Size Heterogeneity of Immunoreactive Glucagon During Bile-duct Obstruction in the Rabbit

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Abstract

The influences of bile-duct obstruction upon basal plasma glucagon levels and the relationships between glucagon-like substance in bile and postobstructive plasma glucagon were examined in the rabbit. Immunoreactive glucagon (IRG) was measured with antiseraum 30 K (Unger).

Bile-duct obstruction was followed by a prompt rise within 60 minutes in plasma IRG which was four times the basal value, but had little influence on plasma immunoreactive insulin and blood sugar.

The biliary IRG and the elevated plasma IRG during bile-duct obstruction were filtrated with a Bio-Gel P-10 column. Most of the postobstructive plasma IRG appeared in the void volume area (plasma large IRG), while almost all of the biliary IRG was recovered in the position equivalent to approximately 2000 daltons (biliary IRG 2000). Both IRGs of different molecular sizes revealed similar dilution curves in radioimmunoassay to that with porcine glucagon.

After incubation of bile with preobstructive plasma, the IRG elution profile of the mixture contained an increased amount of large molecular size IRG similar to that of postobstructive plasma in regard to 30K specificity and elution position. The disappearance of IRG in the void volume area was observed when the bile-plasma mixture or the postobstructive plasma was filtrated with acidic buffer.

These results suggest that plasma large IRG contributing to hyperglucagonemia during bile-duct obstruction may be derived from biliary IRG 2000.

Glucagon secreted by A-cells into the portal blood plays an essential role in regulating the plasma glucose concentration by way of its stimulating effects on glycogenolysis (Sokal, 1966) and gluconeogenesis (Miller, 1960) in the liver, its primary and main target organ. At the same time, the liver serves as an important site for the degradation of glucagon (Buchanan et al., 1968a; Assan, 1972; Dorner et al., 1961). Jaspan et al. (1977) showed that the liver was the major metabolic site of the 3500 molecular weight glucagon.

On the other hand, Buchanan et al. (1968b) found in the dog that immunoreactive glucagon could be detected in bile which served as an excretory medium for glucagon. Assan (1972) showed in man that a rise in blood glucagon level, e.g. induced by pancreozymin or arginine, was quickly followed by an increase in the excretion of glucagon into bile. However, little is known about the influence of bile-stasis on plasma glucagon levels.

In this study, the effect of bile-duct obstruction on plasma immunoreactive
glucagon (IRG) levels and the possible relationship between plasma IRG during bile-stasis and biliary IRG were examined in the rabbit.

Materials and Methods

Animal Models of Bile-duct Obstruction

After overnight fasting, male white rabbits weighing 3.0-3.5 kg were subjected to artificial bile-duct obstruction under pentobarbital anesthesia. The common bile-duct was ligated at a position about 1 cm away from the pars cranialis duodeni. For the sham operation, the common bile-duct was exposed by similar procedures, but was not ligated in order to maintain normal bile flow. In another group of rabbits, ligation of the common bile-duct was relaxed after 3 hours of obstruction to restore the normal bile flow.

Blood samples were taken from the auricular vein by heparinized syringes just before the bile-duct ligation or the sham operation and 30, 60 and 180 min after the respective procedures. In rabbits whose bile-duct obstruction was removed, blood samples were also taken 3 hours after the liberation. Bile was collected under normal flow conditions through the polyethylene catheter (5 French Size. ATOM Co. Ltd. Tokyo, Japan) inserted into the common bile-duct from rabbits No.1 and No.2 which were later subjected to bile-duct obstruction.

In rabbits No. 3 and No. 4, bile was sampled before and every 5 min after the injection of porcine glucagon (100 µg or 200 µg) as a bolus into the portal vein via the mesenteric vein.

After overnight fasting, intravenous arginine infusion was performed in rabbits No. 5 and No. 6. Under pentobarbital anesthesia, the jugular vein was cannulated and an intravenous infusion of L-arginine hydrochloride (0.5 g/kg) was given through the cannula over 20 min. Blood samples were taken through the same cannula just before and 20 min after the beginning of the infusion.

Blood samples were put into chilled tubes containing 500 KIU/ml Trasylol® (Bayer, Leverkusen, Germany) with 2.5 mg/ml EDTA, and bile samples were collected into chilled tubes containing 500 KIU/ml Trasylol®. Both plasma and bile samples were stored at -20°C until the time of assay and gel filtration.

Gel Filtration

A half ml of the sample was applied on a Bio-Gel P-10 column (1×45 cm). The column was calibrated by blue dextran, 125I-insulin, 125I-glucagon and 131I-Na. For estimation of the molecular species of biliary IRG, column calibration was also carried out using porcine insulin (mol. wt. 6000) and porcine glucagon (mol. wt. 3500). Elution was carried out under gravity at 4°C with 0.2 M glycine buffer (pH 8.8) containing 500 KIU/ml Trasylol® and 0.25% bovine serum albumin (BSA). For the acidic condition, eluting buffer was adjusted to pH 2.8 with HCl. The flow rate was about 12 ml/hour and the fraction size was one ml.

Assay Procedures

IRG was measured using antiserum 30K by the polyethylene glycol method described previously (Sanke et al., 1976) with slight modifications. The eluting buffer was used as the assay reagent. The sensitivity of the assay was 5 pg/tube. Glucagon-like immunoreactivity (GLI) was assayed with antiserum K4923 (Heding, 1976). Plasma immunoreactive insulin (IRI) was measured using the Phadebas® Insulin Test (Pharmacia, Sweden). Blood sugar was assayed by the glucose oxidase method.

The porcine glucagon used in this experiment was kindly supplied by Eli Lilly Co. Ltd., Indianapolis, U. S. A. (Lot No. 258-VO16-36), and its contamination with insulin was certified to be 0.001% or less.

Statistical Method

Data are shown as mean±SE. The statistical significance was analysed by the Student's t-test. P value less than 0.05 were considered significant.

Results

Origin and Elution Profile of Biliary IRG

The effect of glucagon injection into the portal vein on the rate of biliary IRG excretion was examined in rabbits No. 3 and No. 4 (Fig. 1). In both rabbits, the biliary IRG excretion rate increased obviously with the peak appearing 20 min after the glucagon injection. Intravenous infusion of saline maintained the rate of bile flow constantly throughout the course of the experiments except for each initial phase of drainage.

In rabbit No. 3 (solid line in Fig. 1), bile was pooled during the three intervals shown in the frames. The mean IRG at (1), (2) and (3) were 38.0 ng/ml, 23.5 ng/ml and 86.2 ng/ml, respectively. A half ml each of the pooled bile was filtrated with a Bio-Gel P-10 column. The major com-
Fig. 1. Changes in biliary IRG excretion rate (upper portion) and rate of bile flow (lower portion) before and after glucagon injection into the portal vein examined in rabbit No. 3 (solid line) and rabbit No. 4 (dotted line). Bile was collected at 5 min intervals. The black or the shaded region indicates the rate of biliary IRG excretion after glucagon load. Pooled bile collected from rabbit No. 3 during the three intervals represented by rectangular zones containing numbers was subjected to gel filtration and results are presented in Fig. 2.

Component of IRG was detected as a peak obviously behind the $^{125}$I-glucagon marker [Fig. 2(1)]. Both decreased during bile drainage and increased after the glucagon load of the mean IRG concentration were correlated to each change in the same fractions around fraction No. 43 [Fig. 2(2), (3)]. Most of the biliary IRG obtained from rabbits No. 1 and No. 2 was also eluted in the same fractions as that from rabbit No. 3 [Fig. 5 c)]. This species was named “biliary IRG 2000” because of its estimated molecular weight of approximately 2000 daltons. These results indicate that plasma glucagon is excreted into bile where it is transformed into 2000 dalton IRG.

In radioimmunoassay using antiserum 30K, a dilution effect identical to that seen on porcine glucagon was indicated on the biliary IRG 2000 (Fig. 6).

Glucagon immunoreactivity in the three different bile samples was assayed with both antiserum 30K and K4023 (Table 1). Glucagon immunoreactivity in bile was shown to be mainly composed of biliary IRG 2000 (71-85%), with specificity to the antiserum 30K.
Table 1. Comparison of glucagon immunoreactive values in bile and chromatographically identified biliary IRG 2000.

<table>
<thead>
<tr>
<th>Rabbit No.</th>
<th>In bile</th>
<th>In component corresponding to biliary IRG 2000</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IRG (ng/ml)</td>
<td>GLI (ng/ml)</td>
</tr>
<tr>
<td>No. 1</td>
<td>24.8</td>
<td>0.22</td>
</tr>
<tr>
<td>No. 2</td>
<td>19.6</td>
<td>0.21</td>
</tr>
<tr>
<td>No. 3</td>
<td>38.0</td>
<td>0.26</td>
</tr>
<tr>
<td>mean</td>
<td>27.5 ± 4.5</td>
<td>0.23 ± 0.01</td>
</tr>
</tbody>
</table>

IRG: Assayed with 30K (Unger)  
GLI: Assayed with K4023 (Heding)

Influences of Bile-duct Obstruction upon Plasma IRG, IRI and BS

In the ten rabbits with bile-duct obstruction, the plasma IRG was elevated significantly from the basal (preobstructive) level of 220±25 pg/ml to 848±80 pg/ml 60 min (p<0.05), and to 1524±140 pg/ml 180 min after the obstruction (postobstructive) (p<0.001). In another five rabbits, the plasma IRG did not change during 180 min after the sham operation. Both the bile-duct obstruction and the sham operation had little influence on the plasma IRI and BS (Fig. 3). In three rabbits among another group of six whose bile-duct obstruction was removed, elevated plasma IRG returned to the preobstructive level within 3 hours after the liberation. In the three remaining rabbits, plasma IRG was still elevated 3 hours after the liberation, but fell to the preobstructive level within three days (Table 2). These results indicate that hyperglucagonemia during bile-duct obstruction is reversible.

Plasma GLI assayed in three rabbits (No. 7, No. 8 and No. 9) was not altered

Fig. 3. Changes in plasma levels of IRG, IRI and BS during the initial 3 hours after ligation of the common bile-duct and sham operation. Values indicate mean±SE. Significance of the difference from basal level: *p<0.05, **p<0.001.
Table 2. Effect of liberation of bile-duct obstruction on plasma IRG level.

<table>
<thead>
<tr>
<th>Rabbit No.</th>
<th>Before</th>
<th>After</th>
<th>3</th>
<th>12</th>
<th>24</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-1</td>
<td>300</td>
<td>675</td>
<td>270</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-2</td>
<td>355</td>
<td>660</td>
<td>350</td>
<td>320</td>
<td>310</td>
<td></td>
</tr>
<tr>
<td>0-3</td>
<td>320</td>
<td>880</td>
<td>400</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-4</td>
<td>300</td>
<td>1400</td>
<td>850</td>
<td>310</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-5</td>
<td>410</td>
<td>1800</td>
<td>1100</td>
<td>500</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-6</td>
<td>380</td>
<td>1515</td>
<td>980</td>
<td></td>
<td>600</td>
<td>405</td>
</tr>
</tbody>
</table>

Before: Before bile-duct obstruction.
After: Three hours after bile-duct obstruction.

Table 3. Comparison of glucagon immunoreactive values in plasma obtained before and 180 min after bile-duct obstruction.

<table>
<thead>
<tr>
<th>Rabbit No.</th>
<th>Before</th>
<th>After</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. 7</td>
<td>167</td>
<td>410</td>
</tr>
<tr>
<td>No. 8</td>
<td>200</td>
<td>535</td>
</tr>
<tr>
<td>No. 9</td>
<td>440</td>
<td>565</td>
</tr>
<tr>
<td>Mean</td>
<td>336</td>
<td>503</td>
</tr>
<tr>
<td>± SE</td>
<td>±80.2</td>
<td>±32.5</td>
</tr>
</tbody>
</table>

Before: Before bile-duct obstruction.
After: 180 min after bile-duct obstruction.

IRG: Assayed with 30K (Unger)
GLI: Assayed with K4023 (Heding)
* significant increase from the mean level before bile-duct obstruction (p<0.05)
† no significant increase from the mean level before bile-duct obstruction (p>0.05)

Table 4. Changes in chemical data during the initial 3 hours after ligation of the common bile-duct and comparison with results of the sham operation.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>30</th>
<th>60</th>
<th>180</th>
</tr>
</thead>
<tbody>
<tr>
<td>Icterus index</td>
<td>OB</td>
<td>1.4±0.2</td>
<td>1.3±0.2</td>
<td>1.8±0.3</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>1.2±0.2</td>
<td>1.0±0.2</td>
<td>1.0±0</td>
</tr>
<tr>
<td>GPT (Ka-U)</td>
<td>OB</td>
<td>15±2</td>
<td>42±11</td>
<td>75±20</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>20±5</td>
<td>18±3</td>
<td>12±4</td>
</tr>
<tr>
<td>ALP (KA-U)</td>
<td>OB</td>
<td>5.4±1.4</td>
<td>11.1±2.9</td>
<td>12.7±4.2</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>5.4±1.6</td>
<td>5.4±1.6</td>
<td>7.4±1.3</td>
</tr>
<tr>
<td>Amylase (somogyi-U)</td>
<td>OB</td>
<td>88±12</td>
<td>94±8</td>
<td>105±14</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>92±6</td>
<td>103±11</td>
<td>95±15</td>
</tr>
</tbody>
</table>

OB: Bile-duct obstruction (n=10)
S: Sham operation (n=5)

significantly during the 180 min of bile-duct obstruction in contrast to the pronounced elevation of plasma IRG (Table 3).

As shown in Table 4, there was significant elevation of the plasma alkaline phosphatase (ALP) and icterus index (I.I.) levels, the indices of cholestasis, 180 min after the obstruction. But the elevation at 60 min was not significant. Plasma glutamic pyruvic transaminase (GPT) as an index of the liver cell damage did not show any serious elevation for the first 60 min.
Gel Filtration Profile of Plasma IRG

Elution profiles of normal basal plasma IRG were examined in four rabbits [Fig. 4 (dotted line) and Fig. 5a)]. Three IRG components were detected. The first one was found in the void volume area (Vo. peak). The next one was eluted in the region of $^{125}$I-glucagon marker (G peak). The last one, eluted behind $^{125}$I-glucagon

![Image](https://example.com/image.png)

Fig. 4. Elution profiles of plasma IRG taken before and after intravenous arginine infusion. Half ml lots of plasma were filtrated through a Bio-Gel P-10 column (1×45 cm). Vo., $^{125}$I-I and $^{125}$I-G refer to the position for void volume, $^{125}$I-insulin and $^{125}$I-glucagon, respectively.

- ... ... ... : before arginine infusion
- ... ... ... : 20 min after arginine infusion

a) : rabbit No. 5, b) : rabbit No. 6

![Image](https://example.com/image.png)

Fig. 5. Elution profiles on a Bio-Gel P-10 column (1×45 cm) of plasma and biliary IRG. Vo., $^{125}$I-I and $^{125}$I-G refer to the position for void volume, $^{125}$I-insulin and $^{125}$I-glucagon, respectively.

- ... ... ... : rabbit No. 1
- ... ... ... : rabbit No. 2

a) : Profile of preobstructive plasma.
b) : Profile of postobstructive plasma at 180 min for rabbit No. 1 and at 60 min for rabbit No. 2.
c) : Profile of biliary IRG.

Table 5. Plasma IRG levels and distribution of glucagon immunoreactivity before and after intravenous arginine infusion.

<table>
<thead>
<tr>
<th>Rabbit No.</th>
<th>Whole plasma (pg/ml)</th>
<th>Vo. peak (pg/ml)</th>
<th>G. peak (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td>Before</td>
</tr>
<tr>
<td>No. 5</td>
<td>315</td>
<td>410</td>
<td>274</td>
</tr>
<tr>
<td>No. 6</td>
<td>370</td>
<td>650</td>
<td>240</td>
</tr>
</tbody>
</table>

Vo. peak : Peak near the void volume.
G peak : Peak near the $^{125}$I-glucagon marker.
Before : Before arginine infusion.
After : 20 min after arginine infusion.
Column recovery : No. 5; Before 95.6%, after 90.2%
No. 6; Before 80.7%, after 78.5%
Table 6. Plasma IRG levels and the distribution of glucagon immunoreactivity obtained pre and post bile-duct obstruction.

<table>
<thead>
<tr>
<th>Rabbit No.</th>
<th>Whole plasma (pg/ml)</th>
<th>Vo. peak (pg/ml)</th>
<th>G peak (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
</tr>
<tr>
<td>No. 1</td>
<td>225</td>
<td>1980</td>
<td>188</td>
</tr>
<tr>
<td>No. 2</td>
<td>160</td>
<td>740</td>
<td>176</td>
</tr>
</tbody>
</table>

Nd: Not detectable.
Pre: Preobstructive.
Post: Postobstructive (at 180 min for rabbit No. 1 and at 60 min for rabbit No. 2).
Vo. peak: Peak near the void volume.
G peak: Peak near the 125I-glucagon marker.
Column recovery: No. 1; pre 88.0%, post 72.9%
No. 2; pre 110%, post 91.9%

marker, was detected only in rabbit No. 6 and its elution position coincided with that of biliary IRG 2000.

After the arginine infusion, G peak was obviously increased while other components were not altered (Fig. 4 and Table 5).

Elution profiles of pre and postobstructive plasma IRG and biliary IRG taken from the two rabbits are shown for comparison in Fig. 5. The Vo. peak was markedly raised while the G peak was not altered after the obstruction [Fig. 5a, b) and Table 6]. The elevated IRG component in Vo. peak after the obstruction was tentatively named “plasma large IRG”.

In radioimmunoassay using 30K, dilution effects identical to those observed with porcine glucagon were seen with plasma large IRG (Fig. 6).

**Relationships between Plasma Large IRG and Biliary IRG 2000**

Bile and preobstructive plasma were mixed in a volume ratio of 1:4 (bile-plasma mixture) and incubated at 37°C for 3 min or 15 min and the incubated mixture was then filtrated with the neutral buffer (pH 8.8). After incubation for 3 min [Fig. 7b]), IRG appeared in the Vo. area and for 15 min [Fig. 7c], the Vo. IRG was obviously increased. When bile was incubated with buffer alone, IRG did not appear in the Vo. area [dotted line in Fig. 7b, c]). Although the preobstructive plasma subjected to the mixing had Vo. IRG [dotted line in Fig. 5a]), it was quantitatively negligible compared with that of the bile-plasma mixture.

The disappearance of IRG in the Vo. area was observed when the postobstructive plasma or the bile-plasma mixture was filtrated with acidic buffer (pH 2.8) (Fig. 8).
Discussion

It has already been reported that hyperglucagonemia is present in patients with severe diabetes (Unger et al., 1970), liver cirrhosis (Marco et al., 1973; Sherwin et al., 1974; Seino et al., 1975), renal failure (Bilbrey et al., 1974; Lefebvre and Luyckx, 1976) and various stress states (Bloom, 1973; Lindsey et al., 1974). Using antiserum 30K, the present study revealed rapid and pro-
nounced hyperglucagonemia following bile-duct obstruction in the rabbit.

Neurological stimulation of glucagon secretion (Unger and Orci, 1977) was not affected by surgical treatment since plasma IRG did not increase after the sham operation. Bile-duct obstruction had no influence on the exocrine pancreatic function since plasma amylase remained normal after the obstruction and this effect can be recognized in the rabbit whose pancreatic duct and the common bile-duct open separately into the intestine.

The most plausible reason for hyperglucagonemia during bile-duct obstruction is the disturbance of bile flow rather than hepatocellular dysfunction, because plasma IRG increased significantly 60 min after the obstruction while plasma GPT did not develop noticeably.

The elevated plasma IRG after bile-duct obstruction consisted of Vo. IRG (plasma large IRG). On the other hand, IRG was detected in bile in accord with the findings of Buchanan et al. (1968b) and Assan (1972). Most biliary IRG consisted of approximately 2000 mol. wt. material (biliary IRG 2000). Both plasma large IRG and biliary IRG 2000 revealed similar dilution curves in radioimmunoassay to that with porcine glucagon. Recent studies showed that antiserum 30K had crossreactivities not only with pancreatic glucagon but also with bile acid such as cholic acid and deoxycholic acid (Kodaira et al., 1981; Sanke et al., 1981). However, we (Sanke et al., 1981) reported that small mol. wt. IRG could be extracted from bile by Kenny’s method (1955). Moreover, biliary IRG 2000 was increased after injection of porcine glucagon into the portal vein (Fig. 1 and Fig. 2). From these pieces of evidence, biliary IRG 2000 is thought to be derived from circulating glucagon, although the possibility still remains that it includes bile acid.

In order to examine the relationships between plasma large IRG and biliary IRG 2000, bile was mixed and incubated with preobstructive plasma in vitro. The IRG elution profile of the mixture was similar to that of postobstructive plasma in regard to the increase amount of Vo. IRG. The Vo. IRG in both bile-plasma mixture and postobstructive plasma disappeared when filtrated with acidic buffer. The elevated plasma glucagon during bile-duct obstruction was detected only by antiserum 30K, and biliary glucagon also consisted solely of 30K-reactive materials. These facts strongly suggest that plasma large IRG contributing to hyperglucagonemia during bile-duct obstruction is derived from biliary IRG 2000.

Plasma IRI and BS did not change during bile-duct obstruction despite the existence of hyperglucagonemia. This seems to indicate that plasma large IRG has no biological activity. However, we have shown previously (Miyamura et al., 1978) that paradoxical IRG responses accompanying glucose intolerance were observed during oral glucose load in patients with obstructive jaundice and also in the rabbit with bile-duct obstruction. To elucidate the biological significance of the plasma large IRG and biliary IRG 2000, further studies will be required.

Recent studies have pointed out that plasma glucagon immunoreactivity is heterogeneous (Valverde et al., 1970; Valverde et al., 1974; Weir et al., 1975; Valverde and Villanueva, 1976; Kuku et al., 1976; Jaspal et al., 1977) and has as many as four fractions when determined with antisera 30K. There is at present little information regarding the immunoreactive nature of Big Plasma Glucagon (BPG) (Valverde et al., 1974) and 2000 mol. wt. IRG. We found three IRG fractions in the fasting plasma of the rabbit and confirmed that only the G peak was modified under physiologic stimulation such as intravenous arginine. The plasma large IRG which appeared during bile-stasis is distinguishable.
from BPG by its unstable nature in an acidic condition. The \textit{biliary IRG 2000} is, incidentally, similar in size to 2000 mol wt. IRG in the circulation. Although the chemical nature and the biological significance of these substances are still uncertain, the possibility remains that these two IRGs are in fact the same molecules.

We conclude that: 1) Mechanically induced bile-duct obstruction is rapidly followed by an increase in plasma IRG, 2) Most postobstructive IRG appears in the Vo. area (\textit{plasma large IRG}) while most of the biliary IRG is recovered in the 2000 mol. wt. fractions (\textit{biliary IRG 2000}) and this IRG is derived from circulating glucagon, 3) Hyperglucagonemia consisting of \textit{plasma large IRG} during bile-duct obstruction may be derived from \textit{biliary IRG 2000}.

**Acknowledgements**

The author is greatly indebted to Prof. K. Miyamura and Assoc. Prof. K. Iwo in our department for their direction and valuable suggestions and to Dr. K. Nanjo and Dr. M. Kondo for their kind cooperation throughout this study. Elaborate technical assistance by Dr. H. Koike and Dr. Y. Moriyama during the animal experimentation is deeply appreciated.

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