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INTERFERENCE WITH LYSOSOMAL PROTEOLYSIS FAILS TO REDUCE CARDIAC MYOSIN DEGRADATION

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SUMMARY

Chloroquine (a lysosomotropic agent) and leupeptin (an inhibitor of thiol proteinases including lysosomal cathepsins B, H, and L) reduced the rate of total protein degradation in cultured fetal mouse hearts by 25% ($p < 0.01$). Simultaneously, the rate of degradation of myosin was not diminished by either agent. In contrast, insulin reduced the rate of total protein degradation by 16% and of myosin degradation by 13% ($p < 0.01$ for both). These results indicate that primary interference with lysosomal proteolytic processes fails to inhibit myosin degradation; it is suggested that the degradation of myosin (and perhaps other myofibrillar proteins) is normally accomplished via nonlysosomal mechanisms.

INTRODUCTION

General lysosomotropic agents (e.g., chloroquine) and inhibitors of individual lysosomal proteinases (e.g., leupeptin) are known to decrease the rate of total protein degradation in heart (1-4). It has not been known, however, if lysosomal processes are involved in the turnover of all cardiac proteins or, instead, if certain subclasses of protein are normally degraded by nonlysosomal mechanisms. Myofibrillar proteins are of special interest in this regard: although lysosomal cathepsins possess the ability to degrade myofibrillar proteins (5-7), fragments of myofibrils have rarely if ever been observed in autophagic vacuoles of normal cardiac or skeletal myocytes. Accordingly, to determine the importance of lysosomal processes in the breakdown of a major myofibrillar protein, we tested the influence of chloroquine and leupeptin on the degradation of myosin and of total protein in hearts of fetal mice in organ culture. For comparison, similar experiments were performed using insulin.

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MATERIALS AND METHODS

Isolated, intact hearts of 19-20 day fetal mice were maintained in organ culture as described previously (8). For each experiment, twelve matched hearts from a single litter were allowed to stabilize overnight in "medium 199" (Grand Island Biol. Co.), after which they were pulsed for five hours with [^{3}H]phenylalanine (150 μCi/ml, Schwarz/Mann). After three one-hour-long washes in non-radioactive medium containing excess cold phenylalanine (3.0 mM), the hearts were divided into three matched groups. The first group was frozen in liquid nitrogen and stored at -65^{o} for later biochemical analyses; the second group was given fresh control "medium 199" containing excess cold phenylalanine and allowed to remain in culture for 24 hours before being frozen and stored; the third group was given an identical medium supplemented with 0.1 mM chloroquine, 50 μg/ml leupeptin, or 50 μg/ml insulin for 24 hours and then frozen and stored. These three agents have been observed in previous studies (1,4,9) to reduce the rate of total protein degradation by 15-35% in organ-cultured hearts. Each heart was homogenized in 100 volumes of 100 mM $Na_4P_2O_7$, 5 mM EGTA, 15 mM 2-mercaptoethanol, 10% glycerol, pH 8.8 at 2^{o} (10) in a microhomogenizer (Radnoti Glass, Arcadia, CA). After a 30 min incubation at 2^{o}, an aliquot of the homogenate was precipitated with 10% trichloroacetic acid (TCA) for measurements of radioactivity in the total protein fraction (9,11).

A second aliquot was centrifuged at 5,000 x g for 20 min, and the supernatant fraction was electrophoresed for 5 hrs on 3.3% polyacrylamide gels for purification of myosin (10). The high concentration of pyrophosphate used in these experiments results consistently in solubilization and recovery of >95% of total myosin. A single band of protein was identified as myosin after polyacrylamide gel electrophoresis, and it possessed Ca^{++}-activated ATPase activity (10). Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate in a second dimension demonstrated the presence of heavy chain and 2 types of light chain subunits of myosin. No other proteins were apparent (10). The

polyacrylamide gel slice containing myosin was incubated in NCS (Amersham) for 2 hrs at 50^{0} and the radioactivity was measured (9,11).

For each litter, the rate of degradation of total labeled protein and of myosin was calculated for control and experimental hearts, as described previously (9,11). Differences between groups were analyzed by Students t test for paired samples, using values for the matched groups of hearts within each litter.

RESULTS

As observed previously in fetal mouse hearts (1,2,4) as well as in other systems (12), chloroquine and leupeptin produced significant decreases in proteolysis. As shown in Table I, the fractional rate of degradation of total labeled protein was decreased from 51% per day in control hearts to 37% per day in matched hearts maintained in the presence of chloroquine (a 27% reduction). For hearts incubated in the presence of leupeptin, the rate of loss of phenylalanine from total protein was reduced by 25%, from 48% per day to 36% per day.

Neither chloroquine nor leupeptin had any effect on the rate of degradation of myosin. In the same hearts in which chloroquine had inhibited total proteolysis by 27%, the rate of myosin degradation was undiminished (63% per day in controls vs 68% per day in the presence of chloroquine; see Table I). Similarly, the rate of myosin breakdown was not altered by leupeptin (59% vs 61%; see Table I), even though total proteolysis was reduced by 25%. In all experiments, the fractional rate of myosin degradation was faster than the fractional rate of degradation of total labeled protein.

For comparative purposes similar experiments were performed using insulin, a well-characterized inhibitor of protein degradation in heart (9,13). As is apparent in Table I, insulin produced a quantitatively similar decrease in the degradation of total protein (–16%) and myosin (–13%).

DISCUSSION

Chloroquine is a "lysosomotropic" agent (14) that is selectively concentrated in lysosomes where it decreases proteolytic capacity by raising the

intralysosomal pH above the optimum of most lysosomal proteinases and also by directly inhibiting lysosomal cathepsin B activity (1,15). Leupeptin decreases proteolytic capacity by inhibiting certain endogenous proteinases, especially those of the thiol class; along with some nonlysosomal proteinases, several important lysosomal enzymes (e.g., cathepsins B, H, and L) are inhibited by the agent (16). As in other tissues (12), inhibition of these enzymes in heart decreases the rate of total protein degradation (2-4).

Although the action of chloroquine and leupeptin clearly include production of major defects in lysosomal proteolytic capacity, it remains possible

Table I. Effects of chloroquine, leupeptin, and insulin on the rate of loss of radioactive phenylalanine from total protein and myosin of fetal mouse hearts in organ culture.

	Total protein degradation (% per day)	Myosin degradation (% per day)
A. Control	51 ± 4	63 ± 4
B. + Chloroquine	37 ± 4	68 ± 2
Difference (B-A)	-14 ± 2*	+5 ± 2
Percent difference $(\frac{B-A}{A} \times 100)$	-27%	+8%
A. Control	48 ± 4	59 ± 5
B. + Leupeptin	36 ± 5	61 ± 5
Difference (B-A)	-12 ± 2*	+2 ± 1
Percent difference $(\frac{B-A}{A} \times 100)$	-25%	+3%
A. Control	50 ± 3	63 ± 2
B. + Insulin	42 ± 3	55 ± 4
Difference (B-A)	-8 ± 2*	-8 ± 2*
Percent difference $(\frac{B-A}{A} \times 100)$	-16%	-13%

Each value represents the mean ± 1 SEM of 24 hearts from 6 litters. Concentrations for each agent are given in the text. * = $p < 0.01$, with comparisons made between matched littermates.

that some of their effects might not be mediated via lysosomal processes. For example, chloroquine in large concentrations can also produce some nonspecific toxic effects on other organelles including mitochondria (17); leupeptin, although remarkably free of nonspecific toxicity (2), does inhibit some nonlysosomal proteinases that are thought to be of importance in heart. Thus, if chloroquine and leupeptin had been found to reduce myosin degradation, one could not have concluded with absolute certainty that myosin was broken down within lysosomes. On the other hand, the absence of any inhibitory effect of these agents on the rate of myosin degradation implies strongly that the normal degradation of this molecule is accomplished via nonlysosomal mechanisms. These results also imply that none of the other proteinases which leupeptin is known to inhibit, including Ca^{++}-activated neutral proteinase (18) and ATP-dependent proteinase (19), are of importance in degrading myosin.

It remains possible, of course, that after the initial steps of myosin breakdown, subfragments of the molecule could still be degraded within lysosomes. In these experiments, intact myosin was extracted quantitatively, and peptide products of the molecule's cleavage were not measured. Thus, myosin subfragments might secondarily be hydrolysed within lysosomes without affecting the observed results. Nevertheless, it seems clear that the critical step of the degradation of the myosin molecule itself is not a lysosomal event.

To see if myosin degradation is also immune to the action of other agents that are known to inhibit cardiac proteolysis, we tested the effect of insulin. Insulin inhibited the degradation of myosin to the same extent as that of total protein, thus indicating that the breakdown of myosin is indeed subject to physiological regulation. It has often been suggested that insulin's inhibition of proteolysis in heart and in other tissues might be the result of its effects on lysosomes (9,13,20,21). In view of the differences observed in the present study between the inhibitory effects on myosin degradation of insulin and of agents that are known to act primarily on lysosomes and lysosomal enzymes, it

seems likely that insulin possesses important nonlysosomal effects in addition to its putative effects on lysosomal function.

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