is unknown.

A means was sought to determine whether redox-dependent protein degradation can be reversibly inactivated by intervention with endogenous metabolites under noninjurious conditions. Several recent reports suggested that DHA might be a particularly interesting endogenous metabolite with which to investigate intracellular sulfhydryl inactivation–reactivation (see Discussion). Under normal intracellular conditions, endogenous AA spontaneously oxidizes to DHA, which is then reduced back to AA by several reductases or possibly by direct reaction with glutathione [10, 14–19]. Thus, AA/DHA are an endogenous cycling redox couple, maintained largely in the reduced form. Extracellular DHA is readily taken up into cells [20]. After extracellular DHA is taken up, it can either directly react with intracellular thiols or other reductants, or be reduced enzymatically to AA by several enzymes [14–19]. Therefore, elevation of extracellular DHA above the normal concentration of approximately 5 μM should exceed the ability of intracellular reductants and/or reductases to reduce it at some supraphysiologic concentration.

DHA exhibits structural features and some biochemical actions in common with the classic experimental protein thiol oxidant alloxan [reviewed in Refs. 12 and 13]. Both DHA and alloxan contain ring vicinal multiketone oxygens that are highly reactive with sulfhydryls, causing oxidation and perhaps direct adducts with some enzyme active site sulfhydryls [12, 13, 15, 18]. It has been reported that alloxan directly inactivates the reduced form of the thiol proteinase cathepsin B [21]. After inactivation of cathepsin B by alloxan, the proteinase activity can be restored by subsequent DTT [21]. The actions of DHA on thiol proteinases or intracellular protein degradation have not been described.

It is presently reported that the physiologic extracellular concentration of DHA (5 μM) caused no appreciable change in myocardial protein degradation. However, a slight elevation of extracellular DHA caused selective inhibition of the same pathways of proteolysis inhibited by diamide. In contrast, some protein degradation was simultaneously insensitive to both DHA and diamide. Correspondingly, purified reduced cathepsin B was inactivated by DHA and reactivated by DTT.

MATERIALS AND METHODS

Heart Perfusion

Hearts were perfused as Langendorff preparations under a constant flow of 7.0 mL/min and low perfusion pressure of 40 mm Hg by methods previously described [1, 22–25]. Nonrecirculating perfusate contained Krebs–Henseleit salts, glucose (11 mM), citrate (0.1 mM), pyruvate (0.1 mM), lactate (0.3 mM), complete physiologic amino acid concentrations, bovine serum albumin (0.2%), and 95% O_2–5% CO_2, adjusted to a final pH of 7.42. Concentrated solutions of experimental agents (100- to 1000-fold) were infused above the heart immediately following dissolution, resulting in exposure within 30 sec at this flow rate. Materials were from the Sigma Chemical Co. (St. Louis, MO) as previously described, except for DHA which was obtained from ICN Biochemicals (Irvine, CA). Alloxan and DHA were dissolved in water, and the infusate was maintained at 4° to minimize spontaneous hydrolysis [26].

Measurement of Protein Degradation

Proteins were biosynthetically labeled by infusion of L-4,5-[^{3}H]leucine (40–60 Ci/mmol, 4.5 μCi/mL) for 10 min. Then nonradioactive leucine (1.5 mM) was added to prevent reincorporation of label. The nonrecirculated effluent was collected at 2-min intervals in a fraction collector, and trichloroacetic acid (TCA) soluble radioactivity was determined. Quantitative characteristics of basal [^{3}H] leucine release, computer extrapolation of the baseline, and measurement of percent changes have been described previously in detail [1]. A two-component equation describing the progress of macromolecular [^{3}H] leucine remaining in myocardial protein over 5 hr has been described: $Y = 0.30\, e^{-1.04t} + 0.70\, e^{-0.031t}$. The present data illustrate the rate of [^{3}H] leucine release per minute, or the differential of this equation. A comparison of the rate of [^{3}H] leucine release with the simultaneous progress of remaining macromolecular [^{3}H] leucine has been illustrated previously [1]. Under constant intracellular degradative conditions, the rate of total [^{3}H] leucine release declines continually in proportion to the declining amount of undegraded proteins remaining. The declining curvilinear control baseline of [^{3}H] leucine release represents the total degradation of diverse proteins with heterogeneous half-lives. The percent inhibition of protein degradation is proportional to the percent downward displacement of the baseline as statistically extrapolated over the transition time to a new steady-state (described and discussed in Ref. 1). Control values of protein degradation are presently illustrated as either the declining baseline of [^{3}H] leucine release or, alternatively, as the normalized 100% value of the declining baseline at each time point. The percent downward displacement of the curvilinear baseline is identical using either method of presentation; however, representation of data as percent inhibition enhances visualization of the time–course of transition to steady-state inhibition.

Immediately after a 10-min incorporation period, 80–90% of subsequent [^{3}H] leucine release was derived from a well-known, rapid-turnover subcomponent that was degraded by 3 hr [see Fig. 1 (A and B) and Ref. 1]. After a 3-hr degradation period, three additional subcomponents of total [^{3}H] leucine release can be distinguished experimentally (data not shown; see Ref. 1). Following 3 hr of preliminary degradation, chloroquine and/or insulin nonadditively inhibit 35–40% of the remaining [^{3}H] leucine release, consisting of the lysosomal subcomponent [25]. Adrenergic agonists inhibit 35% of proteolysis additively with chloroquine or insulin consisting of a nonlysosomal

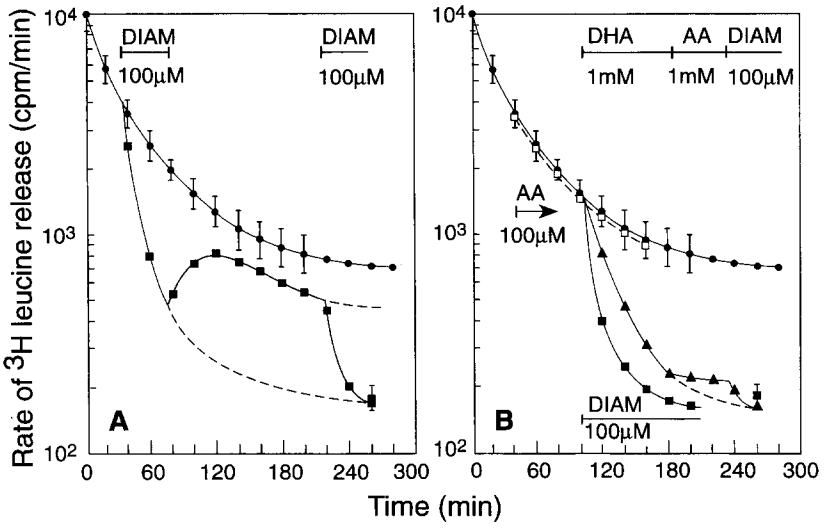

FIG. 1. (A) Effect of interrupted diamide infusion on myocardial protein degradation. Zero time corresponds to 20 min after termination of a 10-min labeling period. The mean of control baselines of [^{3}H] leucine release from four hearts (●) ± SD is shown. Beginning at 220 min, the control baseline was extrapolated as confirmed from separate experiments [see Ref. 1]. The mean initial radioactivity released (1.3×10^4 cpm/min) has been normalized to 10^4 cpm/min. Radioactivity in all preparations was measured at 2-min intervals; the means of ten determinations is shown at 20-min intervals. The effects of continuous diamide exposure (■, ± SD) or longer diamide discontinuation (dashed traces) represent more than four separate experiments each. (B) Effect of AA and DHA on proteolysis. Release of [^{3}H] leucine under basal conditions (●) was insignificantly different when AA (100 μM) was infused (□, ± SD, dashed trace 40–140 min, $N = 3$, $P > 0.1$). The final inhibition caused by DHA (▲) was insignificantly different from the 75 ± 4% inhibition caused by continuous diamide (■, ± SD, $N = 4$, $P > 0.1$).

subcomponent; thus, 70–75% of proteolysis is controlled by these two additive hormone systems after elimination of rapid turnover proteolysis [23–25]. Twenty-five percent of proteolysis remains uninhibited by combined insulin and adrenergic agonists or thiol-reactive agents, thereby defining a fourth nonlysosomal hormone-unresponsive process [1]. The first three proteolytic processes are inhibited reversibly by diamide; however, the fourth, hormone-unresponsive process was not inhibited by diamide for at least several hours (Fig. 1A, lower dashed trace). Diamide action was 80–90% reversible at any time from 0.5 to 5 hr post-labeling (Fig. 1A). Approximately 5–10% of total proteolysis was not accounted for under this experimental precision.

Assay of Inhibitory Actions on Purified Cathepsin B Activity

Cathepsin B activity (EC 3.4.22.1) was assayed in quadruplicates, using hemoglobin radiolabeled by acetylation with [^{3}H] acetic anhydride [3, 22]. ^{3}H-Acetylated hemoglobin was acid denatured with HCl (pH 1.8) and EDTA (50 mM), and then dialyzed extensively against NaCl (100 mM, pH 7). The cathepsin B assay contained 50 μL of [^{3}H] hemoglobin (4 mg/mL) and 50 μL of enzyme solution added to 100 μL of assay buffer (100 mM, sodium acetate and 100 mM EDTA, pH 4.5), and the additions described below. Incubation was for 1 hr at 37°, followed by the addition of 100 μL of 7% nonradioactive hemoglobin and 1 mL of 6% TCA. Following centrifugation, the hydrolysis of [^{3}H] hemoglobin to TCA-soluble fragments was determined by subtracting parallel substrate blanks incubated without added enzyme. This preliminary determination enabled the adjustment of the added enzyme activity to that which caused less than approximately 2% substrate hydrolysis. In this range of substrate hydrolysis, the reaction was linear with time over 1 hr and proportional to added enzyme activity [22]. To reduce purified cathepsin B (bovine Cathepsin B, Sigma), the enzyme was dissolved in 50 mM sodium acetate buffer, pH 6, combined with 5 mM DTT, and incubated for 1 hr at 20° [21]. The enzyme solution was then dialyzed extensively to remove DTT [22].

RESULTS

Proteolytic Inhibitory Action of DHA and Nonadditivity with Diamide Action

Infusion of excess DHA (1 mM) at any time from 20 min to 4 hr after labeling caused a marked 75–80% inhibition of proteolysis, similar to diamide (Figs. 1 and 2). The 75% proteolytic inhibitions caused by either DHA (1 mM) or diamide (100 μM) alone were nonadditive beyond 75% when both agents were simultaneously combined (Fig. 1B); such nonadditivity is consistent with inhibition of the same proteolysis by either agent. Conversely, the 25% of total proteolysis resistant to diamide was also uninhibited by simultaneously combined DHA (Fig. 2B). Maximal DHA action was slightly slower than diamide action (Fig. 1B). Infusion of DHA (1 mM) at 40 min caused inhibition of [^{3}H] leucine release from rapid turnover proteolysis similar to that illustrated for diamide in Fig. 1A (data not shown). The exact DHA concentration–response relationship was difficult to determine at low submaximal DHA concentration because DHA undergoes spontaneous hydrolysis and ring opening with a half-time of less than 0.5 hr in aqueous solution [26]. Nonetheless, a clear 20% inhibition of proteolysis was observed after a 2-hr DHA exposure (3–5 hr postlabeling) when the infusate was renewed each 0.5 hr with freshly dissolved 25 μM DHA infused immediately after dissolution (Fig. 2B). Sustained infusion of 100 μM DHA approached the maximal 75% proteolytic inhibition caused by diamide with a time–course of approximately 3.0

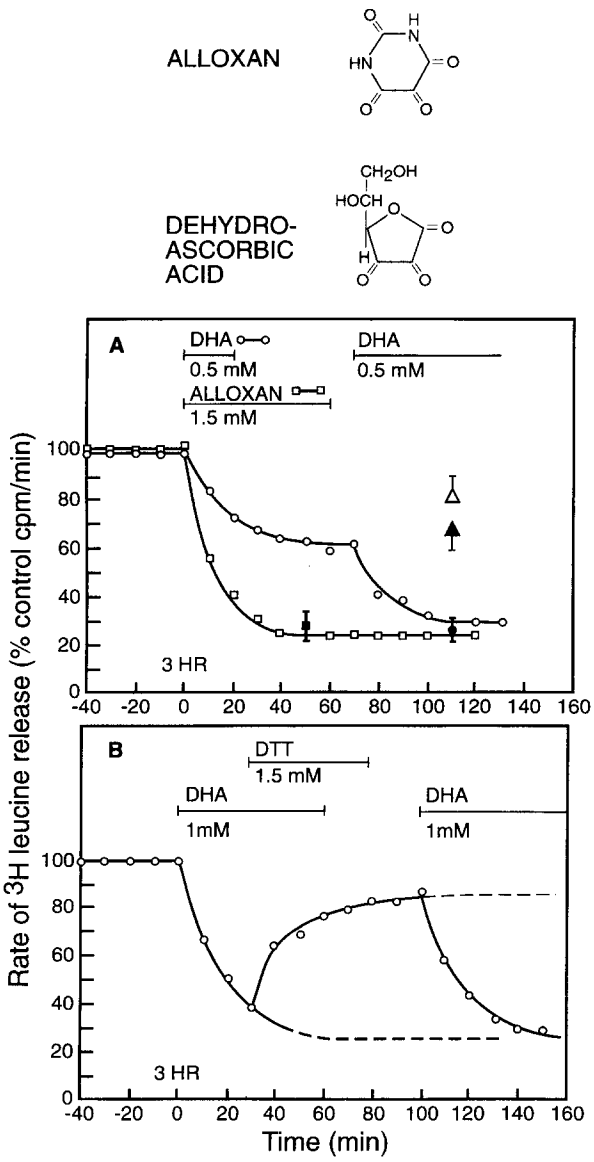

FIG. 2. (A) Inhibition of protein degradation by DHA and alloxan following a 3-hr preliminary degradation period. To eliminate rapid turnover proteins, the experimental period shown was preceded by a 3-hr preliminary degradation period following biosynthetic labeling with [^{3}H] leucine corresponding to 0–180 min in Fig. 1. The curvilinear baselines from single preparations were normalized to 100% control. Total 100% [^{3}H] leucine release consists of the three subcomponents described in the text. The 72% maximal proteolytic inhibitory action caused by alloxan (■) did not exceed significantly the 74% inhibition caused by DHA (●) (NS, $P > 0.1$, ± SD, N = 4). The effects of lesser DHA concentrations of 25 μM (△) and 100 μM (▲) are also shown (± SD, N = 3). (B) Reversal of the proteolytic inhibitory action of DHA by concurrent DTT. The indicated reversal of DHA action by concurrent DTT was sustained for 20 min after discontinuation of both agents in this single experiment and for more than 1 hr in two separate experiments, as indicated by the upper dashed trace. The action of sustained DHA is shown from separate similar experiments by the lower dashed trace. Each point shown at 10-min intervals, in both panels, represents the average of five sequential measurements at 2-min intervals measured continuously over the indicated control and experimental time periods.

hr (Fig. 2B, shown at 2 hr, and additional data not shown). Infusion of physiologic DHA (5 μM) caused no significant change in proteolysis although the exact resulting sustained DHA concentration is uncertain due to its instability in aqueous solution (NS, $P > 0.1$, $N = 3$) (data not shown; see Discussion). A marginal but significant proteolytic inhibition of approximately 10% could be clearly observed after 1 hr of 15 μM DHA infusion ($P < 0.01$, $n = 3$; data not shown).

The exact action of extracellular AA on proteolysis is difficult to interpret because extracellular AA is reportedly not readily taken up into cells [20]. Moreover, AA can spontaneously oxidize to DHA in aqueous solution [26]. Nonetheless, it was determined that 100 μM AA exerted no appreciable action on basal proteolysis (Fig. 1B, 40–160 min).

Reversal of the Proteolytic Inhibitory Action of DHA under Basal Conditions and under DTT Infusion

When infusions of DHA were interrupted, the progress of inhibition stopped at a stable submaximal level within approximately 5–10 min (Fig. 2A). When DHA infusion (0.5 mM) was continued at 65 min, the progress of proteolytic inhibition continued to the range of the 75% maximal steady inhibition by 110 min (Fig. 2A). However, in contrast to diamide action (Fig. 1A), the proteolytic inhibitory action of DHA was reversed either slowly or not at all under present conditions and times of observation (Fig. 2A).

Because AA is reportedly not transported [20], the significance of extracellular AA exposure is uncertain (see above); however, in two experiments, infusion of AA (1 mM) did not cause reversal of prior proteolytic inhibition caused by DHA (1 mM) (Fig. 1B).

DTT is known to cause rapid direct reduction of DHA to AA as well as presumptive reduction of some oxidized targets of DHA action [12, 16, 21, 26]. DTT is readily membrane-permeant. When DTT (1.5 mM) was infused simultaneously in excess of supramaximal DHA (1 mM), the prior proteolytic inhibitory action of DHA was largely reversed (by 80–90%); therefore, DHA action is redox-related. If DHA infusion (1 mM) was discontinued prior to DTT infusion, then the proteolytic inhibition could be reversed by a much lower DTT concentration of 150 μM; however, the time–course of approach to steady reversal was 1–2 hr as compared with the 20 min shown in Fig. 2B (data not shown). The reversal of DHA action by DTT was sustained indefinitely when both agents were discontinued (Fig. 2B and data not shown). The preparations of Fig. 2 (A and B) were not injured; however, exposure to DTT (1.5 mM) for longer than 0.75 hr caused nonlethal weakening of contractile function. Following reversal of DHA action, a second infusion of DHA (1 mM) caused a second 75% inhibition of [^{3}H] leucine release identical to the first (Fig. 2B).

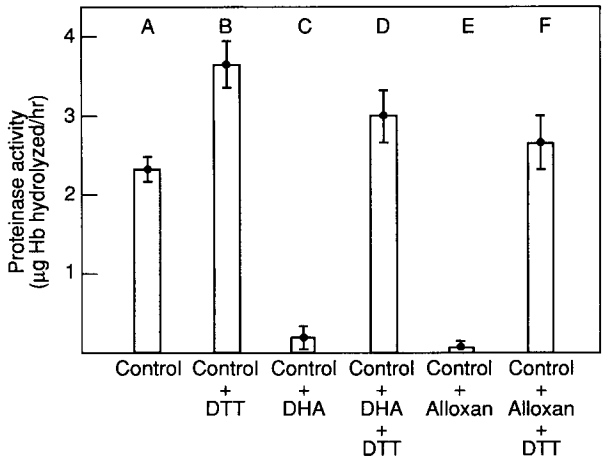

FIG. 3. Inactivation of previously reduced cathepsin B activity by DHA or alloxan and subsequent reactivation by DTT. The control hemoglobinolytic cathepsin B activity (A) was reduced previously by DTT followed by removal of DTT by dialysis. Conditions B–F represent separate assays of the identical enzyme solution as in A, treated and assayed in parallel with experimental protocols described below. (A) The reduced control was maintained for 80 min without treatment and then was assayed for 1 hr for hemoglobinolytic activity in parallel with assay of the following solutions. (B) To a second enzyme solution was added nothing for 40 min, followed by (DTT) 5 mM for 40 min. (C) To a third group was added nothing for 40 min followed by DHA (1 mM) for 40 min. (D) To a fourth solution was added DHA (1 mM) for 40 min followed by stoichiometric excess DTT (5 mM) for 40 min. (E) To a fifth solution was added nothing for 40 min followed by alloxan (1 mM) for 40 min. (F) To a sixth solution was added alloxan (1 mM) for 40 min followed by excess DTT (5 mM) for 40 min. The assay of solutions B, D, and F contained DTT at a 5 mM final concentration.

Inactivation of Thiol Cathepsin Activity by DHA and Alloxan and Reactivation by DTT

A thiol oxidant can inhibit sulfhydryl enzymes by direct action on the enzyme active site or reaction with thiol cofactors required for reduction, or both [12, 13]. To determine the direct action of DHA on cathepsin B, a previously described method for determination of direct alloxan action on cathepsin B was adapted [21]. Purified cathepsin B was first reduced with DTT, followed by the removal of DTT by dialysis (see Materials and Methods). The enzyme solution was then divided into separate portions, and all treatments shown in Fig. 3 contained either the indicated added agents or an equal volume of buffer to maintain equal enzyme concentration. As shown by a comparison of panels A and B of Fig. 3, the previously reduced control enzyme solution was further stimulated by the addition of DTT, suggesting some loss of activity during the 4-hr dialysis to remove DTT and/or the subsequent 1-hr assay. As shown by a comparison of solutions A vs C and A vs E in Fig. 3, both DHA and alloxan inactivated the proteinase essentially completely. Comparison of solutions C vs D and E vs F indicates that treatment with DTT subsequent to either DHA or alloxan reactivated most of the previously inhibited proteinase activity. Similar results have been reported previously for alloxan action on cathepsin B [21]; however, DHA has not been described.

DISCUSSION

Exposure to physiologic levels of DHA (approximately 5 μM) does not change protein degradation, presumably because the intracellular mechanisms are able to reduce the extracellular DHA taken up at the prevailing rate [20]. However, lysosomal and two nonlysosomal pathways of protein degradation are inhibited when the oxidized member of an endogenous redox couple is experimentally elevated above the level that can be reduced by the overall intracellular reducing mechanisms. Cathepsin B inactivation can account for no more than 35–40% of DHA action consisting of lysosomal proteolysis (see Ref. 1); additional nonlysosomal targets and mechanisms are unknown. Moreover, enzymes other than proteinases may also be involved in DHA action [1].

Correspondence of the present experimental oxidative imbalance to the *in vivo* control of proteinases or proteolysis is unknown; however, several features of AA/DHA suggest that future studies may reveal an influence of this couple under some conditions. It is generally accepted that reduced AA and reduced glutathione serve to protect and maintain reduced cellular sulfhydryls [reviewed in Refs. 2 and 27]. The presently observed inactivity of AA in reversing DHA action is expected in view of the known properties of this agent. AA is not a direct thiol reductant, but rather serves to protect sulfhydryls against oxidation by virtue of its "antioxidant" action on various reactions, including free radical mechanisms [2, 27]. The exact range of intracellular fluctuations in the AA/DHA redox couple has not yet been described; however, the present response to 25 μM extracellular DHA is closer to the reported physiologic 5 μM DHA when the instability of the dissolved DHA is taken into account [20, 26].

Due to the strong reactivity of ring triketones with enzyme sulfhydryls, it has been suggested that DHA might be a link in some cell thiol metabolism [16]; however, this interesting speculation remains to be verified. The oxidative inactivation of sulfhydryl proteinases by DHA could involve direct action at the enzyme active site and also indirect action via interference with reducing enzyme chains providing proteinase reduction [2, 8, 16–29]. The sustained *in vitro* reversal of DHA action caused by DTT could simultaneously involve direct reduction of proteinase active sites (Fig. 3), direct reduction and repletion of various components of the endogenous redox system, and direct reduction of residual DHA to AA, all of which are known reactions of DTT. The *in vivo* maintenance of reduced active sulfhydryl proteinases is uncharacterized; however, those reductases known to maintain reduced AA may serve to prevent oxidative inactivation of sulfhydryl proteinases by maintaining low intracellular DHA oxidant levels and high AA antioxidant levels [2]. However, results

do not exclude additional oxidative factors, other than DHA, that might inactivate sulfhydryl enzymes.

Despite remaining uncertainties, the total protein degradation presently observed can be partitioned into redox-sensitive and redox-insensitive processes [1]. Although diamide-sensitive protein degradation is presumably mediated by multiple enzymes, sulfhydryl proteinases are apparently required at some step in the proteolytic–peptidolytic reaction sequences [1]. Because 25% of total protein degradation is insensitive to the harsh thiol-reactive agent *N*-ethylmaleimide, or a thiol proteinase inhibitor over several hours [1], it appears likely that this protein degradation is mediated by enzymes other than sulfhydryl proteinases. It is conceivable that the three diamide-sensitive pathways might be regulated independently under adequate cell reducing equivalents; however, coordinate limitation of these pathways could be imposed by unknown conditions or controls related to the cell redox status. The opposing thiol antioxidant action of AA versus the thiol oxidant action of DHA [2, 27] may be among the factors selectively modulating intracellular proteolytic pathways under some conditions [2, 27–29]. Large changes in AA levels are known, including 96% fluctuation of endogenous millimolar levels under prolonged cell culture [30] as well as human nutritional alterations. It remains to be determined whether the balance between reductive activation and oxidative inactivation of proteolysis represents true proteolytic controlling mechanisms; however, results demonstrated large reversible redox modulations of proteolysis under noninjurious metabolic intervention.*

This work was supported by a grant from the American Heart Association, Ohio Affiliate.

References

1. Thorne DP and Lockwood TD, Distinctions between four major proteolytic processes of myocardium, one insensitive to thiol-reactive agents and thiol protease inhibitor. *Am J Physiol* **265:** E10–E19, 1993.
2. Meister A, Glutathione-ascorbic acid antioxidant system in animals. *J Biol Chem* **269:** 9397–9400, 1994.
3. Barrett AJ, *Proteinases in Mammalian Cells and Tissues.* Elsevier, Amsterdam, 1977.
4. Cleland WW, Dithiothreitol: A new protective agent for sulfhydryl groups. *Biochemistry* **3:** 480–485, 1964.
5. Fernando RM, Nanri H, Yoshitake S, Nagata-Kuno K and Minakami S, Thioredoxin regenerates proteins inactivated by oxidative stress in endothelial cells. *Eur J Biochem* **209:** 917–922, 1992.
6. Holmgren A, Thioredoxin: Structure and functions. *Annu Rev Biochem* **54:** 237–271, 1985.
7. Miller RM, Park EM and Thomas JA, Reduction (dethiolation) of protein-mixed disulfides; Distribution and specificity of dethiolating enzymes and *N,N'*-bis(2-chloroethyl)-*N*-nitrosourea inhibition of an NADPH-dependent cardiac dethiolase. *Arch Biochem Biophys* **287:** 112–120, 1991.
8. Stephen AG, Powls R and Beynon RL, Activation of oxidized cysteine proteinases by thioredoxin-mediated reduction *in vitro*. *Biochem J* **291:** 345–347, 1993.
9. Kosower NS and Kosower EM, Diamide: An oxidant probe for thiols. *Methods Enzymol* **251:** 123–133, 1995.
10. Biaglow JE and Nygaard OF, The use of the oxidant diamide for studying the nonmitochondrial reducing capacity of Ehrlich ascites tumor cells. *Biochem Biophys Res Commun* **54:** 874–881, 1973.
11. Grimm LM, Collison MW, Fisher RA and Thomas JA, Protein-mixed disulfides in cardiac cells: *S*-Thiolation of soluble proteins in response to diamide. *Biochim Biophys Acta* **844:** 50–54, 1985.
12. Lenzen S and Munday R, Thiol-group reactivity, hydrophilicity and stability of alloxan, its reduction products and its *N*-methyl derivatives and a comparison with ninhydrin. *Biochem Pharmacol* **42:** 1385–1391, 1991.
13. Lenzen S and Panten U, Alloxan: History and mechanism of action. *Diabetologia* **31:** 337–342, 1988.
14. Basu S, Som S, Deb S, Mukherjee D and Chatterjee IB, Dehydroascorbic acid reduction in human erythrocytes. *Biochem Biophys Res Commun* **90:** 1335–1340, 1979.
15. Rose RC and Bode AM, Tissue-mediated regeneration of ascorbic acid: Is the process enzymatic? *Enzyme* **46:** 196–203, 1992.
16. Wells WW, Xu DP, Yang YF and Rocque PA, Mammalian thioltransferase (glutaredoxin) and protein disulfide isomerase have dehydroascorbate reductase activity. *J Biol Chem* **265:** 15361–15364, 1990.
17. Winkler BS, Unequivocal evidence in support of the nonenzymatic redox coupling between glutathione/glutathione disulfide and dehydroascorbic acid. *Biochim Biophys Acta* **1117:** 287–290, 1992.
18. Winterbourne CC and Munday R, Glutathione-mediated redox cycling of alloxan. *Biochem Pharmacol* **38:** 271–277, 1989.
19. Coassin M, Tomasi A, Vannini V and Ursini F, Enzymatic recycling of oxidized ascorbate in pig heart; One-electron vs two-electron pathway. *Arch Biochem Biophys* **290:** 458–462, 1991.
20. Vera JC, Rivas CI, Fischborg J and Golde DW, Mammalian facilitative hexose transporters mediate the transport of dehydroascorbic acid. *Nature* **364:** 79–82, 1993.
21. Mikasa E and Tappel AL, Inhibition studies of cathepsins A, B, C, and D from rat liver lysosomes. *Comp Biochem Physiol* **38B:** 661–662, 1971.
22. Lockwood TD, Minassian IA and Roux LR, Protein turnover and proliferation: Turnover kinetics associated with the elevation of 3T3 cell acid proteinase activity and cessation of net protein gain. *Biochem J* **206:** 239–249, 1982.
23. Lockwood TD, Minute to minute neuroendocrine control of a major nonlysosomal pathway of myocardial protein degradation by β-receptor occupancy. *In: Intracellular Protein Catabolism* (Eds. Khairallah EA, Bond JS and Bird JNC), pp. 619–622. Alan R. Liss, New York, 1985.
24. Lockwood TD, Simultaneous response of myocardial contractility and a major proteolytic process to β-adrenergic receptor occupancy in the Langendorff isolated perfused rat heart. *Biochem J* **231:** 299–308, 1985.
25. Lockwood TD, Distinctions between major chloroquine-inhibitable and adrenergic-responsive pathways of protein degradation and their relation to tissue ATP content in the

* Lockwood TD, Antimalarial drug actions reveal metabolic reducing requirement for some mammalian cell proteolytic pathways. *Biology of Proteolysis, 1997.* Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Langendorff isolated perfused rat heart. *Biochem J* **251:** 2341–2346, 1988.
26. Washko PW, Welch RW, Dhariwal KR, Wang Y and Levine M, Ascorbic acid and dehydroascorbic acid analysis in biological samples. *Anal Biochem* **204:** 1–14, 1992.
27. Martensson J and Meister A, Glutathione deficiency decreases tissue ascorbate levels in newborn rats: Ascorbate spares glutathione and protects. *Proc Natl Acad Sci USA* **88:** 4656–4660, 1991.
28. Welch RW, Wang Y, Crossman A, Park JB, Kirk KL and Levine M, Accumulation of vitamin C (ascorbate) and its oxidized metabolite dehydroascorbic acid occurs by separate mechanisms. *J Biol Chem* **270:** 12584–12592, 1995.
29. Park JB and Levine M, Purification, cloning and expression of dehydroascorbic acid-reducing activity from human neutrophils: Identification as glutaredoxin. *Biochem J* **315:** 931–938, 1996.
30. Bergsten P, Amitai G, Kehrl T, Dariwhal KR, Klein HG and Levine M, Millimolar amounts of ascorbic acid in purified human mononuclear leukocytes: Depletion and reaccumulation. *J Biol Chem* **265:** 2584–2587, 1990.