The effect of different hyperglucagonemic states on monooxygenase activities and isozymic pattern of cytochrome P-450 in mouse

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The continuous infusion of a low dose of glucagon (35 μg/kg/d, for 5 d) constitutes, in view of glucose-6-phosphatase and phosphoenolpyruvate carboxykinase activities, a reliable experimental model of hyperglucagonemia. By conjunction of monooxygenase assays and immunoquantitation of specific isozymes of cytochrome P-450, the actual inducing ability of glucagon has been shown and it might explain some of the modifications of the drug metabolizing system in diabetic mice. The isozymic pattern of cytochrome P-450 of liver microsomes from diabetic mice appears very different from that produced by classical inducers.

We have previously described some modifications of the mixed function oxidase (M.F.O.) system produced by streptozotocin-diabetes in the liver of mice, and shown that these modifications did not result from an inducing effect of streptozotocin since they were completely corrected by insulin (1-2). In the rat, Past and Cook (3) also reported diabetes-induced modifications of the monooxygenase system, which were ascribed to the induction of peculiar forms of cytochrome P-450. However, these data did not make it possible to determine whether the described modifications resulted from decreased insulin secretion, or increased blood glucagon which is usually observed in insulin-dependent diabetes. In order to delineate the respective roles of these hormones, the total level and isozymic pattern of cytochrome P-450, and the associated monooxygenase activities, were compared in another situation involving increased glucagon/insulin level (starvation), and in the hyperglucagonemic state produced by continuous infusion of the hormone.

Materials and Methods

10-week-old male mice of the C57BI/6 strain were obtained from the Centre d'élevage du CNRS, Orléans, France. The insulin-dependent diabetes was produced in mice by a single intraperitoneal (i.p.)

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injection of streptozotocin (STZ) (200 mg/kg freshly dissolved in NaCl pH 4.5); then the glucosuric mice were killed 2 weeks later. Sodium phenobarbital (PB) or 3-methylcholanthrene (3MC) were given i.p. at a daily dose of 80 mg/kg for 3 d in saline for PB or in corn oil for 3MC. Some mice received a continuous infusion of glucagon (Sigma) (35 μg/kg/d for 5 d) by means of Alzet osmotic minipumps (vol. 0.2 ml) implanted subcutaneously in the animals' backs, under light ether anaesthesia; glucagon was dissolved in 1.4% glycerol pH 3.0 as indicated by Gruppuso et al. (4) and sterilized by passing through a 0.22 μm Millex GV filter. Control mice for this experiment were sham operated (implanted with minipumps containing the vehicle only).

Plasma glucose was determined by the glucose oxidase method using a Beckman analyser. Glucose-6-phosphatase and phosphoenolpyruvate carboxykinase activities were determined as described respectively by Swanson (5) and Marsac et al. (6). Cytochrome P-450, 7-ethoxycoumarin-O-deethylase and benzphetamine-N-demethylase were assayed as previously reported (1-2). The isozymic pattern of treated mice was investigated with anti-rat cytochrome P-450 antibodies, which exhibit some cross reactions with homologous mouse isozymes (7). The cytochrome P-450 isozymes were measured by immunoquantitation (Western blots) as described by Guengerich et al. (8). It was checked by immunochemical techniques that isozyme preparations of cytochrome P-450 UT-A, PB-B and BNF-B were similar to those described by Guengerich (8). Cytochrome P-450 UT-A was formerly called A NI and cytochrome P-450 PB-B, B-PB (9). Antisera against our cytochrome P-450 isozymes were raised in rabbits as previously indicated (10). Anti PB-B was rendered monospecific by affinity chromatography with glutardialdehyde activated absorbent from (and as indicated by) Boehringer with liver microsomes from untreated male rats. It recognized rat cytochrome P-450 BP-B and microsomes from PB-treated rats as a single band. It cross reacted with rat cytochrome P-450 PB-B as reported by Guengerich (11). Anti UT-A recognized rat cytochrome P-450 UT-A and untreated microsomes as a single band. Anti BNF-B recognized cytochrome P-450 BNF-B and cytochrome P-450 ISF-G as single bands which differed in their apparent molecular weight; two different bands were recognized in BNF microsomes from BNF-treated rats. 6-naphthoflavone (BNF) treatment of animals leads to similar induction as 3-methylcholanthrene (12).

**Results and Discussion**

In order to substantiate the hyperglucagonemic states, we measured blood glucose and two inducible gluconeogenic enzymes in three situations: starvation, STZ-diabetes and glucagon infusion. Blood glucose was moderately decreased during starvation and was increased 4-fold by STZ-diabetes: glucose-6-phosphatase (G-6-Pase) and phosphoenolpyruvate carboxykinase (PEPCK) were constantly elevated in all situations (Table 1). Numerous reports have well documented both the glucagon level and the G-6-Pase and PEPCK activities in STZ-diabetic and starved animals. As an example, several groups (13-15) observed both a decrease of plasma immunoreactive insulin (IRI) and an increase of immunoreactive glucagon (IRG) in rats, 10 d
Table 1. Blood glucose and inducible enzyme activities in hyperglucagonemic mice

<table>
<thead>
<tr>
<th>Results are means ± S.E. for 3 to 6 animals.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
</tr>
<tr>
<td>Control, sham-operated</td>
</tr>
<tr>
<td>Glucagon-infused</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>STZ-diabetic</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Starved (72 h)</td>
</tr>
</tbody>
</table>

*aResults expressed as µg of phosphate liberated x min⁻¹ x mg⁻¹ of microsomal proteins.

*bResults expressed as nmol of product formed x min⁻¹ x mg⁻¹ of microsomal proteins.

* P < 0.10; ** P < 0.05; *** P < 0.001 as compared with respective control mice.

after STZ injection, with a 4-fold elevation of blood glucose. Under the same conditions G-6-Pase and PEPCK were increased 2- and 4-fold respectively (16). Starvation produced roughly similar variations: IRI decreased 5-fold and IRG increased 2-fold in rats starved for 6 d (13), while G-6-Pase and PEPCK activities were enhanced 3- to 4-fold after 3 d of starvation (16). It has been clearly demonstrated that especially PEPCK induction in diabetic and starved animals is a result of synthesis stimulated by the increased glucagon level (17). Thus, despite the lack of IRG measurement in our experiments, the results shown in Table 1 substantiate the hyperglucagonemic state as shown by the increased activities of gluconeogenic enzymes in starved, STZ-diabetic and glucagon-infused mice, as compared with published results.

The microsomal concentration of cytochrome P-450 was decreased in glucagon-infused mice, but increased in starved and STZ-diabetic animals. The molecular activities of tested monooxygenases were generally increased, though with different patterns: glucagon infusion produced the highest effects, especially at the level of benzphetamine-N-demethylase (6-fold) and aniline hydroxylase (3.5-fold); modifications induced by STZ-diabetes were less intense, and especially involved benzphetamine-N-demethylase (3.4-fold) and 7-ethoxycoumarin-O-deethylase (2.3-fold) activities as previously reported (1-2); starvation increased 7-ethoxycoumarin-O-deethylase and aniline hydroxylase activities only about 2-fold (Table 2), in agreement with the already reported increases of other monooxygenases (18-19). In a preliminary experiment with continuous infusion of about half dose of glucagon (16 µg/kg/d, for 5 d), increased monooxygenase activities were also noticed, although to a lesser degree (data not shown). On the other hand, the addition of somatostatin (250 µg/kg/d, for 5 d) to glucagon infusion (35 µg/kg/d), so as to prevent glucagon-induced insulin secretion, showed a slightly higher increase of aniline hydroxylase and benzphetamine-N-demethylase (results not shown). Thus, low concentrations of infused glucagon appear to act on monooxygenase activities directly, and not through modifications of insulin secretion. Moreover, preliminary experiments had shown that a dose of glucagon...
Table 2. Effect of various hyperglucagonemias on the level of cytochrome P-450 and activities of some related monooxygenases in mouse liver

Results are means ± S.E. for 3 to 4 animals.

<table>
<thead>
<tr>
<th>Mice</th>
<th>Cytochrome P-450&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Aniline hydroxylase&lt;sup&gt;b&lt;/sup&gt;</th>
<th>4-Nitroanisole-0-demethylase&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Benzphetamine-N-demethylase&lt;sup&gt;b&lt;/sup&gt;</th>
<th>7-Ethoxyeoumarin-O-deethylase&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.06 ± 0.78</td>
<td>8.15 ± 0.43</td>
<td>2.29 ± 0.15</td>
<td>2.58 ± 0.14</td>
<td></td>
</tr>
<tr>
<td>sham-operated</td>
<td>0.02 ± 0.06</td>
<td>8.01 ± 0.37</td>
<td>1.21 ± 0.53</td>
<td>1.57 ± 0.43</td>
<td></td>
</tr>
<tr>
<td>Glucagon-infused</td>
<td>0.69 ± 0.06</td>
<td>2.87 ± 3.77</td>
<td>13.01 ± 1.31</td>
<td>3.92 ± 0.67</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.01 ± 0.05</td>
<td>8.64 ± 0.64</td>
<td>3.90 ± 0.35</td>
<td>3.61 ± 0.45</td>
<td></td>
</tr>
<tr>
<td>STZ-diabetic</td>
<td>1.99 ± 0.28</td>
<td>12.31 ± 0.46</td>
<td>13.23 ± 1.00</td>
<td>8.54 ± 0.21</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.37 ± 0.15</td>
<td>7.33 ± 0.43</td>
<td>5.74 ± 0.57</td>
<td>2.05 ± 0.44</td>
<td></td>
</tr>
<tr>
<td>Starved (72 h)</td>
<td>2.60 ± 0.36</td>
<td>6.81 ± 0.52</td>
<td>6.29 ± 0.64</td>
<td>4.27 ± 0.78</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Results expressed as nmol x mg<sup>-1</sup> of microsomal proteins.

<sup>b</sup> Results expressed as nmol of product formed x min<sup>-1</sup> x nmol<sup>-1</sup> of cytochrome P-450.

* P < 0.05; ** P < 0.02; *** P < 0.01 as compared with respective control mice.

of 0.5 mg/kg/d failed to produce significant modification of gluconeogenic enzymes, when the hormone was injected twice daily. A higher dose was necessary to obtain their induction than that of the mixed-function oxidases. Thus, a continuous delivery of glucagon by implanted osmotic minipumps makes it possible to realize reliable experimental situations with low concentrations of the hormone (35 µg/kg/d).

The results shown in Table 2 clearly show that this experimental hyperglucagonemic state does not mimic exactly either the STZ-diabetic state or the starvation state (in which the overall cytochrome P-450 concentration is increased). Thus, glucagon may be one but not the only factor which triggers the modifications of the mixed-function oxidase system in physiopathological situations. Other factors such as decreased growth hormone in diabetic animals (20-21), or nutritional factors in starved mice, may be also partly responsible for the observed modifications.

An immunological study of the isozymic pattern of cytochromes P-450 in the various situations studied as compared with the effects of classical inducers should give a better insight into the hormonal effects on the monooxygenase system. The lack of a sufficient spectrum of purified mouse cytochromes P-450 and corresponding antibodies prompted us to use some anti-rat cytochrome P-450 antibodies, since Kaminsky et al. (7) had shown that immunoreactive cytochrome P-450 PB-B, BNF-B and UT-A are present both in mice and rat, though at different levels: higher in mice for cytochrome P-450 UT-A and lower for PB-B and BNF-B. Fig. 1 and Table 3 report such immunoquantitations: the level of cytochrome P-450 UT-A is well increased (50%) in glucagon-infused mice, is more moderately increased in diabetic mice (20%) and is decreased in starved mice to a similar extent as in PB-treated mice (about 20%). No significant difference appeared in the liver microsomal content of cytochrome P-450 ISF-G between control and any of the hyperglucagonemic mice,
Fig. 1. Immunoquantitation of cytochrome P-450 UT-A in liver microsomes from differently treated mice. For each cytochrome P-450, a standard curve was drawn from the relative areas quantitated by densitometry of known amounts of the antigen (rat cytochrome P-450), from 0.5 to 5.0 pmol. Then the quantity of this immunorecognized isozyme in mouse microsomes was deduced from the standard curve made on the same gel. Ag: Rat antigen. R: Untreated rat microsomes. 1: Normal mice. 2: Glucagon-injected mice. 3: Glucagon-infused mice. 4: Starved mice. 5: STZ-diabetic mice (3 to 5 μg of microsomal protein).

but its level was 2.5-fold increased in 3MC-treated mice. Moreover, in these mice cytochrome P-450 BNF-B was induced. Phenobarbital treatment of mice led to a 10-fold increase of cytochrome P-450 PB-B, which was not detected in any hyperglucagonemic mice (see legend to Table 3).

Though we had shown in a previous paper the appearance of 'PB-like' forms of cytochrome P-450 in the liver of STZ-diabetic mice (9), no increase of cytochrome P-450 PB-B was observed in the present study either in STZ-diabetic or in glucagon-infused and starved animals. This apparent discrepancy may be due to the fact that the antiserum used here was immunopurified and recognized only the PB-B form, whereas in PB-treated mice four PB-induced isozymes have been identified, only one of which reacted with rabbit anti-rat cytochrome P-450 PB-B antiserum (7).

It has been reported that, in the rat, cytochrome P-450 PB-B supports the demethylation of both p-nitroanisole and benzphetamine while cytochrome P-450 UT-A supports the hydroxylation of aniline (10). The high p-nitroanisole-O-demethylase and benzphetamine-N-demethylase activities in liver microsomes from glucagon-infused and STZ-diabetic mice might be due to the increase of other PB-induced isozymes of cytochrome P-450 which are not recognized by our immunopurified antiserum: in a previous study, we had shown that a
Table 3. Immunoquantitation of individual isozymes of cytochrome P-450 in liver microsomes from various hyperglucagonemic or treated mice

Results from 2 animals (means in parentheses) are expressed as nmol of cytochrome P-450/mg of microsomal proteins. Each value is the mean of two determinations. Cytochrome P-450 PB-B in microsomes from control and PB-treated mice was respectively 0.02 and 0.21 nmol/mg; it could not be detected in glucagon-infused, STZ-diabetic and starved mice. Cytochrome P-450 BNF-B was only detected in SMC.

<p>| Mice           | Cytochrome P-450       | Cytochrome P-450       |</p>
<table>
<thead>
<tr>
<th></th>
<th>UT-A</th>
<th>ISF-G</th>
<th>UT-A</th>
<th>ISF-G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.07-1.12</td>
<td>0.19-0.26</td>
<td>1.09</td>
<td>0.22</td>
</tr>
<tr>
<td>Glucagon-infused</td>
<td>1.49-1.58</td>
<td>0.03-0.21</td>
<td>1.53</td>
<td>0.12</td>
</tr>
<tr>
<td>STZ-diabetic</td>
<td>1.08-1.36</td>
<td>0.05-0.21</td>
<td>1.22</td>
<td>0.13</td>
</tr>
<tr>
<td>Starved (72 h)</td>
<td>0.65-0.85</td>
<td>0.05-0.16</td>
<td>0.75</td>
<td>0.10</td>
</tr>
<tr>
<td>PB-treated</td>
<td>0.75-0.85</td>
<td>ND</td>
<td>0.80</td>
<td>ND</td>
</tr>
<tr>
<td>SMC-treated</td>
<td>ND</td>
<td>0.50-0.51</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>
| ND: Not determined.

non-purified antiserum against rat PB-induced isozymes was able to immunoinhibit diabetes-induced benzphetamine-N-demethylase activity in mouse microsomes (2). On the other hand, the increased activity of aniline hydroxylase in glucagon-infused and STZ-diabetic mice seems to correlate with their enhanced content in cytochrome P-450 UT-A. However, in starved mice, the presence of another isozyme presenting an overlapping substrate specificity but no immunological cross-reactivity with cytochrome P-450 UT-A might be responsible for the increased aniline hydroxylase activity.

Thus, the various hyperglucagonemic situations studied certainly promote the synthesis of specific cytochrome P-450 which differ from those induced by chemical inducers, and remain to be characterized. The only clear-cut modification shown by the immunoquantitation of mouse cytochrome P-450 with the anti-rat antiserum concerns cytochrome P-450 UT-A. Such variations of this 'constitutive' cytochrome P-450 may have significant biological effects, since it is involved in the metabolism of endogenous substrates (steroids, vitamin D3, prostaglandins and fatty acids) (22-26).

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