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INCREASED RELEASE OF δ-AMINOLEVULINIC ACID FROM PROTEIN DURING INHIBITION OF PROTEIN SYNTHESIS IN HEART: EVIDENCE FOR THE EXTENSIVE REUTILIZATION OF HEME IN CARDIAC PROTEIN METABOLISM

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SUMMARY

Inhibition of protein synthesis with cycloheximide or puromycin caused a *decrease* in total cardiac proteolysis, as measured by the rate of release of radioactive phenylalanine from protein. At the same time, the rate of release of δ-aminolevulinic acid (δALA), which is incorporated specifically into heme-containing proteins, was *increased* significantly. These results suggest that, contrary to previously accepted beliefs, heme is extensively recycled in cardiac protein synthesis; therefore, the release of δALA from cardiac protein should not be used for measurements of the degradation of heme-containing proteins unless reutilization is controlled.

INTRODUCTION

Synthesis of heme-containing proteins involves the incorporation of δ-aminolevulinic acid (δALA) into the heme moiety and its subsequent inclusion in the complete protein. It has often been assumed that when such a protein is degraded, the heme is converted to nonmetabolizable products, and accordingly, the decrease of isotopically-labeled δALA in protein has commonly been used as a measure of the rate of degradation of heme-containing proteins in many tissues, including heart (1-7). In contrast to the general acceptance of this view, however, it has been suggested by some workers (8,9) that there may be extensive reutilization of heme in protein metabolism of skeletal muscle.

To explore this possibility in heart, we used cycloheximide or puromycin to block protein synthesis in fetal mouse hearts in organ culture (10). In

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heart, as in many other tissues, inhibition of protein synthesis secondarily produces an inhibition of protein degradation (11,12). Therefore, it was reasoned that if δALA *is not* reutilized, hearts exposed to cycloheximide or puromycin should display a *decrease* in the rate of release of δALA from TCA-precipitable protein. On the other hand, if δALA from degraded protein *is* normally recycled to a major extent through ongoing protein synthetic pathways the blockade of these pathways by cycloheximide or puromycin should result in less reincorporation of δALA and an *increase* in its rate of release.

MATERIALS AND METHODS

Isolated, intact hearts of 19-20 day fetal mice were maintained in organ culture as described previously (10). 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 (25 μCi/ml, Schwarz/Mann) and [^{14}C]δ-aminolevulinic acid (2.5 μCi/ml, Amersham Corp.). After three one-hour-long washes in non-radioactive medium containing excess cold phenylalanine (3.0 mM) and δ-aminolevulenic acid (3.0 mM), the hearts were divided into three matched groups. The first group was harvested for later assay; the second group was given fresh control "medium 199" containing excess cold phenylalanine and δALA and allowed to remain in culture for 24 hours before being taken for assays; and the third group was given an identical medium supplemented with cycloheximide (10 μM), puromycin (0.5 mM), or insulin (50 μg/ml) for 24 hours and then harvested. Cycloheximide and puromycin in these concentrations had been found in earlier experiments to block protein synthesis by > 90% in this system, whereas insulin had been observed to increase synthesis by ~10% and to reduce degradation by 15-20% (12,13). As described previously (13), each heart was homogenized in 20 mM phosphate buffer, pH 7.3, in a Dounce homogenizer; protein was precipitated with 10% trichloroacetic acid (TCA); and radioactivity in the precipitate was counted on a Packard Tricarb Liquid Scintillation Spec-

trometer (C2425) after treatment with Protosol, toluene, and PPO/POPOP (New England Nuclear). For each litter, the rate of degradation of total protein was calculated for control and experimental hearts as described previously (13), using the difference between [^{3}H] in TCA precipitates of hearts harvested before and after the 24 hour period (i.e., the fractional rate of degradation/ 24 hours = dpm/heart in group 1 minus dpm/heart in group 2 or 3, divided by dpm/heart in group 1). Similar calculations were made for heme-containing proteins from the observed difference in [^{14}C] in the TCA precipitates. 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 (12) as well as in other systems (11,14-16), inhibition of protein synthesis with cycloheximide or puromycin produced a significant decrease in proteolysis. As shown in Table I, the fractional rate of degradation of total protein, as reflected by the rate of loss of radioactive phenylalanine from prelabeled protein, was decreased from 42%/day in control hearts to 32%/day in matched hearts maintained in the presence of cycloheximide (a 24% reduction). For hearts given puromycin, the rate of loss of phenylalanine was reduced by 35%.

In contrast, cycloheximide significantly *increased* the rate of loss of [^{14}C]δALA from cardiac protein by 16% (from 19%/day to 22%/day). Similarly, puromycin caused a 19% *increase* in the release of δALA from the same hearts that simultaneously displayed a *decrease* in total protein degradation. In all instances, with or without cycloheximide or puromycin, the fractional rate of release of δALA was less than that of phenylalanine.

For comparative purposes similar experiments were performed using insulin, a well-characterized agent which is known to produce a decrease in protein degradation without an inhibition of protein synthesis (11,13). As is apparent in Table I, insulin produced a quantitatively similar decrease (15-19%) in the

Table I. Effects of cycloheximide, puromycin, and insulin on the rate of loss of radioactive phenylalanine and δ-aminolevulenic acid from fetal mouse hearts in organ culture.

	Loss of PHE (% per day)	Loss of δALA (% per day)
A. Control	42±1	19±2
B. + Cycloheximide	32±1	22±2
Difference (B-A)	-10±1*	+3±1*
Percent difference ($\frac{B-A}{A}$ X 100)	-24%	+16%
A. Control	43±1	21±3
B. + Puromycin	28±1	24±3
Difference (B-A)	-15±1*	+3±1*
Percent difference ($\frac{B-A}{A}$ X 100)	-35%	+14%
A. Control	43±1	21±1
B. + Insulin	36±1	17±1
Difference (B-A)	-7±1*	-4±1*
Percent difference ($\frac{B-A}{A}$ X 100)	-16%	-19%
A. Control + Cycloheximide	32±1	20±1
B. + Cycloheximide + Insulin	26±2	17±2
Difference (B-A)	-6±2*	-3±1*
Percent difference ($\frac{B-A}{A}$ X 100)	-19%	-15%

Each value represents the mean ± 1 SEM of at least 32 hearts from 8 litters. Concentrations for each agent are given in the text. * = $p < 0.05$, with comparisons made between matched littermates.

rate of loss of phenylalanine and δALA from cultured hearts. This was true both in the presence and absence of cycloheximide.

DISCUSSION

The only known fate of δALA in cardiac metabolism is incorporation into heme and, thereby, into heme-containing proteins (4). The generally accepted mechanism for the degradation of hemoproteins involves proteolysis of the

apoprotein to its constituent amino acids, and conversion of the heme as a unit to bilirubin, which cannot be further metabolized. This nonmetabolizable product of heme would serve to "capture" the radioactive δALA that had originally been incorporated within the heme, preventing its reincorporation into newly synthesized protein.

On the basis of this assumed scenario, several investigators have used the rate of loss of radioactive δALA from cardiac protein as a measure of the rate of degradation of heme-containing proteins of the heart (4-7). The present results suggest strongly that the basis for this practice is erroneous: since blockade of ongoing protein synthesis markedly *increases* the rate of loss of δALA from cardiac protein, at the same time that the overall rate of protein degradation is *decreased*, it seems likely that the normal synthesis of heme-containing proteins involves significant reutilization of δALA. The form of this reutilization seems most likely to be recycling of intact heme units before their conversion to bilirubin, rather than release of free δALA and subsequent reincorporation of it into new heme, but the present data do not allow resolution of this issue. Whatever the form of the reutilization, it is clear that prior assumptions that the loss of δALA from the heart can be used as an index of the degradation of heme-containing cardiac proteins, free from problems introduced by reutilization of label, are incorrect. This conclusion is in agreement with that reached by Booth and Holloszy (9) for skeletal muscle using a different approach.

It follows, then, that under any circumstance in which an experimental intervention might alter reutilization of protein precursors, δALA cannot be used with impunity for measurements of proteolysis. Only if potential changes in reincorporation are prohibited (e.g., by blocking reutilization in both control and experimental hearts with cycloheximide), can δALA achieve its desired usefulness as a specific indicator of the breakdown of heme-containing proteins. In this regard, it is noteworthy that insulin appears to inhibit the degradation of heme-containing proteins to the same degree as it inhibits

total protein breakdown, even when protein synthesis and reincorporation of label are blocked. Finally, it is of interest that the apparent fractional rate of degradation of heme-containing proteins is significantly less than that of the total protein pool.

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REFERENCES

1. Levin, W. and Kuntzman, R. (1969) J. Biol. Chem. 244, 3671-3676
2. Poole, B., Leighton, F. and DeDuve, C. (1969) J. Cell Biol. 41, 536-546
3. Druyan, R., DeBernard, B. and Rabinowitz, M. (1969) J. Biol. Chem. 244, 5874-5878
4. Aschenbrenner, V., Druyan, R., Albin, R. and Rabinowitz, M. (1970) Biochem. J. 119, 157-160
5. Aschenbrenner, V., Zak, R., Cutilletta, A.F. and Rabinowitz, M. (1971) Am. J. Physiol. 221, 1418-1425
6. Albin, R., Dowell, R.T., Zak, R. and Rabinowitz, M. (1973) Biochem. J. 136, 629-637
7. Zak, R., Martin, A.F. and Blough R. (1979) Physiol. Rev. 59, 407-447
8. Terjung, R.L. (1975) Bioch. Bioph. Res. Comm. 66, 173-178
9. Booth, F.W. and Holloszy, J.O. (1977) J. Biol. Chem. 252, 416-419
10. Wildenthal, K. (1971) J. Appl. Physiol. 30, 153-157
11. Rannels, D.E., Kao, R. and Morgan, H.E. (1975) J. Biol. Chem. 250, 1694-1701
12. Wildenthal, K. and Griffin, E.E. (1976) Bioch. Bioph. Acta 444, 519-524
13. Wildenthal, K., Griffin, E.E. and Ingwall, J.S. (1976) Circ. Res. 38, I-138-I-142
14. Woodside, K.H., Ward, W.F. and Mortimore, G.E. (1974) J. Biol. Chem. 249, 5458-5463
15. Fulks, R.M., Li, J.B. and Goldberg, A.L. (1975) J. Biol. Chem. 250, 290-298
16. Amenta, J.S., Sargus, M.J. and Baccino, F.M. (1978) J. Cell Physiol. 97, 267-283