Induction of Urea Cycle Enzymes of Rat Liver by Glucagon*

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PHILIP J. SNODGRASS, RENEE C. LIN, WALTER A. MÜLLER,† AND THOMAS T. AKI

From the Veterans Administration Hospital and the Department of Medicine, Indiana University School of Medicine, Indianapolis, Indiana 46202, the Department of Medicine, Harvard Medical School and the Peter Bent Brigham Hospital, the Elliott P. Joslin Diabetes Foundation, Inc., Boston, Massachusetts 02115

All five urea cycle enzymes of rat liver increased in activity 48 h after subcutaneous administration of crystalline zinc glucagon to male rats and remained elevated after 7 days of continuous glucagon infusion. The maximum ratios of enzyme activities over those of controls were 2.0 for carbamyl phosphate synthetase, 1.3 for ornithine transcarbamylase, 2.7 for argininosuccinate synthetase, 3.2 for argininosuccinase, and 2.2 for arginase. Actinomycin D or puromycin prevented these responses to glucagon. The increase in arginase activity after zinc glucagon treatment was matched by an increase in immunoprecipitable enzyme. All five enzymes were induced by physiological plasma levels of glucagon. Tube feeding of casein hydrolysate for 2 days increased all five enzyme activities 1.5- to 2.2-fold and resulted in plasma glucagon levels similar to those required for induction by exogenous glucagon. Thus, glucagon is an inducer of the entire urea cycle in rat liver and plays a role in the induction of the cycle by protein feeding.

In 1962, Schimke showed that the activities of all five urea cycle enzymes of rat liver adapt to the level of protein in the diet (1). An increase in activity on a high protein diet was associated with an increase in the amount of enzyme protein in the case of ornithine transcarbamylase and of arginase. It appeared that any condition which increased the rate of amino acid degradation and of urea synthesis (high protein diet (1), fasting (2), massive cortisol doses (3)) was associated with coordinate increases in the levels of all five enzymes. An unusual characteristic of urea enzyme induction was its slow response, requiring 5 to 7 days to achieve a new steady state (1) and, in the case of arginase, a half-life of 5 days was measured (4). Because arginase in liver has a slow rate of degradation and a large pool, detectable changes in activity due to an increased rate of synthesis require 24 to 48 h of induction (5).

Specific compounds responsible for this enzyme induction were not defined. Addition to a 15% casein diet of arginine, citrulline, ornithine, urea, or any of eight amino acids in the amounts present in a 60% casein diet did not increase enzyme levels (3). McLean and Novello (6) found that pharmacologic glucagon doses did increase carbamyl phosphate synthetase, argininosuccinate synthetase, and argininosuccinase but not ornithine transcarbamylase or arginase, leaving it questionable whether glucagon played a role in the coordinate induction of the entire cycle by high protein diets.

This study was designed to answer: 1) whether glucagon can increase activities of all five urea cycle enzymes of liver in vivo; 2) whether glucagon is effective at physiologic dose levels; and 3) whether glucagon increases activities by enzyme induction.

EXPERIMENTAL PROCEDURES

Assay Methods for Urea Cycle Enzymes of Rat Liver—The methods used were those of Schimke (1), modified as described (7). One unit of enzyme activity is defined as the amount catalyzing the formation of 1 μmol of product/h at 37°C. Results are given as units/mg of protein to express enzyme concentration. Units/liver/100 g of rat were also calculated to assess changes in total activity per animal (6). Protein was measured in duplicate by the method of Lowry et al. (8) using bovine serum albumin as a standard. All pH measurements were at 37°C. Rats were killed by decapitation, the livers were removed, placed in ice-cold saline, and, within 20 min, blotted dry, and weighed, and samples were taken from three lobes. A 5% homogenate was prepared in ice cold distilled water, using a glass Potter-Elvehjem homogenizer, and kept at 4°C. All five urea cycle enzymes were assayed within 80 min of death.

Animal Diets and Balance Studies—Male Sprague-Dawley rats weighing about 120 g were weighed, placed in cages in groups of two, and fed a standard rat diet containing 15% casein (Nutritional Biochemical Co.). The pelleted diet also contained sucrose, optimal amounts of vitamins, minerals, and a constant amount of corn oil. After 5 days on a 15% casein diet, individual rats were transferred to polyethylene metabolic cages where daily weights and food intake were measured and urine was collected in containers acidified with concentrated HCl to maintain a pH below 3. Urea and creatinine concentrations were measured in urine by standard Auto-Analyzer methods. The ratio of urea/creatinine became constant after 6 days on a given diet. Three experimental animals and one pair-fed control were killed for each set of enzyme assays.

Glucagon Administration—Rats stabilized on a 15% casein diet for 6 days were injected subcutaneously with zinc glucagon, 1 to 100 μl of crystalline suspension (2 mg/ml) made up in sterile normal
Effects of Zinc Glucagon on Urea Cycle Activities—The responses of the urea cycle enzyme activities to subcutaneous administration of crystalline zinc glucagon are shown in Table I. A pharmacologic dose of zinc glucagon (4 mg/kg/day) resulted in maximal increases over controls in the activities of all five urea cycle enzymes of rat liver by 48 h. Expressed as units/mg of protein, the increases over controls injected with saline were 1.9-fold for carbamyl phosphate synthetase, 1.3-fold for ornithine transcarbamylase, 2.5-fold for argininosuccinate synthetase, 3.0-fold for argininosuccinase, and 1.7-fold for arginase. The maximal increments based on units/liver per 100 g of rat were somewhat greater: 2.0, 1.3, 2.7, 3.2, and 2.2, respectively. Carbamyl phosphate synthetase, argininosuccinate synthetase, and argininosuccinase increased (p < 0.001) at the end of 24 h of zinc glucagon administration, whereas ornithine transcarbamylase and arginase responded to glucagon more slowly.

The rats given these high doses of glucagon lost 6% of their body weight due only in part to small (p > 0.1) decreases in food intake on Days 2 and 3. Urea/creatinine ratios were significantly elevated at 24 h by this large dose of glucagon whereas ornithine transcarbamylase and arginase responded to glucagon more slowly.

The results of both groups were compared with seven 300-g rats on 15% casein diet alone, for days 7 and 8, and then killed after 48 h of treatment for liver urea cycle assays. Statistical Methods—In experiments on whole rats, the mean and standard deviation was calculated for each treated group and for its control group. The significance of the difference of the means was calculated by Student's t-tailed unpaired t-test.

Table I

<table>
<thead>
<tr>
<th>Urea Cycle Enzymes</th>
<th>Changes (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbamyl phosphate synthetase</td>
<td>1.9-fold</td>
</tr>
<tr>
<td>Ornithine transcarbamylase</td>
<td>1.3-fold</td>
</tr>
<tr>
<td>Argininosuccinate synthetase</td>
<td>2.5-fold</td>
</tr>
<tr>
<td>Argininosuccinase</td>
<td>3.0-fold</td>
</tr>
<tr>
<td>Arginase</td>
<td>1.7-fold</td>
</tr>
</tbody>
</table>

The abbreviations used are: SDS, sodium dodecyl sulfate.
Increases in urea cycle enzyme activities of rat liver during glucagon treatment

Values given are means ± standard deviations. Footnoted data are values versus controls on the same 15% casein diet, injected with the same volumes of diluent at the same times as the glucagon-treated rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Body weight (g)</th>
<th>Food intake (mg)</th>
<th>Urinary urea/creatinine</th>
<th>Carbamyl phosphate synthetase</th>
<th>Ornithine transcarbamylase</th>
<th>Argininosuccinate synthetase</th>
<th>Argininosuccinase</th>
<th>Arginase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zinc glucagon, 4 mg/kg/day, subcutaneously at 0800, 1300, and 1800 h daily</td>
<td></td>
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<td></td>
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<td></td>
<td></td>
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<tr>
<td>Controls</td>
<td>11</td>
<td>142 ± 12</td>
<td>7.1 ± 2.6</td>
<td>31.9 ± 7.1</td>
<td>1.66 ± 0.16</td>
<td>47.7 ± 4.3</td>
<td>0.555 ± 0.116</td>
<td>0.927 ± 0.086</td>
<td>363 ± 103</td>
</tr>
<tr>
<td>Day 1</td>
<td>5</td>
<td>135 ± 10</td>
<td>7.8 ± 2.1</td>
<td>89.4 ± 13.2</td>
<td>2.26 ± 0.17</td>
<td>53.1 ± 3.2</td>
<td>1.09 ± 0.20</td>
<td>2.18 ± 0.24</td>
<td>342 ± 55</td>
</tr>
<tr>
<td>Day 2</td>
<td>9</td>
<td>134 ± 11</td>
<td>6.4 ± 1.5</td>
<td>86.0 ± 7.3</td>
<td>3.14 ± 0.66</td>
<td>55.8 ± 5.9</td>
<td>1.96 ± 0.20</td>
<td>2.78 ± 0.29</td>
<td>672 ± 100</td>
</tr>
<tr>
<td>Day 3</td>
<td>6</td>
<td>134 ± 22</td>
<td>5.2 ± 2.2</td>
<td>83.6 ± 16.4</td>
<td>3.04 ± 0.81</td>
<td>57.3 ± 5.1</td>
<td>1.39 ± 0.26</td>
<td>2.70 ± 0.42</td>
<td>576 ± 60</td>
</tr>
<tr>
<td>Soluble glucagon, 0.3 mg/kg/day, subcutaneously by continuous osmotic pump infusion</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Controls</td>
<td>8</td>
<td>152 ± 9</td>
<td>8.5 ± 1.1</td>
<td>32.2 ± 9.2</td>
<td>2.18 ± 0.43</td>
<td>52.2 ± 6.1</td>
<td>0.270 ± 0.090</td>
<td>1.12 ± 0.19</td>
<td>329 ± 42</td>
</tr>
<tr>
<td>Day 7</td>
<td>8</td>
<td>155 ± 20</td>
<td>8.7 ± 1.0</td>
<td>57.7 ± 20.4</td>
<td>3.27 ± 0.99</td>
<td>63.3 ± 8.1</td>
<td>0.732 ± 0.159</td>
<td>2.05 ± 0.56</td>
<td>522 ± 46</td>
</tr>
</tbody>
</table>

* Food intakes on zinc glucagon are mean values for the last 2 days of controls and for 1, 2, or 3 days for glucagon rats; food intakes on continuous glucagon are averages of the 7 days of infusion with glucagon or diluent.

b p < 0.001.
c p < 0.01.
d p < 0.05.

The use of the implantable osmotic pumps allowed constant infusion of glucagon for 7 days. The average increments over controls, expressed as units/mg of protein (Table I), were: carbamyl phosphate synthetase, 1.5; ornithine transcarbamylase, 1.2; argininosuccinate synthetase, 2.7; argininosuccinase, 1.8, and arginase 1.6. The increments based on units/liver/100 g of rat were 1 to 9% higher. The urinary urea/creatinine ratios were elevated by 24 h and remained stable for the 7 days on glucagon, indicating a constant hormonal effect.

Dose Responses to Glucagon — The responses of the urea cycle enzymes to zinc glucagon were tested over a range of 27 to 4000 μg/kg/day in rats on a 15% casein diet, to find the lowest dose which would increase all five enzymes. At 27 μg/kg/day carbamyl phosphate synthetase increased by a factor of 1.2, argininosuccinate synthetase by 1.4, and argininosuccinase by 1.3, all of which were significant (p < 0.05). Ornithine transcarbamylase required 40 μg/kg/day to increase from 47.7 ± 4.3 to 54.8 ± 12.8 units/mg of protein (p < 0.05). Arginase was increased by a factor of 1.4 (p < 0.05) at this dose level. Urea/creatinine ratios were increased 1.4-fold (p = 0.01) over controls at the 40-μg dose of zinc glucagon.

Plasma Glucagon Levels after Various Zinc Glucagon Doses — The plasma glucagon levels achieved during the dose-response experiments were assessed by administering 2, 20, or 200 μg of zinc glucagon to rats previously fasted for 2 h and by following the plasma glucagon levels over a period of 12 h, while continuing the fast. As shown in Fig. 1, the plasma glucagon levels were highest at 2 h, the first value measured, and then declined at different rates depending on the dose. The peak attained at the 2-μg dose was 470 pg/ml, at 20 μg was 2400, and at 200 μg was 19,000 pg/ml. Average fasting values were 125 pg/ml. After the lowest dose, plasma glucagon returned to normal between 8 and 10 h; the other doses showed a very slow decline, demonstrating the prolonged release from subcutaneous deposits of crystalline zinc glucagon. The 40 μg/kg/day dose level, the lowest which increased all five enzymes, was achieved by giving 2 μg at 0800, 1300, and 1800 for 2 days.
The intercept is 4036 units/7 ~1 of arginase antiserum. The arginase activity in the glucagon-treated rat liver was 121,000 units/g, compared with 64,000 units/g in the control, a ratio of 1.9:1. To equalize the activities of the two liver extracts, the glucagon liver extract was added to 7 ~1 of anti-arginase antiserum, with the volumes and protein concentrations made constant by addition of MnCl2-buffered bovine serum albumin. After incubation for 45 min at 37°C and then for 18 h at 4°C, the precipitates were collected by centrifugation and the supernatants assayed. The supernatants after the glucagon-treated and control liver extracts (A) (slope = 3.30 ± 0.28c) showed superimposable lines for the equivalence point was not altered when the arginase activity was tested.

Titration of Arginase with Anti-arginase Antiserum—After injecting a 150-g male rat with zinc glucagon, 4 mg/kg/day for 2 days, the arginase activity was 121,000 units/g wet weight of liver compared with 64,000 units/g in a saline-injected control on the same 15% casein diet. Titration of the supernatants from these livers with anti-arginase antiserum showed that the equivalence point was not altered when the arginase activity was decreased from that in seven 300-g controls (p < 0.05). A series of 300-g rats given glucagon alone was not tested.

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Three hundred-gram rats given 200 μg of zinc glucagon plus a relatively low dose of cycloheximide (1 mg/kg/day) showed no urea cycle activity increases over the levels found in those given the inhibitor alone. Cycloheximide did suppress ornithine transcarbamylase below that in seven 300-g controls (p < 0.05). A series of 300-g rats given glucagon alone was not tested.

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Urea Cycle Activities and Plasma Glucagon Levels After Feeding Casein Hydrolysate—A rapid method for effecting dietary urea cycle induction was developed: casein hydrolysate was tube-fed to 150-g male rats at a dose of 0.86 g (105 mg nitrogen) at 0800, 1300, and 1800 h for 2 days. The increments (units/mg of protein) in urea cycle activities over controls tube-fed water while on the same diet were 1.3 for carbamyl phosphate synthetase, 1.2 for ornithine transcarbamylase, 1.6 for argininosuccinate synthetase, 1.2 for argininosuccinase, and 1.1 for arginase (Table II). Based on units/liver/100 g of rat, increments were 1.7, 1.5, 2.2, 1.5, and 1.5, respectively, and p < 0.001. Feeding 0.86 g of casein hydrolysate to rats resulted in an increase of mean plasma glucagon from fasting values of 125 μg/ml to a maximum of 390 at 4 h, slightly elevated values persisted at 8 h.

These studies show that glucagon will increase all five urea cycle enzyme activities in rat liver (Table II). McLean and Novello (6) were only able to increase three of the five enzymes with large subcutaneous doses of soluble glucagon, and we
confirmed their results in preliminary studies. Therefore, it was not previously clear that glucagon might play a role in the coordinate increase in all five enzyme activities which Schimke demonstrated resulted from feeding rats a high protein diet (1). Our eventual success is probably due to the longer duration of action of zinc glucagon (Fig. 1) than of soluble glucagon given subcutaneously (12) and to our use of a constant infusion of glucagon by an osmotic pump.

The second question we asked was whether all five enzyme activities were increased by physiological levels of plasma glucagon. A dose of 40 \( \mu \text{g/kg/day} \) for 9 days gave activity increments over controls of 1.2 to 1.4. This dose level required 2 \( \mu \text{g} \) of zinc glucagon at 0800, 1300, and 1800 h. The lower curve in Fig. 1 shows that the plasma glucagon after a 2-\( \mu \text{g} \) dose is about 200 pg/ml at 5 h. We calculate that three doses 5 h apart would give a stepwise increase to approximately 900 pg/ml after the third dose, with levels returning to normal by 24 h. Glucagon levels in the portal vein of dogs have been reported to be between 500 to 800 pg/ml after meals (13) so we are increasing all five activities by exposing the rat liver to physiologic glucagon levels for 48 h.

The plasma glucagon levels resulting from 7 days of perfusion with the osmotic pump were measured at the end of the experiment, when they were in the high physiologic range. This experiment supports the hypothesis that a constant stimulus to glucagon release should result in persistent elevations of all five enzyme activities.

What role might glucagon play in the induction by high protein feedings? Certain amino acids are known to provoke glucagon release in dogs (14) but the plasma glucagon response to feeding mixed amino acids in rats had not been reported. We found that a modest dose of casein hydrolysate (only 5.7 g/kg) led to a rise in plasma glucagon to a 300 pg/ml level at 4 h. The plasma glucagon response curve after a casein hydrolysate feeding is similar to that achieved with the lowest dose of zinc glucagon which will induce the entire cycle. However, glucagon does not give as great an increase in the enzymes as casein, so we conclude that it is one of a number of factors which are responsible for enzyme induction by protein feeding.

An unusual aspect of this urea cycle induction by glucagon compared to other enzymes is its slow time course. One short exposure to glucagon will increase tyrosine aminotransferase in rat liver or cultured liver cells to a maximum in 4 to 5 h, yet we were not able to show significant increases in urea cycle enzymes until 24 h (Fig. 2). Neither glucagon nor casein hydrolysate increased urea cycle activities at 12 h in our hands, when doses were given at 0 and 6 h. This slow response was observed by Schimke (1) to feeding of high protein diets. His studies suggest that the reasons for the slow changes in activity are the long half-life and large liver pools of the urea cycle enzymes (5). The same increase in rate of synthesis will cause a large increase in tyrosine aminotransferase activity because it has a short half-life and small liver pool, but little change results in arginase activity which has a half-life of 5 days and a large liver pool. Thus we do not see a statistically significant increase in four of the urea cycle activities until glucagon has been continually stimulating enzyme synthesis for at least 24 h, and the new steady state level is not achieved for 48 h. A more sensitive index of the glucagon effect would be to measure the change in rate of synthesis over 2 to 4 h after exposure to glucagon (5).

Whatever protein feeding does to induce the cycle, it is not likely that it acts merely by exposing the liver cells to higher concentrations of amino acids, because we cannot affect urea cycle activities in cultured liver cells (15) with 5-fold differences in amino acid levels of the medium (16). We are presently correlating the relative inducing abilities of 20 different amino acids when fed to rats with their plasma glucagon effects and with their effects on the enzymes when added to cultured cells. If certain amino acids both induce in whole rats and elevate plasma glucagon levels, but do not induce in cultured rat liver cells, the role of glucagon will be strengthened as a major inducer resulting from protein feeding. Another approach would be to show no induction with protein feedings in pancreactomized rats.

The next important question we addressed was whether these increased activities were due to enzyme induction, i.e., an increase in enzyme synthesis or a decrease in degradation which results in an overall increase in the amount of enzyme protein. It is apparent from studies on alkaline phosphatase (17) that an increase in activity with steroid treatment can occur not from induction but from activation of performed enzyme. Therefore, the crucial test is whether or not there is also an increase in immunologically precipitable enzyme when there is an increase in activity. Fig. 3 demonstrates that, when the activity of arginase in the liver supernatant is 1.9 times that of the control, the amount of immunoprecipitable arginase is 1.9 times that of the control. Although our arginase antiserum is not completely monospecific, it is potent and specific enough to show that two liver supernatants diluted to contain the same activities have the same equivalence points on a titration curve (Fig. 3). We will have to do radioactive amino acid incorporation studies to prove whether the increased amounts of arginase protein is due to an increase in synthesis rate or to a decrease in degradation rate or both (4). Prevention of glucagon induction by a low dose of actinomycin D (Fig. 2) is fairly strong evidence that RNA synthesis is required for the glucagon effect. The fact that the inhibitor alone caused no reduction in basal levels of the enzymes is against a toxic effect of actinomycin on protein synthesis (18). We still cannot conclude that glucagon induces via stimulation of transcription until we can show that specific messenger RNAs are produced by its action. Inhibition of the glucagon effect by puromycin or cycloheximide is reliable evidence that protein synthesis is required when the amounts of enzyme after glucagon are shown to be increased by immunotitration (17), as we showed for arginase. The decrease in the basal level of ornithine transcarbamylase, after 24 h of treatment with either of these inhibitors, suggests that its rate of degradation is more rapid than the other four enzymes whose activities were stable for this time period (Fig. 2). The fact that arginase shows an increase in immunoprecipitable enzyme and that all five enzyme activity increases after glucagon are blocked by actinomycin D and puromycin implies that the increased activities of all five enzymes are due to coordinate enzyme induction.

The injection of large doses of glucagon into intact rats is likely to cause many secondary effects, such as release of insulin and growth hormones, a rise in blood glucose, increased amino acid uptake in the liver, and induction of enzymes of carbohydrate and amino acid metabolism (13, 19). Therefore, our induction of the urea cycle in vivo may not have been a direct effect of glucagon upon the liver cells but may have only reflected the actions of one or more of these other substances. In cell culture we can control most of these variables. Preliminary experiments using a monolayer liver cell culture method developed in our laboratory (15) show
small increases in the activities of all enzymes but carbamyl phosphate synthetase (16). The increases in cell culture activities of the four enzymes are not as great in vitro as they are after maximal doses of zinc glucagon in vivo, so we suspect that other hormones are missing from the culture medium which might play a permissive, additive, or synergistic role with glucagon (20). For example, high protein feedings are known to release cholecystokinin, gastrin, insulin, and growth hormone (13, 19).

The physiological importance of glucagon in urea cycle induction is the coordination of gluconeogenesis and ureogenesis by this hormone. In perfused rat livers, glucagon (and cyclic AMP) stimulate gluconeogenesis and ureogenesis without a time lag, apparently due to increasing transport of amino acids into the cell and to increasing conversion of pyruvate to P-pyruvate (19, 21). This "fine control" of these processes does not seem to involve enzyme induction. Glucagon and cyclic AMP also induce a key enzyme of gluconeogenesis, phosphoenolpyruvate carboxykinase in rat liver (20, 22), a slower process of "coarse control" which we have demonstrated also exists for the urea cycle enzymes.

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