Differential regional metabolism of glucagon in anesthetized pigs

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The processing of proglucagon (PG) and secretion of its products have been studied in detail (see Ref. 23 for review); however, the subsequent metabolism of some of these products, once they have been released from the endocrine cells producing them, has not received the same thorough investigation. The postsecretory fate of glucagon (PG-(33–61)) was one of the first to receive detailed attention, but many of these early studies relied on assays that were not well characterized with respect to the cross-reaction of other PG-derived peptides. Thus, although there is general agreement that the kidney is a major site of glucagon degradation in human, dog, rat, and pig (14, 22, 30, 40), the role of the liver is less clear, with some studies showing significant extraction (3, 26), whereas in others hepatic extraction was considered not to be important (16, 42). The significance of assay specificity has recently received renewed interest, since it is being shown that many peptide hormones are themselves the subject of further processing that generates metabolites that are not only immunoreactive but that may also have a biological function. Thus glucagon-like peptide-1 (GLP-1)-(9–36) amide, the degradation product of GLP-1-(7–36) amide, can behave as a receptor antagonist (29).

In recent years, it has been shown that the enzyme dipeptidyl peptidase IV (DPP IV) plays an important role in the in vivo metabolism of two other members of the proglucagon family (GLP-1 and -2) (6, 12, 28) and of the related peptide glucose-dependent insulinotropic polypeptide (GIP) (7, 28). In vitro studies have indicated that high concentrations of glucagon may also be degraded by DPP IV (19, 41), but the significance of this mechanism for glucagon metabolism in vivo has not yet been addressed.

The aim of the present study was, therefore, to examine glucagon metabolism and to identify the sites of its elimination by measurement of arteriovenous concentration differences across organ vascular beds by use of three well-characterized assays specific for different epitopes in glucagon and high-performance liquid chromatography (HPLC). The ability of this approach to reveal metabolic degradation has previously
been validated for GLP-1 and GIP (6, 7, 9, 10). In addition, the effect of the selective DPP IV inhibitor valine-pyrrolidide (37) was investigated to ascertain whether DPP IV has a physiological role in glucagon metabolism.

MATERIALS AND METHODS

**Glucagon stability in vitro.** The stability of glucagon was determined by incubation of 300 pmol/l glucagon in porcine plasma (1 ml of plasma from 4 different animals) at 37°C for 0, 30, 60, 120, 180, and 240 min. Valine-pyrrolidide (0.01 mmol/l, final concentration) or aprotinin [500 kallikrein inhibitory units (KIU/ml)] were added to two additional samples, which were incubated for 240 min. After incubation, samples were placed in an ice bath and immediately extracted with ethanol (70% vol/vol, final concentration) for subsequent RIA measurement using antisera 4305 and 4830, as described below.

Because it is known that two other members of the proglucagon family, GLP-1 and -2, are NH₂-terminally degraded by DPP IV (6, 12, 28), the susceptibility of glucagon was tested. Glucagon (300 pmol/l) was incubated with recombinant human DPP IV (5 mU/ml, a generous gift from Dr. S. Brenner, Novo Nordisk, Bagsværd, Denmark) in Tris·HCl buffer (600 μl, 0.04 mol/l, pH 7.6) at 37°C for 0, 30, and 120 min (n = 3–4 incubations at each time point). Valine-pyrrolidide [a selective DPP IV inhibitor (34), 0.02 mmol/l, final concentration; a gift from Dr. L. B. Christensen, Novo Nordisk] was added to additional samples, which were incubated for 30 and 120 min. After incubation, samples were placed in an ice bath, and the reaction was immediately stopped by the addition of trifluoroacetic acid (TFA, 50 μl, 10% vol/vol; Rathburn, Walkerburn, Scotland). Samples were analyzed by reverse-phase HPLC using a C₈ column, as previously described for GLP-1 (6) except for the use of a modified gradient of acetonitrile (Rathburn) in 0.1% TFA (0–25% over 5 min, followed by 25–45% over 20 min, and 45–75% over 3 min). Fractions were assayed for glucagon immunoreactivity using antisera 4305 and 4830 as described in Hormonal assays.

**Animals and surgical procedures.** The animal studies were in accordance with international guidelines (National Institutes of Health publication no. 85-23, revised 1985, and Danish legislation governing animal experimentations, 1987) and were carried out after permission had been granted by the National Superintendence for Experimental Animals. Danish LYY strain pigs (30–39 kg) were fasted for 24 h before surgery but allowed free access to drinking water. After premedication with ketamine (Ketaminol, 10 mg/kg; Veterinaria, Zurich, Switzerland), the animals were anesthetized with halothane (4% for induction and 1% for maintenance; Halocarbon Laboratories, River Edge, NJ), and maintained with intermittent positive pressure ventilation with an anesthesia ventilator in a semiopen system. Vascular catheters were placed in the right carotid artery for sampling of reference arterial blood and into the pulmonary artery and the left internal jugular, the left femoral, the portal, a hepatic, and the renal veins, as described previously (10). After surgery, animals were heparinized, a saline drip was set up and infused into an ear vein (5 ml/min), and animals were left undisturbed for 30 min.

**Glucagon metabolism in vivo.** To measure the regional balance of endogenous glucagon (n = 9), arteriovenous concentration differences were measured by taking simultaneous blood samples (4 ml) from the carotid artery and the jugular, renal, and femoral veins. A second set of simultaneous samples was taken from the carotid and pulmonary arteries and the hepatic and portal veins. After a 5-min interval, the protocol was repeated to obtain a duplicate set of samples.

After samples in the basal state had been obtained, glucagon (a gift from Dr. Christian Brand, Novo Nordisk) was dissolved in 0.9% NaCl containing, in addition, 1% human serum albumin (Behringwerke, Marburg, Germany) and infused via the right ear vein catheter at a rate of 5 pmol·kg⁻¹·min⁻¹ for 30 min using a syringe pump. Arterial blood samples were taken at 0, 5, 10, and 15 min after the start of the infusion, after which, during minutes 20–30, two duplicate sets of simultaneous samples were taken from all the vascular sites as described above. After 30 min, the infusion was stopped, and additional arterial blood samples were taken at 1, 2, 4, 6, 10, 15, 20, and 30 min. The total amount of blood taken during the entire procedure was 176 ml, which for a 35-kg pig is 5% of the total blood volume, which has previously been shown not to affect heart rate or blood pressure (10).

In separate experiments (n = 5), the effect of the selective DPP IV inhibitor valine-pyrrolidide was examined. After an anesthesia ventilator in a semiopen system. Vascular catheters were placed in the right carotid artery for sampling of reference arterial blood and into the pulmonary artery and the hepatic and portal veins. After a 5-min interval, the protocol was repeated to obtain a duplicate set of samples.
Calculations and statistical analysis. For each animal, the net balance of endogenous glucagon across the brain, hindlimb, kidney, and portal bed was calculated as a ratio, defined as

\[
([\text{glucagon-IR}]_{\text{carotid artery}} - [\text{glucagon-IR}]_{\text{vein}})/[\text{glucagon-IR}]_{\text{carotid artery}}
\]

where \([\text{glucagon-IR}]_{\text{carotid artery}}\) is the concentration of glucagon immunoreactivity in the carotid artery and \([\text{glucagon-IR}]_{\text{vein}}\) is the concentration of glucagon immunoreactivity in the vein.

For the liver, the hepatic blood supply was calculated on the assumption that 75% originates from the portal vein and 25% is of arterial origin. The net balance across the lungs was assumed that 75% originates from the portal vein and 25% is of arterial origin. The net balance across the lungs was defined as

\[
([\text{glucagon-IR}]_{\text{pulmonary artery}} - [\text{glucagon-IR}]_{\text{carotid artery}})/[\text{glucagon-IR}]_{\text{pulmonary artery}}
\]

where \([\text{glucagon-IR}]_{\text{pulmonary artery}}\) is the concentration of glucagon immunoreactivity in the pulmonary artery.

The extraction of exogenous glucagon was calculated for individual animals as above, after subtraction of the mean endogenous glucagon concentration at each site.

The incremental area under the curve (AUC) for glucagon immunoreactivity in the sample taken before the start of the infusion. The plasma half-life \((t_{1/2})\) was calculated by log-linear regression analysis of peptide concentrations in samples collected after termination of the infusion after subtraction of endogenous arterial glucagon concentrations, and the metabolic clearance rate (MCR) was calculated using the formula

\[
\text{MCR} = \text{infusion rate} / ([\text{glucagon-IR}]_{\text{plateau}} - [\text{glucagon-IR}]_{\text{basal}})
\]

where \([\text{glucagon-IR}]_{\text{plateau}}\) is the plateau concentration, defined as the mean of the last four measurements during the glucagon infusion, and \([\text{glucagon-IR}]_{\text{basal}}\) is the concentration of endogenous immunoreactivity in the sample taken before the start of the glucagon infusion.

The volume of distribution \((V_d)\) was calculated using the formula

\[
V_d = \text{MCR}/k, \text{ where } k = 0.693/t_{1/2}
\]

Table 1. Degradation of glucagon by porcine plasma in vitro

<table>
<thead>
<tr>
<th>Minute</th>
<th>% Initial COOH-Terminal Immunoreactivity</th>
<th>% Initial NH2-Terminal Immunoreactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma + glucagon at 37°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>30</td>
<td>89.0 ± 3.3a</td>
<td>97.2 ± 5.2b</td>
</tr>
<tr>
<td>60</td>
<td>87.5 ± 1.7c</td>
<td>85.9 ± 3.1a</td>
</tr>
<tr>
<td>120</td>
<td>86.9 ± 3.6a</td>
<td>81.5 ± 3.0c</td>
</tr>
<tr>
<td>180</td>
<td>77.7 ± 6.3a</td>
<td>73.8 ± 2.4f</td>
</tr>
<tr>
<td>240</td>
<td>70.3 ± 2.5</td>
<td>68.4 ± 3.5</td>
</tr>
<tr>
<td>+ valine-pyrrolidide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>240</td>
<td>78.3 ± 5.4ae</td>
<td>77.4 ± 1.5ae</td>
</tr>
<tr>
<td>+ aprotinin</td>
<td>240</td>
<td>87.6 ± 3.3e,f</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE and were analyzed using GraphPAD InStat software, version 1.13 (San Diego, CA) and Statistica software (StatSoft, Tulsa, OK). Two-factor ANOVA for repeated measures with post hoc analysis was used to analyze time course curves. The \(t_{1/2}\), arteriovenous concentration differences, MCR, and \(V_d\) were analyzed using ANOVA (where appropriate) and two-tailed \(t\)-tests for paired and unpaired data. One-sample two-tailed tests were used to evaluate organ extractions. \(P < 0.05\) was considered significant.

RESULTS

Glucagon stability in vitro. When porcine plasma was incubated at 37°C with glucagon, concentrations measured with COOH- and NH2-terminally directed assays decreased slowly with time (Table 1), giving a \(t_{1/2}\) of 439 ± 42 min for COOH-terminal immunoreactivity and 455 ± 46 min for NH2-terminal immunoreactivity. Addition of valine-pyrrolidide did not significantly affect the rate of degradation determined with either assay, although there appeared to be a trend \((P = 0.0899)\) toward a reduction in NH2-terminal degradation (Table 1). COOH-terminal degradation was significantly reduced but not totally prevented by aprotinin, but aprotinin had no effect on NH2-terminal degradation.

The susceptibility of glucagon to degradation by DPP IV was examined by incubating the peptide with recombinant human DPP IV. After incubation of glucagon alone, HPLC and analysis of the fractions by NH2-terminal RIA, one minor peak (peak 1) and one major peak (peak 2) were obtained (data not shown). The earlier-eluting peak (peak 1) amounted to 28.9 ± 0.7% of the total immunoreactivity and may represent the oxidized form of glucagon (glucagon contains a methionine residue in the COOH-terminal region that is susceptible to oxidation). Both peaks reduced slowly in size after incubation with recombinant human DPP IV, reaching 88.4 ± 4.4% (peak 1) and 75.7 ± 7.1% (peak 2) of their original size after 120 min, respectively. Peak 1 was not detected with the COOH-terminal assay, but a time-dependent decrease in the size of the peak corresponding to intact glucagon (peak 2, to 76.0 ± 11.6% of its original size after 120 min), together with the appearance of an additional peak (peak 3), was detected.

Data are expressed as means ± SE of incubations carried out in plasma obtained from 4 different animals. Plasma was incubated with 300 pmol/l glucagon and analyzed with COOH- and NH2-terminally directed RIAs. Results are expressed in percent glucagon concentration initially added. \(^aP < 0.05\) compared with control incubation for 0 min; \(^bP < 0.001\) compared with control incubation for 0 min; \(^cP < 0.01\) compared with control incubation for 0 min; \(^dP < 0.001\) compared with control incubation for 0 min; \(^e\) not significant compared with control incubation for 240 min; \(^fP < 0.05\) compared with control incubation for 240 min.
The reductions in size of peaks 1 and 2, together with the formation of peak 3, were totally prevented by coincubation in the presence of valine-pyrrolidide. This suggests that peak 3 is generated specifically as a result of DPP IV-mediated catalysis and may be glucagon-(3–29), which has been reported to be formed after incubation of glucagon with purified porcine renal DPP IV (19, 41). Furthermore, peak 3 was not detectable with the NH2-terminal assay, confirming the specificity of antiserum 4830 and its requirement for the intact NH2 terminus of glucagon.

The catalytic activity of the recombinant enzyme was confirmed by carrying out similar incubations with GLP-1(7–36) amide instead of glucagon, and HPLC analysis revealed the expected formation of an additional peak eluting in the position of GLP-1(9–36) amide (data not shown).

Concentrations of glucagon in the basal state. Basal glucagon concentrations differed depending on the assay (ANOVA, *P < 0.02). Levels determined with the midregion assay (58 ± 13 pmol/l) did not differ significantly from those determined with the NH2-terminal assay (34 ± 10 pmol/l) but were significantly higher than levels determined with the COOH-terminal assay (16 ± 3 pmol/l, *P = 0.01). There was no significant difference in the basal NH2-terminal and COOH-terminal immunoreactivities.

Arteriovenous concentrations of endogenous glucagon. Plasma concentrations of glucagon measured with COOH-terminal (4305), NH2-terminal (4830), and midregion (4304) RIAs are shown in Table 2, and the net balance across the various vascular beds is illustrated in Fig. 1. Renal extraction was detected with all three assays. Extraction across the hindlimb was detected with the COOH-terminal assay, whereas the midregion assay detected a modest secretion across the hepatic bed.

Exogenous glucagon extraction. During infusion of glucagon (5 pmol·kg⁻¹·min⁻¹), arterial glucagon concentrations were similar, irrespective of which assay was used (Fig. 2; incremental plateau concentrations: 365 ± 32, 420 ± 54, and 314 ± 28 pmol/l, ANOVA, *P = 0.191; incremental AUC: 11,044 ± 1,040, 11,592 ± 1,386, and 10,967 ± 836 pmol·l⁻¹·min, ANOVA, *P = 0.101, midregion and COOH- and NH2-terminal RIAs respectively).

The plasma concentrations in the different vascular beds achieved during the infusion are shown in Table 3, and the regional extraction is given in Fig. 3. Significant renal extraction was detected with all three assays. Extraction across the hindlimb was detected with the midregion and COOH-terminal assays, with a

Table 2. Concentrations of endogenous glucagon in plasma

<table>
<thead>
<tr>
<th>Assay</th>
<th>Midregion</th>
<th>COOH-Terminal</th>
<th>NH2-Terminal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carotid artery</td>
<td>60.7 ± 14.1</td>
<td>17.7 ± 4.1</td>
<td>35.1 ± 10.6</td>
</tr>
<tr>
<td>Jugular vein</td>
<td>50.2 ± 11.9*</td>
<td>19.5 ± 6.2NS</td>
<td>35.9 ± 11.7NS</td>
</tr>
<tr>
<td>Renal vein</td>
<td>48.2 ± 11.3*</td>
<td>11.3 ± 2.9‡</td>
<td>21.7 ± 5.9*</td>
</tr>
<tr>
<td>Femoral vein</td>
<td>60.7 ± 14.1NS</td>
<td>15.7 ± 4.6NS</td>
<td>33.0 ± 11.7NS</td>
</tr>
<tr>
<td>Carotid artery</td>
<td>54.1 ± 11.3*</td>
<td>19.1 ± 5.2</td>
<td>35.7 ± 12.2</td>
</tr>
<tr>
<td>Pulmonary artery</td>
<td>62.6 ± 14.4*</td>
<td>16.3 ± 2.9NS</td>
<td>43.3 ± 16.1NS</td>
</tr>
<tr>
<td>Portal vein</td>
<td>64.2 ± 13.9*</td>
<td>23.6 ± 4.6†</td>
<td>43.1 ± 13.7NS</td>
</tr>
<tr>
<td>Hepatic blood supply</td>
<td>61.7 ± 13.5</td>
<td>22.5 ± 4.7</td>
<td>41.2 ± 13.3</td>
</tr>
<tr>
<td>Hepatic vein</td>
<td>66.1 ± 13.7NS</td>
<td>19.4 ± 4.0*</td>
<td>41.5 ± 14.0NS</td>
</tr>
</tbody>
</table>

Data are means ± SE of individual plasma concentrations given in pmol/l; *n = 9 pigs. Plasma samples were measured with midregion (4304), COOH-terminal (4305), and NH2-terminal (4383) RIAs for glucagon. Hepatic blood supply was calculated on the assumption that 75% originates from the portal vein and 25% is of arterial origin. NS, not significant. *P < 0.05, ‡P < 0.01 and †P < 0.005, difference from carotid artery and between hepatic vein and hepatic blood supply.

Fig. 1. Regional balance of endogenous glucagon immunoreactivity calculated as the ratio of the arteriovenous concentration difference divided by the arterial concentration. Hepatic blood supply was calculated on the assumption that 75% originates from the portal vein and 25% is of arterial origin. Data are means ± SE of individual balances across each organ, calculated for each animal; n = 9. *P < 0.05, ‡P < 0.01, difference from 0. Positive balance indicates net extraction; negative balance indicates net secretion.

COOH-terminal assays, and there was a trend (P = 0.066) toward a secretion to be detected with the NH2-terminal assay, whereas the midregion assay detected a modest secretion across the hepatic bed.

Fig. 2. Increase in plasma glucagon immunoreactivity in blood sampled from the carotid artery during glucagon infusion (5 pmol·kg⁻¹·min⁻¹ iv), measured with midregion (●), COOH-terminally-directed (▲), and NH2-terminally-directed (○) RIAs. Data are means ± SE; n = 9. Horizontal arrow indicates period of infusion.
The MCR and distribution volumes were similar irrespective of which assay was used (MCR: 14.4 ± 1.1, 13.6 ± 1.7, and 17.0 ± 1.7 ml·kg⁻¹·min⁻¹; Vd: 68.3 ± 9.0, 51.3 ± 8.3, and 56.1 ± 7.0 ml/kg; midregion, COOH-terminal, and NH2-terminal assays, respectively).

**Effect of DPP IV inhibition on glucagon stability in vivo.** DPP IV inhibition had no significant effect on the metabolism of either endogenous or exogenous glucagon in vivo (Fig. 4). Thus basal glucagon immunoreactivity (before the start of each infusion) was not significantly altered by DPP IV inhibition (57 ± 7 vs. 50 ± 8, 8.5 vs. 9 ± 6, and 27 ± 6 vs. 29 ± 7 pmol/l; glucagon alone vs. glucagon + valine-pyrrolidide and midregion, COOH-terminal, and NH2-terminal assays, respectively), and arterial glucagon concentrations remained similar during infusion of glucagon alone and together with valine-pyrrolidide (incremental plateau concentration: 44 ± 5 vs. 43 ± 2, 53 ± 7 vs. 43 ± 1, and 32 ± 2 vs. 30 ± 1 pmol/l; incremental AUC: 1,479 ± 240 vs. 1,352 ± 147, 1,434 ± 261 vs. 1,307 ± 83, and 928 ± 17 vs. 908 ± 98 pmol·l⁻¹·min⁻¹; glucagon alone vs. glucagon + valine-pyrrolidide and midregion, COOH-terminal, and NH2-terminal assays, respectively).

**DISCUSSION**

In the present study, we have examined the metabolism of glucagon in vivo. The use of anesthetized animals ensured that the catheters remained accu-

### Table 3. Plasma concentrations of glucagon during a glucagon infusion

<table>
<thead>
<tr>
<th>Assay</th>
<th>Midregion</th>
<th>COOH-Terminal</th>
<th>NH2-Terminal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carotid artery</td>
<td>435 ± 43</td>
<td>432 ± 49</td>
<td>325 ± 34</td>
</tr>
<tr>
<td>Renal vein</td>
<td>231 ± 33†</td>
<td>217 ± 30‡</td>
<td>160 ± 28‡</td>
</tr>
<tr>
<td>Femoral vein</td>
<td>335 ± 27†</td>
<td>316 ± 52‡</td>
<td>258 ± 23‡</td>
</tr>
<tr>
<td>Carotid artery</td>
<td>423 ± 37</td>
<td>446 ± 67</td>
<td>337 ± 37</td>
</tr>
<tr>
<td>Pulmonary artery</td>
<td>419 ± 39NS</td>
<td>452 ± 56NS</td>
<td>339 ± 45NS</td>
</tr>
<tr>
<td>Portal vein</td>
<td>444 ± 46NS</td>
<td>376 ± 55*</td>
<td>267 ± 36*</td>
</tr>
<tr>
<td>Hepatic blood supply</td>
<td>441 ± 43</td>
<td>388 ± 59</td>
<td>284 ± 35</td>
</tr>
<tr>
<td>Hepatic vein</td>
<td>380 ± 40NS</td>
<td>387 ± 60*</td>
<td>283 ± 34NS</td>
</tr>
</tbody>
</table>

Data are means ± SE of individual plasma concentrations given in pmol/l; n = 9 pigs. Plasma samples were measured with midregion (4304), COOH-terminal (4305), and NH2-terminal (4830) RIAs for glucagon. Hepatic blood supply was calculated on the assumption that 75% originates from the portal vein and 25% is of arterial origin. *P < 0.05, †P < 0.005, difference from carotid artery and between hepatic vein and hepatic blood supply.

![Fig. 3. Regional extraction of glucagon during exogenous infusion calculated as the ratio of the arteriovenous concentration difference divided by the arterial concentration. Hepatic blood supply was calculated on the assumption that 75% originates from the portal vein and 25% is of arterial origin. Data are means ± SE of individual extractions across each organ, calculated for each animal after subtraction of endogenous concentrations at each site; n = 9. *P < 0.05, †P < 0.01, difference from 0. Positive extraction indicates net extraction; negative balance indicates net secretion.](image)

![Fig. 4. Increase in plasma glucagon immunoreactivity in blood samples from the carotid artery, measured with midregion (α), COOH-terminally-directed (●), and NH2-terminally-directed (○) RIAs. Animals received a 30-min infusion of glucagon (1 pmol·kg⁻¹·min⁻¹ iv). Thirty minutes after the end of the first glucagon infusion, valine-pyrrolidide (val-pyd; 300 μmol/kg) was given, and after an additional 10 min, a 2nd glucagon infusion was initiated. Data are means ± SE; n = 5. Horizontal arrows indicate period of glucagon infusions.](image)
rately placed for blood sampling during the experiment and possibly also reduced any stress reaction associated with the presence of intravascular catheters and ligation of catheterized vessels, although it cannot be excluded that the anesthesia itself may have had an effect on the metabolism compared with the conscious animal. The use of three well-characterized assays made it possible to identify qualitatively the type of degradation occurring. The midregion assay detects glucagon fragments with limited degradation from each end of the molecule; a reduction in immunoreactivity detected with this assay, therefore, suggests extensive degradation to disrupt the central sequence of the peptide. A reduction measured with the COOH-terminal assay indicates degradation occurring at the COOH-terminal end of the peptide, since the assay is dependent on the presence of the intact COOH-terminus of glucagon, whereas reduced immunoreactivity measured with the NH2-terminal assay is indicative of loss of the NH2-terminal residues, given the failure of the antibody to recognize the NH2-terminally truncated products of DPP IV-mediated cleavage. However, measurements of glucagon immunoreactivity in the basal state are complicated by the cross-reactivity of the intestinal products of proglucagon processing. Thus the finding that the three assays measure significantly different amounts of glucagon in the basal state (midregion > NH2 terminus > COOH terminus) may simply be explained by the assay specificities. The midregion assay detects not only glucagon but also glicentin and oxyntomodulin from the intestine, and the NH2-terminal assay measures both glucagon and oxyntomodulin, whereas only the COOH-terminal assay is specific for pancreatic glucagon. Nevertheless, it cannot be excluded that part of these differences may reflect differing degrees of degradation from each end of the molecule. However, a comparison of the arterial concentrations measured during the glucagon infusion, when contributions of endogenous moieties to the overall immunoreactivity are reduced, reveals no significant differences between the assays. This would tend to suggest that any degradation of glucagon involves a more complete disruption of the peptide rather than a limited proteolysis from one or the other end of the molecule, so any reduction in immunoreactivity will be detected with all three assays. However, it cannot be excluded that a more limited degradation occurs in the midregion of the peptide beyond residue 15. This could generate two fragments, one of which would be detected with both NH2-terminal and midregion assays and the other of which would be detected only with the COOH-terminal assay, i.e., a degradation that would escape notice by leaving equal concentrations of immunoreactive fragments to be detected with each assay.

When interpreting the organ extractions of glucagon, it should be borne in mind that the calculations of glucagon extraction are expressed as percent extraction relative to the arterial supply. However, because we did not assess individual organ blood flows, we cannot exclude the possibility that these may have changed during the course of the experiment. Thus, although the percentage of glucagon supplied to each organ that is extracted under endogenous and exogenous conditions can be compared directly, it is possible that the individual organs’ contribution to the overall metabolic clearance may vary between the two conditions.

The negative balance (−40%) of glucagon immunoreactivity across the portal bed, detected with all three assays under basal conditions, reflects the net secretion of glucagon from the pancreas and of glicentin and oxyntomodulin from the gut. However, the portal bed also appears to be a site of glucagon removal, since during the infusion an extraction of >10% was detected with the COOH- and NH2-terminal assays. The fact that this was not seen with the midregion assay indicates that the degradation was not extensive, leaving the central sequence of the peptide intact.

The brain and the lung seem not to be major sites of glucagon degradation. Thus, under endogenous conditions, an extraction was detected only with the midregion assay, suggesting either a limited cleavage occurring in the central part of the glucagon molecule or, perhaps, degradation of other endogenous proglucagon products. Given that no extraction of exogenously supplied glucagon was detected in the lung (changes across the brain could not be determined due to a spill-over of the infused peptide into the jugular vein from the contralateral infusion site), this would tend to suggest that the pulmonary extraction seen under endogenous conditions may be due to degradation of glicentin, since it was only detected with the midregion assay. The significance of this is unknown.

Degradation across the hindlimb, representing metabolism by connective, supportive, and muscular tissues, was evident, particularly with the COOH-terminal assay (up to 30%) but also with the midregion assay (20%) during exogenous infusion. This would be consistent with the action of neutral endopeptidase (NEP) 24.11, an enzyme with a widespread distribution (17) that has been reported to degrade glucagon in vitro at cleavage sites located predominantly in the midregion and COOH-terminal half of the molecule (24). In support of this, a preliminary study has indicated that a selective inhibitor of NEP 24.11 improves the metabolic stability of glucagon in vivo (45).

A considerable proportion of both endogenous (~30%) and exogenous (~40%) glucagon was removed during passage across the renal bed. During exogenous glucagon infusion, the renal clearance was calculated after subtraction of the basal endogenous glucagon immunoreactivity, meaning that the given values apply to the extraction of the infused glucagon (assuming that the infusion itself does not affect endogenous glucagon levels). The corresponding values for endogenous glucagon reflect the renal clearance for all the glucagon-containing moieties. Glicentin and oxyntomodulin have longer $t_{1/2}$ values than glucagon (27, 32); so it is likely that their slower metabolic clearance masks the true renal clearance of endogenous glucagon, thus explaining the difference between the calculated values under the two conditions (basal and dur-
ing infusion) in the present study. The fact that similar renal extractions were revealed by all three assays during the infusion is indicative of glucagon being substantially degraded to small or undetectable fragments and not just a limited removal or one or two amino acids from one or the other terminus. This is in agreement with previously reported studies of renal metabolism in a number of species, including humans (14, 22, 30, 40). In the present study, calculation of the total renal clearance of glucagon (assuming that the kidneys receive 25% of the cardiac output) gives a value (∼320 ml/min) that is greater than the glomerular filtration rate. Detailed studies have indicated that this is an active process involving glomerular filtration, proximal tubular uptake with degradation by tubule brush-border enzymes on the luminal membrane, and reabsorption of the liberated amino acids, as well as peritubular uptake (4, 39, 44). The kidney has also been shown to be an important site of elimination for the related peptides GLP-1 and GIP (7, 10), so renal clearance is likely to be the ultimate factor regulating the metabolic stability of these peptides.

In the present study, the liver appeared not to be a major site of glucagon or glucagon-related metabolism, since degradation was not detected with any of the assays under basal conditions. During the infusion, a limited extraction of ∼10% was detected with the midregion assay, indicating that cleavage occurs in the central region of glucagon, which leaves the terminal portions intact. This is based on the assumption that 25% of the hepatic blood supply is of arterial origin; however, the conclusion is not altered if the arterial contribution is taken to be 20 or 33%. Interestingly, a novel, trypsin-like serine proteinase, which is reported to cleave glucagon at Arg-X, Lys-X, and Leu-X bonds (46), i.e., at sites that lie mostly in the midregion of the peptide, has been isolated from the pancreas. Such cleavages would be likely to hamper detection with the midregion assay (directed against the 6–15 sequence) but could result in NH2- and COOH-terminal fragments that would still be detected with the two terminal-specific assays. The human liver has not been reported to degrade glucagon to any measurable extent (16), whereas in other studies the hepatic extraction coefficient varied between 5 (42) and 35% (26) in dogs and sheep (2), with a somewhat higher value in rats (25). Differences between species could account for some of this variation (see Ref. 22), but it should also be noted that the poorly characterized antisera (in terms of cross-reactivity with other PG products) used in many of these earlier studies could contribute to some of these differences. A more recent study in conscious dogs found a hepatic extraction of 18% (11). In that study, glucagon immunoreactivity was determined using a nondiscriminating assay that also detects larger and smaller glucagon-containing moieties that lack biological activity and is thus likely to have a similar specificity to the midregion assay used in the present study. The hepatic extraction of glucagon, calculated after subtracting the cross-reacting portion of total glucagon immunoreactivity (determined by gel filtration), therefore indicates degradation occurring in the midregion of glucagon and that it is of similar magnitude to the 10% detected in the present study using the midregion assay. Hyperglucagonemia is seen in patients with liver cirrhosis (31, 43), but this appears to be due to hypersecretion of glucagon (36, 43) rather than reduced hepatic extraction. Thus the finding that the liver seems relatively unimportant in glucagon metabolism is in contrast to what has been found for the two incretin hormones GLP-1 and GIP, wherein significant hepatic NH2-terminal degradation was detected. For both of these peptides the liver appears to have a major role in regulating their insulinotropic activity (7, 10), since an intact NH2-terminal is required for receptor activation (19, 29).

It has been reported that liver plasma membranes can degrade glucagon, forming miniglucagon [glucagon-(19–29)] (1), which inhibits the hepatic membrane calcium pump (33) and reduces secretagogue-induced insulin secretion (5). Miniglucagon has an extremely short t1/2 (<10 s when incubated with liver membranes) and is apparently not detectable in plasma, suggesting that it is produced locally within target tissues (1). However, the degree of conversion of glucagon to miniglucagon is low (1% of total glucagon added to liver membranes), which could explain why a net extraction of glucagon by the liver was not detected in the present study. It cannot be excluded that the anesthetic used in the present study may have resulted in a change in splanchic blood flow. This would not be expected to change the hepatic fractional extraction of glucagon; however, we cannot totally rule out a more prominent role for the liver, since a change in blood flow may have altered the hepatic contribution to the overall clearance, although, as discussed in Ref. 11, glucagon clearance was comparable in anesthetized and conscious dogs.

The NH2-terminal dipeptide of glucagon (histidine-serine) does not suggest that glucagon would be particularly susceptible to degradation by the ubiquitous aminopeptidase DPP IV (35). However, recent reports indicate that high (micromolar) concentrations of glucagon can be metabolized by the purified enzyme in vitro (19, 41). In the present study, in vitro incubations of small concentrations of glucagon with recombinant human DPP IV also revealed that glucagon can be degraded, but apparently at a slower rate (t1/2 of >120 min) than reported for purified porcine renal DPP IV (t1/2 of ~19 min (19, 41)). Similarly, glucagon has been shown to be NH2-terminally degraded by human serum in vitro (19, 41), but with a t1/2 (~330 min (19)) that is much slower than that for GLP-1 (20 min (6)) or GIP [75 min (9)]. In the present study, too, incubation of picomolar concentrations of glucagon with porcine plasma in vitro revealed a slow rate of NH2-terminal degradation (t1/2 of 455 min). However, in contrast to the studies of Hinke et al. (19) and Pospisilik et al. (41), where the DPP IV inhibitor Ile-thiazolididetotally prevented NH2-terminal degradation, valine-pyrrolidide only marginally affected the degradation, raising the possibility that another NH2-terminally degrading en-
zyme, inhibitible by Ile-thiazolidide but not by valine-pyrrolidide, may be present in plasma. The results of the present study also seem to indicate that glucagon is not particularly susceptible to NH₂-terminal degradation in vivo, since during exogenous infusion NH₂-terminal immunoreactivity made up ~90% of total immunoreactivity (determined using the midregion assay) and 80% of COOH-terminal immunoreactivity. This is in contrast to the situation for GLP-1 and GIP, where NH₂-terminal immunoreactivity makes up only ~20% of total immunoreactivity (7, 10). The plasma t₁/₂ for glucagon was slightly shorter when determined with the NH₂- and COOH-terminal assays compared with the midregion assay, indicating that some degradation occurs at each terminus of the molecule. However, the MCR for glucagon was similar regardless of which assay was used, which suggests that limited degradation from each end of the molecule probably only contributes in a minor way to the overall clearance of the peptide. This limited NH₂-terminal degradation seen in vivo seems unlikely to be due to DPP IV, given the demonstration that glucagon is NH₂-terminally degraded only slowly in vitro (19, 41, present study) compared with GLP-1 and GIP (6, 7, 38), together with the finding that valine-pyrrolidide did not affect glucagon pharmacokinetics in vivo. Moreover, if significant NH₂-terminal degradation of glucagon by DPP IV were to occur in vivo, we would have expected to detect a hepatic extraction with the NH₂-terminal assay, since the liver has a relatively high level of DPP IV activity associated with the hepatocytes (15, 34). Indeed, hepatic extractions of 40% for GLP-1 (10) and 30% for GIP (7) have been reported, in contrast to the total absence of NH₂-terminal degradation of glucagon in the liver found in the present study. The lack of effect of the DPP IV inhibitor in vivo suggests, therefore, that the minor NH₂-terminal degradation of glucagon seen in vivo may be due to another NH₂-terminally degrading enzyme that is not inhibited by valine-pyrrolidide, a suggestion that is supported by the finding that valine-pyrrolidide is unable to prevent completely the NH₂-terminal degradation of GIP in vivo (7). Therefore, despite the results of in vitro kinetic analysis demonstrating that glucagon has a second-order rate constant (Kcat/Km, where Kcat is a catalytic constant) for DPP IV-mediated hydrolysis that is comparable to that for GLP-1 and GIP (19), the results of the present study indicate that DPP IV is less important for glucagon’s metabolic stability in vivo than it is for the two incretin hormones (7, 8). Furthermore, these findings underscore the need for caution when trying to extrapolate the results of in vitro kinetic studies to the situation in vivo, since they are not necessarily predictive of the physiological relevance of the enzyme in question.

The MCRs and volumes of distribution determined in the present study are very similar to those reported for the dog, where steady-state measurements with noncompartmental analysis and compartmental modeling both gave equivalent values, which indicated that glucagon is distributed in only one compartment with approximately the same volume as plasma (11). To verify that all major sites of glucagon elimination have been identified, the individual extractions across each organ were combined (allowing for a cardiac output for a 33-kg pig of ~5 l/min passing through the lungs and distributed with 25% to the kidneys, 33% to the liver, 25% to the extremities, 10% to the portal bed, and the remainder to the brain and taking into account a hematocrit of 45%). This gave the total clearance of glucagon as 0.45 ± 0.17, 0.47 ± 0.13, and 0.43 ± 0.09 l/min, as determined with NH₂-terminal, COOH-terminal, and midregion assays, respectively. These values do not differ significantly from the values calculated directly (0.57 ± 0.07, 0.46 ± 0.06, and 0.49 ± 0.05 l/min of NH₂-terminal, COOH-terminal, and midregion assays, respectively), suggesting that the major sites of glucagon elimination have been identified.

In conclusion, this study has identified the kidney as the major site of glucagon elimination in vivo and indicated that the liver plays little, if any, role in glucagon metabolism. Furthermore, the enzyme DPP IV appears to be unimportant in terms of glucagon’s metabolic stability in vivo, in contrast to its role in the metabolism of the other proglucagon-derived peptides, GLP-1 (6, 28) and GLP-2 (12).

DISCLOSURES

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