Angiotensin-Converting Enzyme in Cultured Endothelial Cells
Synthesis, Degradation, and Transfer to Culture Medium

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Cultured endothelial cells from swine aorta possess the ecto-enzyme angiotensin-converting enzyme (E.C.3.4.15.1) in cell-associated form and also release it in soluble form into the culture medium. Using antibody to purified converting enzyme from swine kidney and incorporation of $^3$H-leucine, we examined the synthesis, degradation, and release of enzyme into the medium. $^3$H-leucine is incorporated into cellular converting enzyme, and later appears in the enzyme in the culture medium. The amount of cell-associated enzyme activity remains constant, and the amount of activity in the medium increases linearly during this period. These data show unequivocally that the appearance of enzyme activity in the cell culture medium is accompanied by synthesis of new enzyme protein.

In pulse-chase experiments, the radioactivity disappeared from the cellular enzyme in two kinetic components with apparent half-lives of 1–2 hours and 20 hours, respectively. The appearance of the radiolabel in the medium enzyme corresponds very closely in rate and amount to the slow disappearance from the cells. There was no apparent uptake of labeled medium enzyme by the cells. The data suggest that the pool of active enzyme on the cell surface is the immediate precursor of the medium enzyme. The effects of culture conditions on the turnover of angiotensin-converting enzyme were also examined. The incorporation of $^3$H-leucine into both cell and medium enzyme was greater when cells were maintained in medium containing serum than when they were maintained in serum-free medium. The rate of degradation of the cellular enzyme was similar under the two culture conditions.


Angiotensin-converting enzyme (E.C.3.4.15.1) is a dipeptidyl carboxypeptidase. It catalyzes the conversion of angiotensin I to angiotensin II, which is the most potent vasoconstrictor known. This enzyme also catalyzes the sequential release of Phe-Arg and Ser-Pro from the carboxyl terminus of bradykinin, thereby inactivating this potent vasodilator molecule. Using immunocytochemical techniques, investigators have shown that the enzyme is located on the luminal surface of the plasma membrane of vascular endothelium. The enzyme is also found in soluble form in plasma.

We have shown that cultured endothelial cells from swine aorta not only possess angiotensin-converting enzyme in cell-associated form, but also release it in soluble form into the culture medium. Cell-associated and medium enzyme exhibit similar catalytic and immunological properties. Further, the carbohydrate composition of the cell-associated and medium enzyme differ from each other, as judged by lectin affinity studies. The cell-associated form behaves on lectin affinity columns like purified enzyme from kidney, and the soluble, released form, like enzyme from plasma.

We present here a study of the incorporation of $^3$H-leucine into angiotensin-converting enzyme, and the rates of degradation and release of enzyme in cultured arterial endothelial cells from swine. The data are consistent with the hypothesis that the medium enzyme is shed from the cell surface. We find that...
the radiolabel disappears from the cellular enzyme in two kinetic components, one of which corresponds to the release of enzyme to the medium. Finally, we find that the incorporation of \(^3\)H-leucine into new enzyme is higher when cells are maintained in medium containing serum than when they are maintained in serum-free medium.

**Methods**

**Materials**

Hippuryl-histidyl-leucine (Hip-His-Leu) was purchased from Sigma Chemical Company, St. Louis, Missouri. Collagenase was CLS II from Worthington Biochemical Corporation, Freehold, New York. Media and other reagents for cell culture were from Gibco, Grand Island, New York. Goat antirabbit IgG immunobeads were obtained from Bio-Rad, Richmond, California. \(^3\)H-leucine (47 Ci/mmol) was from New England Nuclear, Boston, Massachusetts. The purified angiotensin-converting enzyme from swine kidney and the rabbit antienzyme IgG used are the preparations described by Ching et al.\(^8\)

**Enzyme Assay**

Angiotensin-converting enzyme activity in the cell extract and in the medium concentrate was measured by the fluorimetric method of Friedland and Silverstein.\(^9\) The assay mixture contained 5 mM Hip-His-Leu, 0.1 M sodium borate, pH 8.3, 0.3 M NaCl, and enzyme in a total volume of 0.25 ml. A unit of enzyme activity is defined as the amount catalyzing generation of 1.0 \(\mu\)mol His-Leu from Hip-His-Leu per minute at 37°C.

**Cultured Endothelial Cells**

Endothelial cells were isolated and cultured from swine aorta according to the methods described previously\(^10\) with the following modifications: handling of the aortas was less gentle than previously described to increase the initial yield of suspended cells. The vessel was pinched between the fingers, and vigorous mechanical stress was used in pipetting the collagenase solution against the inside vessel wall to dislodge cells. The suspended cells from the collag enase digest were planted in growth medium containing nystatin and 20% fetal bovine serum in T-25 flasks. At the time of the first passage when the cells were transferred to a T-75 flask, and in all subsequent passages, endothelial cells were planted in growth medium containing 20% swine plasma-derived serum.\(^10\) Subcultures were made at a split ratio of 1:2. Cells were used at passage 4 to 10.

**Incorporation of \(^3\)H-Leucine**

We prepared cell culture media that contained all the usual components except serum and leucine. Cell monolayers in T-75 flasks were washed twice with Hank’s balanced salt solution, then incubated in medium containing no leucine, 10 ml/flask, for 15 minutes to deplete the cellular pool of leucine. This medium was then replaced with 10 ml medium containing \(^3\)H-leucine in the concentrations given for the various experiments. Incorporation was terminated by replacing the labeling medium with normal cell culture medium that contained unlabeled leucine, 0.8 mM, (with or without serum according to the conditions of the individual experiments) or by the addition of unlabeled leucine to the labeling medium at 5 mM.

**Isolation of Radiolabeled Angiotensin-Converting Enzyme from Cultured Cells and Culture Medium**

Culture medium was removed from cells, centrifuged in a desk-top centrifuge to remove any detached cells, and concentrated to one-tenth of its original volume by ultrafiltration with an Amicon PM-30 membrane. Cells were collected by trypsinization as described before.\(^7\) The cell pellet from each flask was extracted with 0.5 ml of buffer containing 0.5% NP-40, 10 mM sodium phosphate, pH 7.4, 0.15 M NaCl and 1 mM phenylmethylsulfonylfluoride. The extract was centrifuged at 23,000 \(\times\) g for 30 minutes and the supernatant solution was used for isolation of the enzyme. The reaction mixture for enzyme isolation contained rabbit anti-enzyme IgG (20 \(\mu\)g), labeled cell extract, or medium concentrate (150 \(\mu\)l), BSA (1 mg), and purified converting enzyme from swine kidney (5 \(\times\) \(10^4\) units) in a total volume of 200 \(\mu\)l, adjusted with the same buffer used for the cell extraction. After incubation at 4°C overnight, goat anti-rabbit IgG immunobeads (0.5 mg) were added to adsorb the soluble antigen-antibody complexes. The immunobeads with adsorbed antigen-antibody complexes were washed three times with 1 ml of buffer containing 1% deoxycholate, 1% triton X-100, 0.5% BSA, 10 mM sodium phosphate, pH 7.4, and 0.15 M NaCl. Labeled enzyme adsorbed to the immunobeads was solubilized in 100 \(\mu\)l buffer containing 2% SDS and 1 mM sodium phosphate, pH 7.4. After centrifugation at 5,000 \(\times\) g for 5 min, the supernatant was either applied to SDS-polyacrylamide gel electrophoresis or added to 5 ml Biofluor (New England Nuclear) for a measurement of radioactivity.

**Binding Studies of Anti-Enzyme IgG to Labeled Enzyme**

In these studies, the procedures and reaction mixtures used were similar to those used for the isolation of labeled enzyme from its source as described above, except that various amounts of anti-enzyme IgG were used and there was no purified enzyme in the reaction mixtures. The antigen-antibody complexes were adsorbed to the goat anti-rabbit IgG immunobeads. After centrifugation at 5,000 \(\times\) g for 5 min, the residual enzyme activity in the supernatant was measured. The radioactivity in the labeled enzyme adsorbed to the immunobeads was measured as described above.
SDS-Polyacrylamide Gel Electrophoresis

Electrophoresis was carried out in the presence of 0.1% SDS on a slab gel containing a 6% to 15% acrylamide gradient as described by Baum, et al. To determine the distribution of precipitated radioactivity on SDS-polyacrylamide gel electrophoresis, the gel was stained with Coomassie blue and then sliced into 1 mm slices. Each slice was solubilized with 90% protocol (200 µl per slice) in a scintillation vial. After incubation at 37°C overnight, 5 ml of Biofluor and 100 µl of 3N HCl were added and radioactivity was counted.

Measurement of Radioactivity In Total Protein

To determine the radioactivity in the total newly synthesized proteins in the cell extract, 5 µl of the cell extract was put on a square of Whatman 3 MM filter paper (1 x 1 cm) and air dried. The protein in the square of filter paper was then precipitated with 10% trichloroacetic acid in a scintillation vial (2 ml). The paper was washed three times with 5% trichloroacetic acid containing 0.2% leucine, 2 ml for each wash, and then 5 ml of Biofluor was added and radioactivity was measured with a Beckman LS 100C scintillation counter.

Protein Measurements

Protein concentrations in the cell extract and in the medium concentrate were measured by the method of Bradford.

Calculations

Rate constants for disappearance of ³H from labeled enzyme were estimated as below. It was assumed that after termination of labeling, radioactivity in the enzyme would obey the relationship:

\[ \frac{d³H}{dt} = -k_D³H + k_R \]

where \( k_D \) is a first-order rate constant for degradation (or transfer) and \( k_R \) is the rate of reincorporation of label. Integrating (1) gives:

\[ ³H = Ce^{-kt} + \frac{k_R}{k_D} \]

The rate constants are computed using a program for fitting experimental data to a single exponential function kindly provided by David Hayes.

Results

Effect of Leucine Starvation on ³H-Leucine Incorporation into Newly Synthesized Proteins

Experiments were done to establish conditions for labeling which gave maximum incorporation of ³H-leucine into total acid precipitable material. Cultured endothelial cells in T-25 flasks were washed twice with Hank's balanced salt solution, then incubated for different times in leucine-free medium to deplete the cellular pool of leucine. The medium was then replaced with labeling medium, which contained ³H-leucine (0.3 µM, 47 Ci/mmol), and incubation was continued for times up to 2 hours. As shown in Figure 1, the incorporation of ³H into trichloroacetic acid precipitable material was linear with the time of incorporation for at least 1 hour and comparable in rate, when the cells were preincubated with leucine-free medium for 15-30 minutes. In subsequent experiments for short-term labeling, cells were preincubated in leucine-free medium for 15 minutes and then incubated with ³H-leucine at concentrations equal to or greater than 0.3 µM, as indicated in the individual experiments.

Figure 1. The effects of leucine starvation on ³H-leucine incorporation into newly synthesized proteins. Cultured endothelial cells in T-25 flasks were incubated for different times in leucine-free medium to deplete the cellular pool of leucine. (▪ = 0 minutes; □ = 15 minutes; ○ = 30 minutes; ● = 60 minutes). At the times indicated, cells were collected by trypsinization and radioactivity in total trichloroacetic acid precipitable materials was determined.
protein (data not shown). The enzyme activity in cultured endothelial cells and the culture medium bound similarly to this IgG. Radiolabeled enzyme was isolated from labeled cell extract or medium concentrate by the double immunoprecipitate method described in the Methods section. Less than 10% of the applied enzyme activity was found in the unbound form in the supernatant. Precipitated immune complexes were then subjected to SDS-polyacrylamide gel electrophoresis together with additional purified enzyme. The gel was stained with Coomassie blue and sliced, and the radioactivity in each slice was measured. The distribution of radioactivity on the gel is shown in Figure 2. Over 85% of the radioactivity in the anti-enzyme precipitate coincided with the purified enzyme on the gel. The remainder was in a single lower molecular weight band which was also the location of most of the radioactivity in the control precipitate.

In binding studies of antienzyme IgG to the labeled enzyme (Figure 3), the ratio of the enzyme activity to the radioactivity precipitated by the antienzyme IgG was constant over an eightfold range of the amount of IgG used.

**Pulse-Chase Experiments**

Cultured cells in T-75 flasks were labeled with \(^{3}H\)-leucine (0.45 \(\mu\)M, 47 Ci/mmol) for 60 minutes; and the incorporation of \(^{3}H\)-leucine into the newly synthesized enzyme was terminated by adding the excess unlabeled leucine (final concentration, 5 mM). The medium and the cells were collected separately at various times after the termination of the label incorporation, and the amounts of enzyme activity and radioactivity in the angiotensin-converting enzyme
were determined. The total radioactivity in the cell-
associated enzyme reached a peak at 3 hours, while
for the medium enzyme the radioactivity did not
reach its maximum until at least 13 hours after the
termination of labeling (Figure 4 A). Approximately
20% of the \(^{3}\)H in the cell-associated enzyme ap-
peared eventually in the medium enzyme. The re-
mainder disappeared with a half-life of 1 to 2 hours
(Figure 4 A). During the chase period, there was a
linear accumulation of enzyme activity in the medi-
um, while the enzyme activity associated with the
cells did not change significantly (Figure 4 B). The
specific radioactivity of the cell-associated enzyme
also peaked at 3 hours. The specific radioactivity
of the medium enzyme was greatest between 8 and 13
hours (Figure 4 C).

To obtain better measurements of the slow com-
ponent of turn-over and to gain further insight into the
mechanisms of transfer of newly synthesized en-
zyme to the cell surface and to the medium, we con-
ducted long-term labeling experiments. We meas-
ured the incorporation of \(^{3}\)H-leucine into the newly
synthesized enzyme in the cells and medium during
a labeling period of 24 hours using cells in T-25
flasks that were incubated with \(^{3}\)H-leucine (3.7 \(\mu\)M,
12 Ci/mmol). After a short lag, the radiolabel in the
cell-associated enzyme increased continuously dur-
ing the labeling period (Figure 5). Again there was a
significant delay before the appearance of measur-
able amounts of radiolabeled medium enzyme.

Cells in T-75 flasks were labeled with \(^{3}\)H-leucine
(3.7 \(\mu\)M, 12 Ci/mmol) for 24 hours and the incorpora-
tion of \(^{3}\)H-leucine into newly synthesized enzyme
was terminated by adding unlabeled leucine (final
concentration: 5 mM). At the same time, we pre-
pared another set of flasks of cells under the same
culture conditions except that we replaced the \(^{3}\)H-
leucine by the same concentration of unlabeled leu-
cine. When the excess unlabeled leucine was added
in both sets of flasks, the radiolabeled medium (con-
taining by this time a substantial amount of radiola-
beled converting enzyme) was exchanged with the
medium that had been sitting over the unlabeled
cells; incubation was continued for another 60 hours.
The label in the rapidly degraded component of the
cell-associated enzyme had disappeared by 20
hours after the exchange (Figure 6 A). The remain-
ing radiolabeled cellular enzyme disappeared very
slowly, accompanied by an increase of labeled en-

Figure 5. Long-term labeling of angiotensin-converting
enzyme in cultured endothelial cells. • = radioactivity in
cell-associated enzyme; o = radioactivity in medium
enzyme.

Figure 6. Long-term labeling and exchange experiments
for angiotensin-converting enzyme. Cells and medium
were collected at the times indicated, and the radioactivity
in angiotensin-converting enzyme and the amount of en-
zyme activity were determined. • = cells; o = me-
dium. A. Radioactivity in enzyme in flasks initially con-
taining labeled cells and unlabeled medium. B. Enzyme
activity. The amount of activity in the medium is the amount
in excess of that found at the time of termination of label-
ing. C. The radioactivity in enzyme in the flasks initially
containing unlabeled cells and labeled medium.
zyme in the culture medium. The sum of the radioactivity in the cell-associated and the medium enzyme was constant, within experimental error, after the 20th hour of the chase (Figure 6 A). This component of labeled cellular enzyme has an apparent half-life of 26 hours during the exchange period. There was a linear release of enzyme activity into the culture medium with a release of $5.7 \times 10^{-5}$ units of enzyme per hour per flask. The enzyme activity associated with cells rose very slightly during this period (Figure 6 B). When unlabeled cells were incubated with labeled medium, there was neither uptake nor degradation of the medium enzyme by the cells during the experimental period (Figure 6 C).

### Effects of Culture Conditions on Enzyme Turnover

Our previous work on the release of enzyme activity by cultured cells into medium was done using confluent cells in serum-free medium, since serum contains angiotensin-converting enzyme and some inhibitors of its activity. The use of radiolabel and antibody allows us to examine enzyme synthesis in cells maintained in the presence of serum and, therefore, in growing cells.

It is clearly shown in Figure 7 that the rate of $^3$H-leucine incorporation into cell-associated enzyme is much higher for subconfluent cells growing in the presence of serum than for confluent cells maintained in serum-free medium. For the subconfluent cells the peak of radioactivity appeared one hour earlier than that of the confluent cells. The half-lives for degradation of the enzyme during the rapid turnover phase were estimated by measuring the $^3$H in the cellular enzyme at 2, 3, 4, 7, and 8 hours for subconfluent cells, and 3, 4, 5, 7, and 8 hours for confluent cells (Table 1). The medium was sampled at 10 hours for subconfluent cells grown in serum. There were 1800 cpm in the total medium enzyme per flask when the cells had been maintained in serum, and 600-800 cpm for cells maintained in serum-free medium.

To measure the rates of total protein degradation and of the disappearance of $^3$H-leucine from angiotensin-converting enzyme under various culture conditions, we labeled cultured cells with $^3$H-leucine (3.7 nM, 12 Ci/mmol) for 24 hours. Incorporation was terminated by replacement of the labeling medium with the appropriate test medium containing 0.8 mM unlabeled leucine. The radioactivity in total cell protein and cell-associated enzyme was measured at 20, 25, 30, and 40 hours after termination of labeling, and the half-time for the disappearance of the label from each of these pools was estimated. The results are shown in Table 1. For both growing and confluent cells, the radiolabel disappears from the converting enzyme in two kinetic components, a phase of rapid degradation of enzyme, and a slower phase of transfer of enzyme to the culture medium.

### Discussion

We have reported that cultured endothelial cells from swine aorta possess the ectoenzyme angiotensin-converting enzyme in cell-associated form, and also release it in soluble form into the culture medium. Here we present evidence that $^3$H-leucine is
incorporated into the cellular converting enzyme, and later appears in the enzyme in the culture medium. The amount of cell-associated enzyme activity remains constant, and the amount of activity in the medium increases linearly during this period. These data show unequivocally that the appearance of enzyme activity in the cell culture medium is accompanied by a synthesis of new enzyme protein.

Using the turnover data together with previously reported information about converting enzyme in cultured endothelial cells, we can address two issues. We can consider the mechanism by which cellular enzyme is released into the medium, and since converting enzyme is a plasma membrane protein, we can also draw some inferences about the turnover of plasma membrane proteins. We propose three different models by which the newly synthesized enzyme can be transferred to the cell surfaces and released into the culture medium: 1) Both forms may be secreted first into the culture medium, and then the appropriate one can be reattached to the outer surface of the cells. 2) A fraction of the membrane-bound enzyme may undergo further processing and be released into the culture medium. 3) The two forms of enzyme may be independently synthesized and transferred to their final locations. There is a precedent for each of these models. Lysosomal enzymes can be secreted from fibroblasts into the extracellular space and then taken back to the cells.\(^1\,15\,17\) Miller et al.\(^18\,19\) clearly showed that the soluble glycoproteins (named collectively epiglycanin) released by TA3 Ha tumor cells are shed from a surface-bound pool. Finally, Rogers et al.\(^20\) and Alt et al.\(^21\) have shown that the membrane-bound and secreted form of the \(\mu\) chain of IgM are coded for by different mRNA's and hence must be independently synthesized.

The exchange experiment (Figure 6 C), the time course of appearance of radiolabel in cell and medium enzyme, and the relative specific activities of the two pools after pulse chase (Figure 4) unequivocally rule out the possibility that the principal relationship between the cell and the medium enzyme is a reattachment of a subset of molecules to the cell surface from the pool of molecules identified as medium enzyme. Hasilik et al.\(^22\) have shown that human umbilical vein endothelial cells reinternalize lysosomal enzymes very poorly compared to smooth muscle cells and fibroblasts, although they secrete forms of the enzymes that are readily taken up by fibroblasts.

The principal site of the active converting enzyme is on the cell surface\(^23\,24\) (also verified in this laboratory). The time course of the appearance of the label, and the relative specific radioactivities of the cell and the medium enzyme are consistent with the hypothesis that newly synthesized enzyme is first transferred to the cell surface, with the enzyme appearing in the medium as a consequence of shedding from the surface. In this model, preexisting surface enzyme provides a pool that dilutes the radioactivity of the newly synthesized enzyme before shedding.

The rate of release of enzyme activity into the medium can be described as follows:

\[
\frac{\Delta E_{\text{medium}}}{\Delta t} = E_{\text{precursor}} \cdot k_f
\]

where \(\Delta E_{\text{medium}}/\Delta t\) is the rate of appearance of enzyme activity in the medium; \(E_{\text{precursor}}\) is the pool of enzyme molecules available for release, and \(k_f\) is the rate constant for transfer from cells to medium. If we assume that all the cell-associated activity is available for release, then from Figure 6 B, \(E_{\text{medium}}/t = 5.7 \times 10^{-5}\) units. hr\(^{-1}\) and \(k_f\) is therefore 0.033 hr\(^{-1}\). This is in good agreement with the first order rate constant found for the transfer of \(^3\)H-labeled enzyme from cells to medium (\(k = 0.026\) hr\(^{-1}\), Table 1).

In experiments using \(^3\)H-leucine to determine the rate of turnover of cellular proteins, reutilization of radiolabeled amino acid can cause the apparent fractional rate of disappearance of radiolabel to be significantly different from the true fractional catabolic rate. The analysis used to compute rate constants for the two phases of the disappearance of \(^3\)H from the cellular enzyme pool takes account of reutilization. However, it assumes that the rate of reutilization of isotope is constant over the time period of observation, which may not be the case. However, since the slow phase of the disappearance of the label from the cells is well accounted for by the transfer of labeled material to the medium and the transfer of radiolabel to the medium enzyme during the phase of rapid degradation of labeled cellular enzyme is very slow, it must be the case that the two kinetic phases represent qualitatively different processes. The second phase cannot be simply an artifact of reutilization.

The time lag after the chase and before the maximum incorporation of \(^3\)H-leucine into the cellular enzyme is very similar to the time course observed by others for the transit of newly synthesized plasma membrane proteins to the cell surface.\(^15\,25\,28\) However, the immunoprecipitation reaction is carried out on a detergent extract of whole cells, so that the internal pools should also be detected, if they are immunoreactive. Therefore, we conclude that the antiserum used responds to forms of the enzyme that have undergone significant processing.

The time courses observed for the initial incorporation of radiolabel into cellular enzyme in the pulse chase experiments, and for its eventual release into the medium, are similar to those observed by Miller et al.\(^18\,19\) for the labeling and shedding of the surface glycoproteins (called collectively, epiglycanin) by TA3 Ha tumor cells. Newly synthesized epiglycanin appeared in cell membrane with a half-life of 0.75 hours. There was a slow release of epiglycanin into the medium (\(t_{1/2} = 70\) hours). Based on surface labeling studies, and also on experiments with cells whose surface had been completely stripped of epiglycanin using trypsin, these authors concluded that
the slow release was shedding from the cell surface. Unlike converting enzyme, newly labeled epiglycancin also appeared in medium in an early, rapid component of release which was attributed to secretion.

Recently, it has been reported that angiotensin-converting enzyme activity associated with cultured bovine endothelial cells increases substantially after the cells reach confluence, as compared to growing cells. This observation suggests that the growth rate of cells can affect the net amount of enzyme activity associated with cells, either through effects on the rate of synthesis and degradation of enzyme, or by regulation of its activity. It is clear from the data in Figure 7 and Table 1 that the populations of dividing cells from swine aorta can carry out rapid synthesis of converting enzyme and that degradation is not accelerated during growth, although release may be. It is possible, indeed likely, that the quantitative behaviors of the cultured cells from the two species (bovine and swine) are not the same. The responses of various membrane marker enzymes to culture conditions is quite different in bovine and swine arterial smooth muscle cells.

In summary, we conclude that cultured arterial endothelial cells make new angiotensin-converting enzyme and release the newly synthesized enzyme into the culture medium both in the presence and absence of serum in the medium. Our results also suggest that the membrane-bound enzyme on the cell surface undergoes further processing and is released into the culture medium.

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