Induction of Urea Cycle Enzymes of Rat Liver by Glucagon*

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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.

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saline, on Days 7 and 8, and were killed on Day 9. The micrograms of zinc glucagon administered subcutaneously at various times and the resulting daily doses in 150-g rats were as follows: 2 μg at 0800 and 1800 h (27 μg/kg/day); 2 μg at 0800, 1300, and 1800 h (40 μg/kg/day); 2 μg at 0800, 1300, and 1800 h (400 μg/kg/day); and 200 μg at 0800, 1300, and 1800 h (4000 μg/kg/day).

An instantaneous infusion of soluble glucagon over 7 days, Alzet osmotic minipumps (Alza Corp., Palo Alto, Calif.) were implanted subcutaneously after filling the chamber with 0.17 ml of 2 mg/ml of glucagon (Lilly) dissolved in a pH 7.2 diluent (Lilly) containing 0.2% phenol and 1.6% glycerol or with diluent alone for the total amount delivered was calculated by weight difference. Plasma glucagon concentration was measured at time of death. The pumps were specified to deliver 1 μg/h of isotonic saline at 37°C for 7 days. All contained less than 0.01 ml of glucagon fluid after 7 days.

Glucagon Assays—Plasma was obtained by decapitation and drainage of blood into a tube or by anesthetizing the rats with ether, opening the abdomen, and performing transdiaphragmatic cardiac puncture. The blood for glucagon was collected in cold EDTA- Vacutainer tubes containing 1000 units of Trasylol (FBA Pharmaceuticals Ltd., N. Y.), mixed, and spun at 2000 rpm. The plasma was removed and frozen at –20°C until the glucagon was assayed by the method of Ungcr (9), using an antisera, 30K, which is highly specific for pancreatic glucagon.

Urea Cycle Enzymes—Purified rat liver arginase was derived from control rabbit γ-globulin showed the bands at 67,000, 46,000, 25,500 and 22,000 weight seen above, as well as a band at 35,000 weight. No 31,500-weight band was seen.

Immunological titration of arginase in liver supernatants was carried out (4) by mixing 7 μl of antisera with increasing amounts of 105,000 x g supernatant, brought to a total volume of 1.0 ml with 0.05 M MnCl₂, 0.005 M Tris/HCl (pH 7.4), and 1 mg/ml of bovine serum albumin. The mixture was kept at 37°C for 45 min and then at 4°C for 18 h. The tubes were centrifuged for 30 min at 27,000 x g and the supernatants and precipitates were assayed for arginase activity. A 5% homogenate of liver in the above MnCl₂/Tris buffer, centrifuged 30 min at 105,000 x g gave complete recovery of arginase activity in the supernatant. The livers were derived from one rat given 4 μg/kg/day of zinc glucagon subcutaneously for 2 days and from a control rat on 15% casein, given 0.1 ml saline subcutaneously four times a day for 2 days.

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 3.1, 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 and body weight during glucagon administration. The small fasting for 48 h in five rats caused an average weight loss of 7.4 g/day and only increased the enzymes slightly (p > 0.05) between feeding and fasting rats. Thus the small decrease in food intake and body weight during glucagon treatment did not account for the enzyme and urea/creatinine ratio changes. Injection of controls with saline did not affect basal enzyme activities; compared with 30 uninjectected rats on

1 The abbreviation used is: SDS, sodium dodecyl sulfate.
**Table I**

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 intakes*</th>
<th>Urinary urea/creatinine</th>
<th>Carbamyl phosphate synthetase</th>
<th>Ornithine transcarbamylase</th>
<th>Argininosuccinate synthetase</th>
<th>Argininosuccinate lyase</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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<tr>
<td>Controls</td>
<td>11</td>
<td>145 ± 12</td>
<td>7.1 ± 2.6</td>
<td>31.9 ± 7.1</td>
<td>47.7 ± 4.3</td>
<td>5.055 ± 0.116</td>
<td>0.927 ± 0.086</td>
<td>363 ± 103</td>
<td></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>7.25 ± 0.17b</td>
<td>43.1 ± 3.2</td>
<td>1.09 ± 0.20b</td>
<td>2.18 ± 0.24b</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 ± 7.3a</td>
<td>3.11 ± 0.66a</td>
<td>55.8 ± 5.9a</td>
<td>1.96 ± 0.20a</td>
<td>2.78 ± 0.29b</td>
<td>672 ± 100a</td>
</tr>
<tr>
<td>Day 3</td>
<td>3</td>
<td>134 ± 22</td>
<td>5.2 ± 2.2</td>
<td>83.6 ± 16.4a</td>
<td>3.04 ± 0.81a</td>
<td>57.5 ± 5.1c</td>
<td>1.39 ± 0.26b</td>
<td>2.70 ± 0.42a</td>
<td>576 ± 60a</td>
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<tr>
<td>Soluble glucagon, 0.3 mg/kg/day, subcutaneously by continuous osmotic pump infusion</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>52.2 ± 6.1</td>
<td>0.270 ± 0.090</td>
<td>1.12 ± 0.19</td>
<td>329 ± 42</td>
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</tr>
<tr>
<td>Day 7</td>
<td>8</td>
<td>155 ± 20</td>
<td>8.7 ± 1.0</td>
<td>57.7 ± 20.4a</td>
<td>3.27 ± 0.99a</td>
<td>63.3 ± 8.1e</td>
<td>0.732 ± 0.159a</td>
<td>2.05 ± 0.56a</td>
<td>532 ± 46a</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 < 0.01.

c p < 0.001.

d p < 0.05.

e p < 0.001.

**Fig. 1.** Plasma glucagon levels after administration of various doses of zinc glucagon subcutaneously. Male rats, 150 g, were fasted 2 h, injected subcutaneously with various doses of zinc glucagon, and killed by decapitation at 0 to 12 h; blood was collected in EDTA/Trasylo and plasma was separated at 4°C and frozen at -20°C for 3 weeks before radioimmunoassay. Each point is derived from the pooled plasma of two rats.

The same 15% casein diet for 8 days, none of the activities were different (p > 0.1).

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, 1.2; 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 arginase activity in the glucagon-treated rat liver was 121,000 units/g, compared with 64,000 units/g in a saline-injected control, a ratio of 1.9:1. To equalize the activities of the two liver extracts, the glucagon liver was titrated with anti-arginase antiserum. The supernatants and precipitates assayed. The supernatants after centrifugation at 105,000 g for 1 h. The resulting clear supernatants contained 20,000 units/ml each.

After 7 days of glucagon infusion with the osmotic pumps, the plasma glucagon levels ranged from 560 to 960 pg/ml while those given saline had values of 150 to 200 pg/ml at the time of death.

Prevention of Glucagon Stimulation by Actinomycin D, Pyrouron, and Cycloheximide—In Fig. 2 the five enzyme activities over the means of 30 control rat liver activities, were increased (units/mg of protein) in urea cycle activities over controls tested. A series of 300-g rats given glucagon alone was not treated.

Tritration 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 derived from a rat liver treated with glucagon compared with that from a control liver (Fig. 3).

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 1.1, respectively, all 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 pg/ml to a maximum of 330 at 4 h, slightly elevated values persisted at 8 h.

**DISCUSSION**

These studies show that glucagon will increase all five urea cycle enzyme activities in rat liver (Table I). 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 μg/kg/day for 3 days gave activity increments over controls of 1.2 to 1.4. This dose level required 2 μg of zinc glucagon at 0800, 1300, and 1800 h. The lower curve in Fig. 1 shows that the plasma glucagon after a 2-μg 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 300 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 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.

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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