Inhibition of cardiac proteolysis by colchicine

Selective effects on degradation of protein subclasses

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1. The effect of colchicine (2.5 μM) on cardiac protein turnover was tested with foetal mouse hearts in organ culture. 2. Colchicine had no effect on protein synthesis, but inhibited total protein degradation by 12–18%. Lumicolchicine, which lacks colchicine's ability to disaggregate microtubules, but shares its non-specific effects, did not alter protein degradation. 3. The colchicine-induced inhibition of protein degradation was accompanied by significant changes in cardiac lysosomal enzyme activities and distribution. 4. Colchicine inhibited the degradation of organellar proteins, including mitochondrial cytochromes, more than that of cytosolic proteins. 5. Colchicine decreased the rate of myosin degradation and the rate of proteolysis of the total protein pool to a similar extent. Since the regulation of myosin degradation does not involve lysosomes, this suggests that colchicine affects non-lysosomal as well as lysosomal pathways. 6. Release of branched-chain amino acids from colchicine-treated hearts was disproportionately decreased, suggesting that colchicine increased their metabolism. 7. It is concluded that colchicine, via its actions on microtubules, exerts important inhibitory effects on cardiac proteolysis. Colchicine is especially inhibitory to the degradation of organellar proteins, including mitochondrial cytochromes. Its inhibitory effects may be mediated in part via lysosomal mechanisms, but non-lysosomal mechanisms are probably involved as well.

Disaggregation of the microtubular system induced by colchicine and similar drugs interferes with the normal degradation of exogenous endocytosed proteins (Kolset *et al.*, 1979; Ostlund *et al.*, 1979; Brown *et al.*, 1980; Ose *et al.*, 1980; Grinde & Seglen, 1981). In cultured fibroblasts (Amenta *et al.*, 1976, 1977) and hepatocytes (Seglen *et al.*, 1980; Marzella & Glaumann, 1980b; Grinde & Seglen, 1981), degradation of endogenous proteins is also inhibited by these agents under conditions in which autophagy is activated, but not under baseline conditions (Amenta *et al.*, 1976, 1977). The degradation of endocytosed protein appears to involve lysosomal enzymes after fusion of endocytic vacuoles with primary lysosomes. The normal movement of some organelles, including lysosomes, is inhibited when microtubules are disaggregated by colchicine (Freed & Lebowitz, 1970; Parr, 1979).

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Thus circumstantial evidence suggests that the decrease in proteolysis caused by microtubular disruption is linked in some way to interference with lysosomal function. This hypothesis is supported by experiments which have shown that the effects of lysosomotropic agents and those of anti-microtubular agents, both of which inhibit protein degradation, are not additive (Seglen *et al.*, 1980; Grinde & Seglen, 1981).

Lysosomes are relatively sparse in heart, and it has been suggested that lysosomal proteolysis is quantitatively less important in heart than in most other tissues. Furthermore, the breakdown of cardiac myosin appears not to be regulated by lysosomal mechanisms (Wildenthal *et al.*, 1980). Therefore it seemed possible that interference with microtubular function would have less effect on protein degradation in heart than in other tissue, and might have different effects on the degradation of some classes of myocardial protein than on others. Accordingly, the present study was undertaken to test the effects of colchicine-induced disaggregation

of microtubules on cardiac protein degradation. Experiments were made with intact beating organ-cultured hearts of late-foetal mice, a model that provides long-term contractile and metabolic stability of myocardial cells under precisely controlled conditions *in vitro*, without overgrowth of non-myocardial cells.

Experimental

Organ culture of foetal mouse hearts

Hearts from 18–20-day foetal mice were maintained in culture as described in detail previously (Wildenthal, 1971). Matched littermates were divided into two groups, which were cultured under identical conditions except for the presence or absence of 2.5 μM -colchicine (Sigma Chemical Co.) in the culture media. Preliminary experiments established that exposure to this concentration of colchicine produced maximal effects on protein balance and altered the staining pattern observed microscopically with fluoresceinated anti-tubulin antibody, but had no observed effect on contractile function. To ascertain whether colchicine's actions were due to disaggregation of microtubules, or were caused by non-specific toxic properties of the drug, parallel experiments were performed with lumicolchicine, a mixture of colchicine isomers produced by exposure of colchicine to u.v. light for 72 h. Lumicolchicine does not bind to or cause depolymerization of tubulin, but it does retain the non-specific toxic properties of colchicine (Borisy & Taylor, 1967; Mizel & Wilson, 1972). In some experiments cycloheximide (2.5 $\mu\text{g}/\text{ml}$; Sigma) was added to the culture media of both control and experimental hearts to inhibit protein synthesis and prevent reutilization of radiolabelled amino acids.

Measurements of protein degradation

Protein degradation was measured by two separate techniques. In the first, hearts were stabilized in

degradation and that reutilized for protein synthesis. When cycloheximide (2.5 $\mu\text{g}/\text{ml}$) is added to the culture medium, protein synthesis is decreased by more than 90% (Wildenthal & Griffin, 1976), so that phenylalanine release becomes an index only of protein degradation. Although cardiac proteolysis is inhibited somewhat by cycloheximide, this effect was present in both control and experimental hearts, and differences in phenylalanine release between these groups can be reasonably attributed to the actions of colchicine on proteolysis in the treated hearts.

In the second technique, protein degradation was measured from the release of radioactive amino acid from prelabelled protein under conditions designed to minimize reutilization of labelled amino acid (Wildenthal *et al.*, 1976). This approach avoids the potential complicating influence of cycloheximide. After stabilization for 18–24 h in chemically defined medium 199 (Grand Island Biological Co.) hearts were exposed to medium containing L-[*side chain*-2,3- ^3H]phenylalanine (15–100 $\mu\text{Ci}/\text{ml}$, 50 Ci/mmol ; Schwarz-Mann Chemical Co.) for 4 h. In some experiments, the pulsing medium also contained 5-amino[4- ^{14}C]laevulinic acid (2.5 $\mu\text{Ci}/\text{ml}$, 42 mCi/mmol ; Amersham Corp.). After being labelled the hearts were washed in medium containing an excess of labelled phenylalanine (and sometimes δ -amino-laevulinic acid), and then were divided into three groups. One group ('zero-time') was harvested immediately and frozen for subsequent analysis. The second group was cultured in control medium, and the third group was cultured in colchicine-containing medium. After 24 h, hearts in these groups were harvested. All hearts were homogenized and the trichloroacetic acid-precipitable material ('total protein') was collected and its radioactivity measured in a Packard 2425 Tri-Carb liquid-scintillation counter. Protein degradation during the 24 h culture period was determined as described previously (Wildenthal *et al.*, 1976) from the loss of radioactivity, by using the following equation:

$$\text{Protein degradation (\%/24 h)} = \frac{\text{radioactivity at 'zero-time'} - \text{radioactivity after 24 h culture}}{\text{radioactivity at 'zero-time'}} \times 100$$

Earle's salt solution (Grand Island Biological Co.) and then transferred to fresh medium containing cycloheximide with or without colchicine. After 24 h in culture, hearts were harvested and the media collected. The concentrations of 14 amino acids in the media were measured with a Durrum amino acid analyser. Since phenylalanine is not metabolized by heart (Morgan *et al.*, 1971), its release reflects the difference between amino acid freed during protein

Myosin degradation

In order to calculate the rate of myosin degradation, the protocol described above for radioactive phenylalanine labelling was followed. After the hearts had been harvested and homogenized, half of the homogenate was used to measure total protein degradation and half for myosin degradation. Myosin was isolated by a modification of the method of Hoh *et al.* (1978), as previously

described (Wildenthal *et al.*, 1980). This method, which involves the use of pyrophosphate/polyacrylamide-gel electrophoresis, results in consistent recovery of more than 95% of the total myosin with virtually no contamination (Hoh *et al.*, 1978; Wildenthal *et al.*, 1980). The gel slices containing myosin were incubated in NCS (Amersham) and the myosin radioactivity was measured (Wildenthal *et al.*, 1980). The fractional rate of myosin degradation was calculated as described above.

Haem protein degradation

Degradation of haem proteins was measured in hearts exposed to δ -amino[^{14}C]laevulinic acid plus [^3H]phenylalanine. The experimental protocol was identical with that described above for total protein, except that ^{14}C radioactivity and ^3H radioactivity in the trichloroacetic acid precipitate were measured simultaneously. Calculations of the fractional rate of degradation of haem protein were as described above, by using the ^{14}C radioactivity (Ord & Wildenthal, 1980). Because of the extensive reutilization of δ -aminolaevulinic acid in heart tissue (Ord & Wildenthal, 1980), cycloheximide was added to the media of control and colchicine-treated hearts during the degradation period in some experiments.

Organelle protein degradation

In some experiments, hearts prelabelled with [^3H]phenylalanine were homogenized in 0.25 M-KCl/1 mM-EDTA, pH 7.3, and fractions of the homogenate were separated by differential centrifugation. A 500 g pellet (5 min) ('nuclear' fraction), 5000 g (20 min) pellet ('mitochondrial' fraction), and the 5000 g supernatant ('supernatant' fraction) were obtained. The 'nuclear' fraction contained some unbroken cells and large fragments, nuclei, aggregates of organelles, and 'heavy' mitochondria. The 'mitochondrial' fraction was composed largely of mitochondria, with some large lysosomes, other organelles and unidentified membrane fragments. The 'supernatant' fraction included small membrane fragments, sarcoplasmic reticulum, lysosomes and soluble proteins. When this protocol was used, the soluble proteins included myofibrillar proteins; in foetal hearts, however, myofibrillar proteins make up only 5–10% of the total protein, so that most of the 'supernatant' fraction was composed of other cytosolic and interstitial proteins. Approximately half of the total protein was recovered in the 'supernatant' fraction.

Protein in each of the three fractions was precipitated with trichloroacetic acid and counted for radioactivity, and the rate of proteolysis for each fraction was calculated as described above for total protein degradation.

Protein synthesis

Protein synthesis was calculated as described previously (Wildenthal *et al.*, 1976) from the incorporation of [^3H]phenylalanine into protein in hearts cultured in medium 199 supplemented with 0.40 mM-phenylalanine.

Enzyme activities

The activities and distribution of the lysosomal enzymes β -acetyl-N-glucosaminidase, acid phosphatase and cathepsin D were measured in pooled groups of three to six hearts by modifications of Barrett's methods (Barrett & Heath, 1977), as described previously (Wildenthal *et al.*, 1975). Creatine kinase activity was measured by the method of Szasz *et al.* (1970) and protein was measured by the method of Lowry *et al.* (1951).

Statistical analysis

There is some variability between foetal hearts of different litters and gestational ages, but results from within a litter are highly reproducible (S.D. < 10%). Therefore statistical comparisons between values obtained from matched littermates under control and experimental conditions were made by Student's *t* test for paired samples. All results were expressed as the mean $\pm$ 1 S.E.M.

Results

Total protein degradation

The release of phenylalanine from cultured foetal hearts was significantly decreased by colchicine. In the presence of cycloheximide, when phenylalanine release serves as an index of protein degradation, colchicine inhibited phenylalanine release by 14% (0.155 ± 0.0041 nmol/h per mg wet wt. in controls, 0.133 ± 0.0019 nmol/h per mg wet wt. in colchicine-treated hearts, $P < 0.001$) (Fig. 1). Lumicolchicine had no significant effect on phenylalanine release in parallel experiments. The effect of colchicine on protein degradation as measured by the loss of radioactivity in the total protein pool is shown in Fig. 2. Protein degradation in control hearts was $44.8 \pm 0.79\%$ /day, compared with $37.5 \pm 1.14\%$ /day in hearts exposed to colchicine. This is a 16% decrease ($P < 0.001$) in the rate of total protein degradation. In similar experiments lumicolchicine did not alter protein degradation.

Myosin degradation

As reported previously (Wildenthal *et al.*, 1980), labelled myosin was degraded at a slightly faster rate in cultured foetal mouse hearts than was the total protein pool (Fig. 3). Colchicine inhibited the rate of myosin degradation by 15%, and the rate of total proteolysis was decreased similarly (13%) in the same hearts ($P < 0.05$ for both).

Fig. 1. Rate of phenylalanine release from hearts cultured in the presence of colchicine or lumicolchicine and in matched controls

Hearts were cultured for 24 h in Earle's salt solution containing cycloheximide (2.5 µg/ml). Each bar represents the mean $\pm$ 1 s.e.m. The rate of phenylalanine release from colchicine-treated hearts was $86 \pm 2.1\%$ of that from matched littermate controls ($n = 12$); phenylalanine release from lumicolchicine-treated hearts was $96 \pm 2.2\%$ of that from matched littermate controls ($n = 7$). ** $P < 0.001$ compared with controls.

Degradation of haem proteins

In double-label experiments the rate of total protein degradation and the rate of degradation of haem protein (as identified by the loss of δ -aminol¹⁴C]laevulinic acid) could be measured in the same hearts. In the absence of cycloheximide, colchicine produced a 31% decrease in the rate of loss of radioactive δ -aminolaevulinic acid and an 18% decrease in the rate of loss of radioactive phenylalanine. Because of the extensive cardiac reutilization of δ -aminolaevulinic acid in this system (Ord & Wildenthal, 1980), this difference between loss of δ -aminolaevulinic acid and of phenylalanine could be due to a difference in the fractional rate of reutilization of these compounds rather than a difference in protein degradation; therefore, to block reutilization, experiments were also performed in the presence of cycloheximide. In these experiments (Fig. 4), colchicine decreased the loss of radioactive phenylalanine by $12 \pm 2.2\%$ and that of δ -aminolaevulinic acid by $31 \pm 4.1\%$ ($P < 0.001$ for both). The 31% inhibitory effect of colchicine on the degradation of haem proteins was significantly greater than its 12% effect on the degradation of the total protein pool in the same hearts (difference = $19 \pm 3.6\%$, $P < 0.001$).

Organellar protein degradation

The rates of degradation of protein in three

Fig. 2. Rate of protein degradation in hearts treated with colchicine and in matched controls

(a) Release of [³H]phenylalanine from trichloroacetic acid-precipitable protein in hearts cultured in medium 199. Each point represents the mean $\pm$ 1 s.e.m. for 39 experiments. (b) The same data as in part (a) expressed as fractional rate of protein degradation. The rate of protein degradation for colchicine-treated hearts was $84 \pm 2.2\%$ of that for matched littermate controls. ** $P < 0.001$ compared with controls.

Fig. 3. Fractional rates of degradation of total protein and myosin in hearts treated with colchicine and in matched controls

Hearts were cultured in medium 199 containing cycloheximide (2.5 µg/ml). Each bar represents the mean $\pm$ 1 S.E.M. for nine experiments. The rate of degradation for colchicine-treated hearts (hatched bars) compared with matched littermate control hearts (open bars) was $87 \pm 4.5\%$ for total protein and $85 \pm 3.1\%$ for myosin. * $P < 0.05$ compared with controls.

Fig. 4. Fractional rates of degradation of total and haem protein in hearts treated with colchicine and in matched controls

Hearts were cultured in medium 199 containing cycloheximide (2.5 mg/ml). Each bar represents the mean $\pm$ 1 S.E.M. for 31 experiments. The rate of degradation for colchicine-treated hearts (hatched bars) compared with matched littermate control hearts (open bars) was $88 \pm 2.2\%$ for total protein and $69 \pm 4.1\%$ for haem protein. ** $P < 0.001$ compared with controls.

Fig. 5. Fractional rates of degradation of 'nuclear', 'mitochondrial' and 'supernatant' protein in hearts treated with colchicine and in matched controls

Hearts were cultured in medium 199 without cycloheximide. Each bar represents the mean $\pm$ 1 S.E.M. for 11 experiments. The rate of degradation for colchicine-treated hearts (hatched bars) compared with matched littermate control hearts (open bars) was $55 \pm 5.9\%$ for 'nuclear' protein, $66 \pm 12.7\%$ for 'mitochondrial' protein, and $98 \pm 5.7\%$ for 'supernatant' protein. ** $P < 0.001$ compared with controls.

fractions of the tissue homogenate obtained by differential centrifugation are displayed in Fig. 5. Colchicine caused a significant decrease in the rates of degradation of 'nuclear' and 'mitochondrial' protein (45% and 34% decreases respectively, $P < 0.05$), but had no significant effect on the degradation of 'supernatant' protein.

Protein synthesis

Protein synthesis was measured from the rate of incorporation of [^3H]phenylalanine into protein. There was no significant difference in the rate of protein synthesis between control and colchicine-treated hearts (phenylalanine incorporation in colchicine-treated hearts was $96 \pm 4.8\%$ of that in matched controls, $P > 0.10$).

Amino acid metabolism

The effect of colchicine on the release of most amino acids was similar to its effect on phenylalanine release (Table 1). For example, in the presence of cycloheximide, phenylalanine release was decreased by 14% by colchicine, and release of tyrosine, lysine, histidine, arginine, alanine and methionine was decreased by 12–15%. Notable exceptions to this pattern included the branched-chain amino acids. Colchicine decreased their release by 25–31%. Serine release was decreased by

Table 1. *Effects of colchicine on net amino acid release from foetal mouse hearts maintained in organ culture in the presence of cycloheximide*

Values are means $\pm$ 1 s.e.m., and results are given as nmol/h per mg wet wt. for the net release of each amino acid into the medium. Column C gives the mean difference $\pm$ 1 s.e.m. for 12 matched samples, and numbers in parentheses indicate the percentage difference between colchicine-treated hearts and controls. All differences were significant ($P < 0.001$) by Student's *t* test for paired samples.

	(A) Control	(B) + 2.5 μ M-Colchicine	(C) Differences (%)
Lys	0.355 $\pm$ 0.0095	0.308 $\pm$ 0.0035	-0.047 $\pm$ 0.0080 (-13)
His	0.089 $\pm$ 0.0022	0.078 $\pm$ 0.0013	-0.012 $\pm$ 0.0017 (-13)
Arg	0.235 $\pm$ 0.0070	0.203 $\pm$ 0.0027	-0.032 $\pm$ 0.0055 (-14)
Thr	0.278 $\pm$ 0.0064	0.246 $\pm$ 0.0034	-0.031 $\pm$ 0.0055 (-11)
Ser	0.362 $\pm$ 0.0100	0.275 $\pm$ 0.0048	-0.086 $\pm$ 0.0086 (-24)
Pro	0.173 $\pm$ 0.0046	0.132 $\pm$ 0.0023	-0.041 $\pm$ 0.0048 (-24)
Gly	0.557 $\pm$ 0.0151	0.516 $\pm$ 0.0074	-0.041 $\pm$ 0.0107 (-7)
Ala	0.703 $\pm$ 0.0213	0.603 $\pm$ 0.0176	-0.100 $\pm$ 0.0170 (-14)
Val	0.216 $\pm$ 0.0074	0.162 $\pm$ 0.0033	-0.054 $\pm$ 0.0064 (-25)
Met	0.114 $\pm$ 0.0030	0.097 $\pm$ 0.0014	-0.017 $\pm$ 0.0023 (-15)
Ile	0.139 $\pm$ 0.0057	0.096 $\pm$ 0.0024	-0.043 $\pm$ 0.0052 (-31)
Leu	0.263 $\pm$ 0.0102	0.191 $\pm$ 0.0045	-0.072 $\pm$ 0.0088 (-27)
Tyr	0.124 $\pm$ 0.0031	0.108 $\pm$ 0.0015	-0.015 $\pm$ 0.0026 (-12)
Phe	0.155 $\pm$ 0.0041	0.133 $\pm$ 0.0019	-0.022 $\pm$ 0.0033 (-14)

Fig. 6. *Activities of cardiac enzymes in hearts treated with colchicine and in matched controls*

Enzymes activities for colchicine-treated hearts (hatched bars) and matched littermate control hearts (open bars) are displayed as a percentage of control values. Each bar represents the mean $\pm$ s.e.m. for 8 experiments. * $P < 0.05$ and ** $P < 0.001$ compared with controls.

24%, but glycine release was decreased by only 7% in colchicine-treated hearts. Similar differences (relative to phenylalanine release) were present without cycloheximide (results not shown).

Cardiac enzymes

Total activities of cathepsin D, glucosaminidase, acid phosphatase and creatine kinase in control and colchicine-treated hearts relative to control values are shown in Fig. 6. Lysosomal enzyme activities

were diminished in the presence of colchicine, whereas creatine kinase activity was not altered. In separate experiments, the effect of colchicine on the distribution of glucosaminidase activity in the tissue homogenate was measured. In hearts treated with colchicine there was a significant decrease in the non-sedimentable proportion of enzyme activity, from $54 \pm 3\%$ in control hearts to $42 \pm 2\%$ in colchicine-treated hearts ($P < 0.05$). Thus most of the decrease in the total activity of glucosaminidase reflected a decrease in non-sedimentable enzyme activity, with little or no change in sedimentable glucosaminidase.

Discussion

The role of microtubules in endogenous proteolysis is poorly understood. In cultured fibroblasts, basal protein degradation is not altered by agents which interfere with normal microtubule function, including colchicine, vincristine or vinblastine (Amenta *et al.*, 1976, 1977). When fibroblasts are cultured in medium lacking serum and insulin, autophagy increases and the rate of protein degradation is accelerated; colchicine and other anti-microtubule drugs inhibit this accelerated rate of proteolysis (Amenta *et al.*, 1976, 1977). When vinblastine is given *in vivo*, hepatic autophagy and protein degradation are accelerated (Arstila *et al.*, 1974; Marzella & Glaumann, 1980a), but proteolysis in isolated rat hepatocytes in culture is inhibited by vinblastine under conditions in which lysosomal proteolysis is active (Seglen *et al.*, 1980; Marzella & Glaumann, 1980b; Grinde & Seglen, 1981). Thus

the role of microtubules in endogenous proteolysis may depend on the type of tissue studied, its condition, and the extent of autophagy and lysosomal proteolysis.

Cardiac protein degradation is less well understood than protein degradation in other tissues. Relatively few autophagic vacuoles are seen in normal myocytes, and the degradation of some cardiac elements, including myofibrillar proteins, appears to be regulated by non-lysosomal mechanisms (Wildenthal *et al.*, 1980; Ord *et al.*, 1982). Lysosomal proteolysis, however, is important in the degradation of much of the rest of the protein pool (Wildenthal & Crie, 1980*a,b*). The possible role of microtubules in cardiac protein degradation has not been studied previously, and accordingly the present study was undertaken to test the effects of microtubular disruption by colchicine on proteolysis in heart.

These experiments document for the first time that colchicine decreases the rate of degradation of endogenous cardiac protein. In several groups of experiments the rate of proteolysis in hearts treated with colchicine was decreased by 12–18% compared with that in matched control hearts. Although all currently used methods for measuring protein turnover depend on assumptions that make the absolute rates of protein turnover potentially inaccurate (Everett & Zak, 1980), in the present study emphasis was placed on differences in the measured rates of proteolysis between matched hearts rather than on the absolute rates of protein degradation. Furthermore, to minimize the possibility of methodological artifact, two different methods for measuring changes in the rate of protein degradation were used, and the effects of colchicine were quantitatively similar with each method. The failure of lumicolchicine to have any effect on protein degradation indicates that the decrease in cardiac proteolysis caused by colchicine are due to its actions on tubulin, and not to non-specific toxicity.

Several investigators have proposed the existence of two separate pathways for protein degradation: the lysosomal and non-lysosomal pathways. Because degradation of endocytosed proteins, which appears to involve lysosomes and lysosomal enzymes, is inhibited by microtubular disaggregation (Kolset *et al.*, 1979; Ostlund *et al.*, 1979; Brown *et al.*, 1980; Ose *et al.*, 1980; Grinde & Seglen, 1981), because disruption of microtubules inhibits normal lysosomal movement (Freed & Lebowitz, 1970; Parr, 1979; Mori *et al.*, 1981), because colchicine decreases endogenous proteolysis in some cells only when lysosomally mediated protein breakdown is presumed to be active (Amenta *et al.*, 1976, 1977; Marzella & Glaumann, 1980*a*), and because the effect of colchicine and that of lysosomotropic agents such as NH_4Cl are not additive (Seglen *et al.*,

1980; Grinde & Seglen, 1981), it has been suggested that microtubules are important specifically in the lysosomal pathway of proteolysis. Theoretically, microtubules might be involved in the intracellular transport of lysosomal enzymes and/or their potential substrates to sites of autophagic vacuole formation, or the processes of vacuole formation and fusion themselves might require normal microtubular function.

The possibility that inhibition of proteolysis by colchicine is somehow linked to alterations in lysosomal function was supported indirectly in this study by the demonstration of significant changes in the activities and distribution of several cardiac lysosomal enzymes (Fig. 6). Decreases in the activities of cathepsin D, glucosaminidase and acid phosphatase were observed in hearts treated with colchicine. Interpretation of this finding is difficult, however, because changes in the rate of protein degradation do not always correlate well with alterations in the activities of cathepsin D and other lysosomal enzymes in the heart (Wildenthal & Crie, 1980*a,b*). Thus it does not necessarily follow that the observed decrease in cathepsin D activity contributed causally to the decrease in proteolysis observed in colchicine-treated hearts. Nevertheless, it is clear that colchicine causes some alteration in lysosomal properties in the heart. The decrease in lysosomal enzyme activity, at least for glucosaminidase, is accounted for entirely by a decrease in the non-sedimentable fraction of the enzyme. A great majority of the glucosaminidase and cathepsin D present *in situ* normally is located within primary and secondary lysosomes (Decker *et al.*, 1980), and 'non-sedimentable' enzyme, as measured biochemically, is accounted for primarily by artifacts of methodology owing to breakage of larger and more fragile lysosomes during tissue homogenization. Thus the decrease in non-sedimentable glucosaminidase activity suggests a decrease in the number of large, fragile lysosomes, and is consistent with the hypothesis that colchicine decreases autophagic-vacuole formation.

There is some evidence that lysosomal and non-lysosomal pathways may have different selectivities for degradation of different classes of protein. For example, work from our laboratory has shown that agents known to interfere selectively with lysosomal proteolysis have no effect on the rate of degradation of most cardiac myofibrillar proteins (Ord *et al.*, 1982), notably myosin (Wildenthal *et al.*, 1980), indicating that this breakdown is regulated by non-lysosomal mechanisms. Thus, if colchicine's actions on proteolysis were mediated entirely via the lysosomal pathway, no effect on the rate of myosin breakdown would be anticipated. In fact, contrary to our expectations, degradation of myosin was inhibited by colchicine to the same

degree as was degradation of the total protein pool. Therefore it would appear that the role of microtubules in cardiac proteolysis must include involvement in some non-lysosomal pathway, in addition to whatever involvement it may have in lysosomal proteolysis.

In fibroblasts and other cell lines, mitochondria appear to be aligned in close proximity to microtubules (Heggeness *et al.*, 1978), suggesting that mitochondrial protein degradation might be especially susceptible to colchicine's influence. This possibility is reinforced by the electron-microscopic observation that colchicine treatment causes a loss of the usual parallel alignment of series of mitochondria in cultured heart (R. S. Decker, personal communication). Also, when autophagic vacuoles are observed normally in heart, they usually contain mitochondrial elements and rarely, if ever, contain contractile filaments. To test for effects of colchicine on the breakdown of mitochondrial protein, the degradation of haem protein was measured by using radiolabelled δ -aminolaevulinic acid. Haem protein degradation was indeed inhibited to a significantly greater extent by colchicine than was total proteolysis. This method measures the degradation of myoglobin, also a haem-containing protein, as well as that of mitochondrial cytochromes, however; therefore, in separate experiments degradation of protein that sedimented between 500 and 5000g (including most mitochondria) was measured and observed to be significantly decreased by colchicine, whereas degradation of protein in the 5000g supernatant (including myoglobin) was not decreased. Thus colchicine does appear to have a special inhibitory effect on the degradation of mitochondrial proteins which is greater than its effect on total proteolysis.

Finally, also of interest is the finding of disproportionately large decreases in the release of the branched-chain amino acids from colchicine-treated hearts (Table 1). The branched-chain amino acids are energy-producing substrates in heart (Buse *et al.*, 1972), and increased metabolism (transamination and oxidation) presumably accounts for this decreased release. Why colchicine might lead to increased breakdown of these amino acids is not clear. One hypothesis that requires testing is that the metabolism of some substrates, such as glycogen and intracellular triacylglycerols, may be dependent, in part, on microtubular transport, and that disruption of microtubules causes an increased requirement for other substrates.

In summary, colchicine decreases protein degradation in cultured foetal mouse hearts, whereas its inactive analogue lumicolchicine has no effect. This suggests that intact normally functioning microtubules are required for normal cardiac proteolysis. The degradation of mitochondrial haem

proteins is affected more than that of the total protein pool, perhaps because microtubules are required for the formation of autophagic vacuoles in which mitochondria are degraded. Myosin breakdown, which is not normally regulated by lysosomal mechanisms, is also inhibited by colchicine. This suggests that microtubules are involved in non-lysosomal as well as lysosomal proteolysis.

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