Effect of Androgens on Turnover of Ornithine Decarboxylase in Mouse Kidney

STUDIES USING LABELING OF THE ENZYME BY REACTION WITH [14C]-α-DIFLUOROMETHYLMORNILNITINE∗

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Treatment of male mice with excess androgens increased the activity of renal ornithine decarboxylase 60-fold in the BALB/c strain and 4-fold in the CD-1 strain. Part of the increase in the activity of ornithine decarboxylase was due to a decreased rate of degradation of the enzyme since activity declined more slowly (t₁/₂ 80 min) in androgen-treated BALB/c mice than in controls (t₁/₂ 20 min) when protein synthesis was inhibited by cycloheximide. When ornithine decarboxylase protein was labeled in vivo by injection of [5-14C]α-difluoromethylornithine, the rate of disappearance of the labeled protein was exactly the same as the rate of loss of ornithine decarboxylase activity when protein synthesis was inhibited by cycloheximide, confirming that ornithine decarboxylase protein does turn over rapidly in vivo. The half-life of another rapidly turning over enzyme important in polyamine metabolism, S-adenosylmethionine decarboxylase, was also increased in the mouse kidney by androgen treatment. These results indicate that steroid hormones can affect the level of certain proteins by changing the rate of degradation and that the labeling of ornithine decarboxylase by reaction with radioactive α-difluoromethylornithine in vivo provides a useful method for studying the degradation of this protein.

1-Ornithine decarboxylase is the first enzyme in the mammalian polyamine biosynthetic pathway and is known to be responsive to a wide variety of stimuli which increase activity manifold within a short period of time (1-6). One of the most interesting properties of mammalian ornithine decarboxylase is its apparently very rapid rate of turnover as indicated by the precipitous fall in activity on exposure to inhibitors of protein synthesis (7-15). These results have been widely accepted as indicating that the enzyme has a high rate of synthesis and degradation and this would be consistent with the rapid fluctuation of enzyme activities in response to stimuli. In some cases, the fall in activity has been found to be accompanied by a loss of protein interacting with antibodies to ornithine decarboxylase (10, 16, 17), but the specificity of these antisera was not firmly established (6, 18, 19). Changes in ornithine decarboxylase activity and apparent half-life have been observed in response to amino acids and other agents (11, 12, 20, 21) and a macromolecular inhibitor of the enzyme accumulates in cells under certain conditions involving exposure to exogenous di- and polyamines (18, 19, 21-23). It is possible, therefore, that inhibitors of protein synthesis might reduce ornithine decarboxylase activity by ways other than prevention of the production of new enzyme. Post-translational modifications of ornithine decarboxylase leading to loss of activity have been reported including transglutamination (24), phosphorylation (25), and unknown modification (26, 27). Despite some interesting pioneering experiments (15, 28, 29), there has been little progress in understanding the factors responsible for the degradation of ornithine decarboxylase and in developing in vitro systems carrying out this reaction (9). Changes in the activity of the degradation system could be as important in regulating ornithine decarboxylase as changes in the rate of synthesis (20) and evidence that this may be the case has been obtained in cell culture (8, 9, 11, 20, 21), in the isolated perfused liver (12), and in hepatotoxin-treated rats (14, 31).

Recently we have reported a method for the specific labeling of ornithine decarboxylase (32, 33). α-Difluoromethylornithine is an enzyme-activated irreversible inactivator of the enzyme (34). When incubated with [14C]-α-difluoromethylornithine, the enzyme protein becomes labeled as activity is lost (32). In the present work, we have used this binding to study the half-life of ornithine decarboxylase in the mouse kidney and the effect of androgens on this process. The mouse kidney is a particularly suitable system for such studies because ornithine decarboxylase is known to be androgen dependent, declining after castration and increasing to levels higher than those of any other mammalian tissue in response to treatment with excess testosterone (35-38).

EXPERIMENTAL PROCEDURES

Materials—DL-[5-14C]-Difluoromethylornithine (60 mCi/mmol) was synthesized by Amersham/Searle, Arlington Heights, IL. Unlabeled α-difluoromethylornithine was a generous gift from Merrell-Dow, Cincinnati, OH. [3-14C]-Ornithine (57 mCi/mmol) and S-adenosyl-[carboxy-14C]-methionine (55 mCi/mmol) were purchased from New England Nuclear, Boston, MA. Other biochemicals were obtained from Sigma Chemical Co.

Animals—Male C57/C1D-1 mice were obtained from Charles River Breeding Laboratories, Wilmington, MA. Male BALB/c mice were obtained from Jackson Laboratories, Bar Harbor, ME. Mice were fed standard laboratory chow and water ad libitum and kept under controlled 12-h light and 12-h dark lighting conditions. Testosterone propionate was given at a dose of 100 mg/kg by subcutaneous injection of a solution of 4 mg/ml in sesame oil 3 days before sacrifice. Cycloheximide was administered at a dose of 10 mg/kg by intraperi-
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tone injection of a solution of 1 mg/ml in 0.9% saline. This dose of cycloheximide inhibited protein synthesis more than 98%. Control animals received the vehicle alone.

Preparation of Tissue Extracts—Mice were killed by cervical dislocation and the kidneys homogenized at 0-4 °C in 2-3 volumes of Buffer A (Tris-HCl, pH 7.5, 2.5 mM dithiothreitol, and 0.1 mM EDTA) and centrifuged at 100,000 × g for 30 min at 2 °C. In experiments where the ability to bind [14C]α-difluoromethylornithine was measured, the extracts were then dialyzed overnight at 4 °C against 100 volumes of Buffer A.

Determination of Ornithine Decarboxylase Activity—Ornithine decarboxylase activity was assayed by following the release of 14CO2 from L-[14C]ornithine (32). The assays contained 0.1-0.2 mg of protein, 0.4 mM L-[14C]ornithine (0.9 Ci/mmol), 2.5 mM dithiothreitol, 40 μM pyridoxal phosphate, and 50 mM Tris-HCl, pH 7.5, and were incubated for 15 min at 37 °C. Results were expressed as units of activity/mg of protein added where 1 unit was equivalent to the release of 1 nmol of 14CO2/30-min incubation at 37 °C. Protein was determined by the method of Lowry et al. (39). All assays were conducted under conditions where the activity measured was proportional to the protein added and to the time of incubation.

Binding of [14C]α-Difluoromethylornithine to Ornithine Decarboxylase in Vitro—The tissue extracts were incubated in the presence of 40 μM pyridoxal phosphate, 2.5 mM dithiothreitol, 50 mM Tris-HCl, pH 7.5, and 5 μM [14C]α-difluoromethylornithine for 60 min at 37 °C. (This period was sufficient to inactivate more than 90% of the total enzyme activity present as determined by subsequent assay of diluted aliquots in the standard assay medium described above.) The incorporation of radioactivity into protein was then determined as previously described (32). Incorporation was not increased by further incubation or by increasing the concentration of α-difluoromethylornithine or of pyridoxal phosphate. Results were expressed as picomoles of α-difluoromethylornithine incorporated per mg of protein added in the extracts.

Labeling of Ornithine Decarboxylase with [14C]α-Difluoromethylornithine in Vivo—Mice were injected with 1 mg/kg doses of [5-14C]α-difluoromethylornithine (60 mCi/mmol) by intraperitoneal injection of a solution in 0.9% NaCl. Thirty minutes later, a dose of 200 μCi of [14C]α-difluoromethylornithine was given via the same route to prevent further binding. The animals were killed at various times later (see Figs. 3 and 4) and tissue extracts prepared as described above. The extracts were dialyzed against 500 volumes (four changes) of Buffer A for 3 days to remove unbound α-difluoromethylornithine or low molecular weight degradation products and aliquots used for determination of protein and of radioactivity.

Polyacrylamide gel electrophoresis under denaturing conditions was carried out using 0.15-ml aliquots of the dialyzed supernatant fractions or from the in vitro labeled ornithine decarboxylase. The samples were heated to 100 °C for 2 min in 2.5% sodium dodecyl sulfate, 5% 2-mercaptoethanol, 20% glycerol, 5 mM dithiothreitol, and 0.001% bromphenol blue. They were then subjected to electrophoresis in 7.5% acrylamide gels (40) which were calibrated using standard proteins of known molecular weight (phosphorylase, 94,000, bovine serum albumin, 67,000; ovalbumin, 43,000; and carbonic anhydrase, 30,000). Gels were divided into 2-mm slices and the radioactivity in each slice determined after solubilization in 1.0 ml of Protosol (New England Nuclear, Boston, MA).

Determination of S-Adenosylmethionine Decarboxylase Activity—This was determined by following the release of 14CO2 from S-adenosyl[14C]methionine (41). The assays contained 1-2 mg of protein, 0.1 mM S-adenosyl[14C]methionine (5 Ci/mmol), 2.5 mM dithiothreitol, 0.75 mM putrescine, and 50 mM Na phosphate buffer, pH 7.5, and were incubated for 30 min at 37 °C. Results were expressed as units/mg of protein where 1 unit represents the release of 1 pmol of CO2/30 min.

RESULTS

Treatment of mice with excess androgens produced a substantial increase in renal ornithine decarboxylase activity (Table I). Experiments were carried out with two strains of mice which had similar activities when treated with androgens (Table I). However, untreated male BALB/c mice had much lower activities than untreated male CD-1 mice so that the activity was stimulated only 4-fold in the latter but increased 60-fold in the BALB/c strain. For this reason, most of the experiments were carried out with the BALB/c mice except where a higher control activity was essential. With both strains there were substantial variations in the control activity from one animal to another which led to a large standard deviation in the activity measurements.

The binding of [14C]α-difluoromethylornithine to protein and the loss of enzyme activity when tissue extracts were incubated with the drug were also investigated with the mouse kidney extracts. Complete inactivation of the enzyme resulted in the incorporation of 13.7 ± 1.1 fmol of α-difluoromethylornithine/unit of enzyme activity lost irrespective of whether the extracts were from control or androgen-treated mice. This result is consistent with the hypothesis that the changes in activity are brought about by changes in the amount of active enzyme protein rather than by an altered catalytic activity of the same amount of enzyme.

When protein synthesis was inhibited by administration of cycloheximide, the activity of ornithine decarboxylase declined very rapidly in control mice with a t1/2 of 18 min (Fig. 1A). The t1/2 in the mice treated with excess androgens increased to about 80 min. Fig. 1B shows similar results for the capacity of the extracts to bind [14C]α-difluoromethylornithine and it can be seen that the decline in this capacity exactly matched the decline in activity with a half-life of 18 min in controls which increased 4-fold after androgen treatment. This finding suggests that the loss in enzyme activity in response to cycloheximide is due to the progressive conversion of the enzyme into a form which is totally inactive under the assay conditions and is, therefore, unable to bind [14C]α-difluoro-

TABLE I

Effect of androgen treatment on ornithine decarboxylase activity

Results shown are mean ± S. D. for estimations on extracts from six or seven separate animals for each point.

<table>
<thead>
<tr>
<th>Source of activity</th>
<th>Ornithine decarboxylase activity</th>
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<tbody>
<tr>
<td></td>
<td>units/mg</td>
</tr>
<tr>
<td>Control BALB/c</td>
<td>4.8 ± 1.9</td>
</tr>
<tr>
<td>Androgen-treated BALB/c</td>
<td>273 ± 44</td>
</tr>
<tr>
<td>Control Crl:CD-1</td>
<td>92 ± 31</td>
</tr>
<tr>
<td>Androgen-treated Crl:CD-1</td>
<td>210 ± 58</td>
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![Fig. 1. Loss of ornithine decarboxylase activity and protein after treatment with cycloheximide. Control (A) and androgen-treated (B) BALB/c mice were treated with 10 mg/kg doses of cycloheximide and the ornithine decarboxylase activity (A) and ability of extracts to bind [5-14C]α-difluoromethylornithine (B) measured as indicated. Results are expressed as per cent of the value at zero time. The zero time ornithine decarboxylase activity was equal to 28 units/mg for androgen-treated animals and 3.3 units/mg for controls. The corresponding values for α-difluoromethylornithine (DFMO) binding were 3.15 and 0.053 pmol/mg. Results shown are mean ± S. D. for separate estimations.](http://www.jbc.org/content/fig/1/15/6077/F1.large.jpg)
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Oromethylornithine. The conversion of the enzyme to a form with some residual activity, but much lower than the original such as described in Physarum polycephalum (26, 43), is ruled out by this experiment unless the alternate form is entirely inactive. (It should be noted that the conditions used for the binding of [14C]-difuoromethylornithine did result in complete loss of the enzyme activity and that no further increase in binding or loss of activity was produced on increasing the concentration of drug or cofactor.) The results in Fig. 1 are, therefore, consistent with the loss of enzyme due to proteolytic degradation, but other mechanisms rendering the enzyme totally inactive could also fit with these results.

In order to test whether the decreased degradation rate was a general phenomenon, the effect of androgen treatment on the turnover of S-adenosylmethionine decarboxylase was measured. This enzyme also turns over rapidly in mammalian tissues (41, 42, 44) and the loss of enzyme activity when protein synthesis was inhibited occurred with a half-life of about 33 min in control mice and 72 min after androgen treatment (Fig. 2). In this experiment, only enzyme activity was measured and the results, therefore, may not reflect enzyme protein, but in previous studies in both rat liver (41) and mouse liver and mammary gland (44), such activity measurements have been confirmed to reflect the protein content by the use of specific antibodies.

In order to investigate the turnover of ornithine decarboxylase protein in vivo, mice were injected with [14C]-difluoromethylornithine, killed at various times later, and the labeled proteins present in the kidney analyzed by polyacrylamide gel electrophoresis (Fig. 3). The results were compared with the protein labeled by incubation with the radioactive drug in vitro. As previously reported (32), only one labeled band was observed after in vitro labeling, corresponding to a molecular weight of about 53,000 (Fig. 3, bottom). The administration of [14C]-difluoromethylornithine in vivo also produced only a single labeled band and this coincided exactly with that formed by inactivation of the enzyme in vitro. Therefore, the interaction of this drug is highly specific and can be used to study the fate of the ornithine decarboxylase protein in vivo. The labeled protein observed after treatment with [14C]-difluoromethylornithine declined with time as the protein was degraded, but no labeled fragments of lower molecular weight accumulated (Fig. 3). The results shown in Fig. 3 are for analysis of the labeled protein present in the 100,000 × g supernatant fraction. This accounted for at least 90% of the perchoric acid-insoluble radioactivity of the kidney homogenates at all times.

There was no indication of a time-dependent increase in the amount of labeled protein in the pellet from the centrifugation which could indicate the buildup of a labeled, macromolecular degradation product (or the enzyme itself) in a subcellular organelle such as lysosomes or nuclei. Because so little radioactivity was present in the pellet, it was not possible to detect any specific labeled protein by polyacrylamide gel electrophoresis except for the 53,000 molecular weight band which could have arisen by contamination of the pellet with some cytosol.

α-Difluoromethylornithine is quite rapidly cleared from the circulation in rodents after a single dose (45). Therefore, ornithine decarboxylase could be labeled in vivo by administration of a single dose of the drug and the half-life for decay of the protein in vivo could be measured. In order to ensure that there was no further incorporation from any [14C]-difluoromethylornithine still remaining after 30 min, a large chase dose of unlabeled α-difluoromethylornithine was given. The results are shown in Fig. 4. In androgen-treated mice, the time course for the loss of this protein occurred with a half-life identical with that of the decline in enzyme activity when protein synthesis was blocked by cycloheximide (Fig. 4A). Results shown in Fig. 4A are for androgen-treated Crl:CD-1 strain mice, but androgen-treated BALB/c mice gave similar results. Unfortunately, the specific activity of the [14C]-difluoromethylornithine was not sufficiently high for measurement of the in vivo half-life of ornithine decarboxylase in the control mouse kidneys of the BALB/c strain. Therefore, only control Crl:CD-1 strain mice could be used for the experiment shown in Fig. 4B since they have significantly greater activity (Table I). As shown in Fig. 4B, the t1/2 for the decline in ornithine decarboxylase activity after cycloheximide treatment in these control mice was about 40 min and the protein labeled by [14C]-difluoromethylornithine in vivo declined at approximately the same rate.

Fig. 2. Loss of S-adenosylmethionine decarboxylase activity after treatment with cycloheximide. Mice were treated as in Fig. 1 and the activity of S-adenosylmethionine decarboxylase measured in extracts from control ( ) and androgen-treated ( ) animals. Results are expressed as per cent of the value at zero time which was 425 units/mg for androgen-treated animals and 124 units/mg for controls.
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Fig. 4. Half-life of ornithine decarboxylase labeled with α-difluoromethylornithine in vivo. Androgen-treated (A) and control (B) mice were treated with cycloheximide (10 mg/kg) and the ornithine decarboxylase activity measured (−−−) at the times shown. Similar mice did not receive cycloheximide but instead were given 1 mg/kg doses of [14C]α-difluoromethylornithine to label the enzyme. After 30 min, a dose of 200 mg/kg of unlabeled α-difluoromethylornithine was given to prevent further incorporation and the decline in the amount of labeled protein was followed as shown (−−−). Results are shown as per cent of the activity at zero time. This was 876 cpn/mg of kidney protein for androgen-treated mice and 77 cpn/mg for controls. The corresponding 100% values for ornithine decarboxylase activity were 247 units/mg and 30 units/mg, respectively. Results are given as mean ± S.D. for at least seven estimations. This experiment was carried out with CrlCD-1 strain mice, but similar results to those shown in A were obtained with androgen-treated BALB/c mice.

DISCUSSION

Androgens are known to have a trophic effect on the mouse kidney producing hypertrophy of the cells of the proximal tubules (46, 47). A small number (at least 10) of kidney proteins are selectively increased by androgen treatment and there is convincing evidence that for some of these, such as β-glucuronidase, the increase is brought about by an increased rate of synthesis (46). Increased production of mRNA for ornithine decarboxylase remains one of the most rapidly turning over mammalian proteins. Our experiments indicate that the half-life measured using cycloheximide to inhibit protein synthesis and following enzyme activity does give valid results in this system. However, the method described here in which loss of labeled protein is followed in vivo without application of a protein synthesis inhibitor removes doubts based on the possibility that cycloheximide might itself affect degradation or bring about loss of enzyme activity in some other way.

At present, the titration of ornithine decarboxylase with radioactive α-difluoromethylornithine provides the only method for quantitating enzyme protein, but this suffers from the disadvantage that only active enzyme can be determined. Immunochemical methods which have proved suitable for such studies with other enzymes (and for yeast ornithine decarboxylase (27)) are difficult to apply to ornithine decarboxylase in mammalian cells because the amount of enzyme is so small (32) and the only antiserum currently available are not fully characterized (6, 18, 19). At present, the titration with α-difluoromethylornithine can only be used in those tissues having very high ornithine decarboxylase activities because of the limitations imposed by the specific activity of the drug, but if a higher specific activity becomes available, the method should become of general application. The specificity of binding of α-difluoromethylornithine to protein is quite remarkable in that the only protein labeled in vivo after administration of the drug was ornithine decarboxylase. The labeled α-difluoromethylornithine can, therefore, be used for autoradiographic localization of the enzyme. It is conceivable that the degradation products derived from ornithine decarboxylase by proteolytic cleavage may also be followed in this way, although only the fragments with α-difluoromethylornithine still attached could be studied and no evidence for macromolecular fragments was obtained in the present work.

It is perhaps somewhat surprising that the α-difluoromethylornithine-labeled ornithine decarboxylase protein turns over at the same rate as the native enzyme. It has been shown that a number of reversible inhibitors of ornithine decarboxylase such as α-methylornithine, α-hydrazino-γ-aminobasic acid, and 1,4-diaminobutane increase the half-life of the enzyme (21, 49–51), but, apparently, the covalent attachment of α-difluoromethylornithine does not have this effect. Also, it is well known that abnormal proteins containing amino acid analogs are degraded very rapidly (52), but the addition of a single molecule of α-difluoromethylornithine does not appear to bring about such a change. This is quite fortunate from an experimental point of view since the present system should provide an excellent model to study the mechanism of degradation of this enzyme. The use of α-difluoromethylornithine-labeled protein and the ability to modify the rate of degradation by androgens should enable the characterization of this system. It is noteworthy that no intermediate degradation products of lower molecular weight were seen in these experiments. This may indicate that after an initial inactivation step, the enzyme is rapidly degraded to acid-soluble materials, but, at present, the possibility that a small fragment which happens to contain the labeled α-difluoromethylornithine is cut off cannot be ruled out. The development of an in vitro system for the degradation of the enzyme should be aided by the use of the α-difluoromethylornithine-labeled protein and should help to clarify these points.

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