Neutral endopeptidase 24.11 is important for the degradation of both endogenous and exogenous glucagon in anesthetized pigs

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Submitted 4 August 2003; accepted in final form 30 April 2004

Trebbein, Ramona, Letty Klarskov, Mette Olesen, Jens J. Holst, Richard D. Carr, and Carolyn F. Deacon. Neutral endopeptidase 24.11 is important for the degradation of both endogenous and exogenous glucagon in anesthetized pigs. Am J Physiol Endocrinol Metab 287: E431–E438, 2004. First published May 4, 2004; 10.1152/ajpendo.00353.2003.—Glucagon has a short plasma t1/2 in vivo, with renal extraction playing a major role in its elimination. Glucagon is degraded by neutral endopeptidase (NEP) 24.11 in vitro, but the physiological relevance of NEP 24.11 in glucagon metabolism is unknown. Therefore, the influence of candoxatril, a selective NEP inhibitor, on plasma levels of endogenous and exogenous glucagon was examined in anesthetized pigs. Candoxatril increased endogenous glucagon concentrations, from 6.3 ± 2.5 to 20.7 ± 6.3 pmol/l [COOH-terminal (C)-RIA, P < 0.05]. During glucagon infusion, candoxatril increased the t1/2 determined by C-RIA (from 3.0 ± 0.5 to 17.0 ± 2.5 min, P < 0.005) and midregion (M)-RIA (2.8 ± 0.5 to 17.0 ± 3.0 min, P < 0.01) and reduced metabolic clearance rates (MCR; 19.1 ± 3.2 to 9.4 ± 2.0 ml·kg⁻¹·min⁻¹, P < 0.02, C-RIA; 19.2 ± 4.8 to 9.0 ± 2.3 ml·kg⁻¹·min⁻¹, P < 0.05, M-RIA). However, neither t1/2 nor MCR determined by NH₂-terminal (N)-RIA were significantly affected (t1/2; 2.7 ± 0.4 to 4.5 ± 1.6 min; MCR, 30.3 ± 6.4 to 28.5 ± 9.0 ml·kg⁻¹·min⁻¹), suggesting that candoxatril had no effect on NH₂-terminal degradation but leads to the accumulation of NH₂-terminally truncated forms of glucagon. Determination of arteriovenous glucagon concentration differences revealed that renal glucagon extraction was reduced (but not eliminated) by candoxatril (from 40.4 ± 3.8 to 18.6 ± 4.1%, P < 0.02, C-RIA; 29.2 ± 3.1 to 14.7 ± 2.2%, P < 0.02, M-RIA; 26.5 ± 4.0 to 19.7 ± 3.5%, P < 0.06, N-RIA). Femoral extraction was reduced by candoxatril determined by C-RIA (from 22.7 ± 2.4 to 8.0 ± 5.1%, P < 0.05) but was not changed significantly when determined using M- or N-RIAs (10.0 ± 2.8 to 4.7 ± 3.7%, M-RIA; 10.5 ± 2.5 to 7.8 ± 4.2%, N-RIA). This study provides evidence that NEP 24.11 is an important mediator of the degradation of both endogenous and exogenous glucagon in vivo.

candoxatril; peptide; enzyme inhibition

THE PANCREATIC PEPTIDE GLUCAGON has a short plasma elimination half-life in vivo (<3 min) in pigs, and there is evidence from many studies to indicate that the kidneys play an important role in determining the peptide’s metabolic clearance (10, 11, 17, 20, 31). Early studies suggested that this is an active process involving glomerular filtration with proximal tubular uptake, degradation by tubule brush-border enzymes on the luminal membrane with reabsorption of the liberated amino acids, and peritubular uptake and subsequent catabolism (2, 30, 42). However, the precise mechanisms and enzymes involved have not yet been determined. Neutral endopeptidase (NEP) 24.11 [EC 3.4.24.11, also known as nephrilysin; see Turner et al. (43) for review] is a membrane-bound zinc metallopeptidase, with a broad substrate specificity, that degrades peptides on the amino terminal side of aromatic or hydrophobic amino acids. This enzyme has a wide-spread distribution and is found in particularly high concentration in the kidney, namely in the glomerulus and the proximal tubule brush-borders and intra-cellularly (13), where it could be speculated to be involved in the renal clearance of peptide hormones. Under in vitro conditions, recombinant NEP 24.11 can degrade members of the glucagon-vasoactive intestinal peptide (VIP) family of peptides (18). Products of the proglucagon (PG) gene [glucagon and glucagon-like peptide 1 (GLP-1)] were shown to be relatively good substrates, whereas the structurally related peptide glucose-dependent insulinotropic polypeptide (GIP) was somewhat less susceptible. However, nothing is yet known about the potential relevance of this enzyme in the metabolism of these peptides in vivo. During another study examining the effect of candoxatril on the metabolism of exogenous GLP-1 in vivo (33), we noticed that concentrations of endogenous glucagon were increased during the period of NEP inhibition. Candoxatril, which is the prodrug of the active compound candoxatrilat, is rapidly absorbed upon oral administration and converted to the active drug by esterases in the plasma (19, 48). It was developed as a potent competitive and selective NEP 24.11 inhibitor (28, 37) and has been used in a number of clinical studies as a pharmacological tool to investigate the efficacy of NEP inhibition in hypertension and heart failure (23, 27, 28, 46). Therefore, the present study was undertaken to investigate whether NEP 24.11 is involved in glucagon metabolism in vivo, using candoxatril to inhibit NEP during an exogenous glucagon infusion. Plasma samples were analyzed using three well-characterized radioimmunoassays (RIAs), specific for different epitopes in the glucagon molecule. The suitability of this approach to reveal metabolic degradation has previously been demonstrated for GLP-1 and GIP (4, 6, 8, 9) and, recently, also for glucagon (7).

MATERIALS AND METHODS

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 experimentation, 1987) and were carried out after permission for animal experimentation, 1987) and were carried out after permission

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had been granted by the Animal Experiments Inspectorate, Ministry of Justice, Denmark.

Overnight-fasted Danish LYY strain pigs (30–35 kg) were used. After premedication with midazolam (0.5 mg/kg Dormicum; Roche, Basel, Switzerland) and ketamine (10 mg/kg Ketaminol; Veterinaria, Zurich, Switzerland), animals were anesthetized with intravenous α-chloralose (66 mg/kg; Merck, Darmstadt, Germany) and ventilated with intermittent positive pressure using N2O-O2. Catheters were placed in the right carotid artery for sampling of arterial blood, in a left ear vein for peptide infusion, and in a right ear vein for candoxatril administration. In addition, catheters were placed in the renal and femoral veins as described previously (9). After surgical preparation, animals were heparinized and left undisturbed for 30 min. Anesthesia was maintained with additional chloralose as necessary.

Six animals were used, each receiving two intravenous infusions of glucagon, one before and one after administration of the selective NEP 24.11 inhibitor candoxatril [(28), a gift from Dr. Jane Lundbeck, Novo Nordisk, Målov, Denmark]. Synthetic glucagon (Bachem, Bubendorf, Switzerland), dissolved in 0.9% NaCl containing 1% human serum albumin (Calbiochem, VWR International, Albertslund, Denmark), was infused at a rate of 1 pmol/kg min−1 for 30 min with a syringe pump, commencing at time 0. Arterial blood samples (4 ml) were taken at −30, −20, −10, 0, 5, 10, 15, 20, 25, and 30 min from infusion. After 30 min of glucagon infusion, blood sampling was stopped, and further blood samples were taken at 1, 2, 4, 6, 10, 15, 20, and 30 min. To allow determination of arteriovenous glucagon concentration differences, simultaneous blood samples (2 ml) were collected from the renal and femoral veins at 20 and 25 min. Thirty minutes after the cessation of the first glucagon infusion, candoxatril (5 mg/kg, dissolved in 0.9% NaCl) was given as a bolus intravenous injection over 2 min. Blood samples were taken at 10, 20, and 30 min, after which the second glucagon infusion was started and the protocol was repeated for blood sampling. The volume of blood taken over the entire procedure amounted to 156 ml, which for a 35-kg pig is ~4% of the blood volume, although after each blood sample was taken the fluid volume was replaced by flushing the catheters with 5 ml of 0.9% NaCl. This protocol has previously been shown not to affect blood pressure or heart rate (9). In the present study, blood pressure and pulse rate as well as electrocardiogram were monitored continuously throughout the experiment and remained stable [mean arterial blood pressure, 113 ± 8 mmHg; pulse rate, 96 ± 9 beats/min (mean ± SD)]. The experimental protocol does not affect the metabolic clearance of a second compared with a first infusion of the related peptides GLP-1 (5) or GIP (4), but to confirm the stability of the preparation with respect to glucagon metabolism, three additional pigs received two successive glucagon infusions as above but without administration of candoxatril. Blood sampling from the carotid artery and veins was as before.

Blood glucose was measured immediately (One Touch II; Lifescan, Lyngby, Denmark). Blood samples were collected into chilled tubes containing EDTA (7.4 mmol/l final concentration) for hormonal analysis and into heparinized tubes for NEP 24.11 enzyme activity determination and kept on ice until centrifugation at 4°C. Plasma was analyzed and stored at −20°C until analysis.

Hormonal assays. Three different RIA’s for glucagon were used. Antiserum 4304 (15, 16) is directed toward the midregion (M) amino acid sequence 6–15 of glucagon. It binds with equal affinity to all peptides containing this sequence, recognizing both COOH- and NH2-terminally truncated [e.g., glucagon-(1–17), (1–18), (29)], and extended [PG-(1–69) (glicentin) and PG-(33–69) (oxyntomodulin)] forms, and can thus be considered as being largely processing independent. It has reduced binding to fragments with substitutions in the central portion of the molecule [25% cross-reactivity with des-His3-Glu3-glucagon–H20 (Sigma)], and to some extent to species containing the 6–15 sequence [e.g., glucagon-(1–12), (13–17), (18–29), (19–29)]. The assay has a detection limit of 2 pmol/l. Antiserum 4305 (15, 16) requires the intact COOH terminus of glucagon and does not cross-react with COOH-terminally truncated [e.g., glucagon-(1–12), (−1–17), (−1–18), (−13–17)], extended (glicentin and oxyntomodulin), or modified (e.g., des-His4-Glu3-glucagon–NH2) molecules, making it specific for fully processed glucagon of pancreatic origin. It does, however, cross-react with fully glucagon fragments truncated from the NH2 terminus [e.g., glucagon-(18–29), (−19–29), (−29)]. The assay has a detection limit of 2 pmol/l. For all assays, the intra-assay coefficient of variation was <6%. Standard and 125I-labeled glucagon were obtained from Novo Nordisk ( Bagsværd, Denmark). Plasma samples were extracted with ethanol (70% vol/vol; final concentration) before assay, giving recoveries of added glucagon or oxyntomodulin of 75%. Insulin immunoreactivity was determined in unextracted plasma by use of antiserum 2004, as previously described (29).

NEP 24.11 activity determination. NEP 24.11 activity was assessed using assay conditions modified from a previously published method (39). This is a two-stage enzymatic reaction, where NEP 24.11 cleaves the substrate (Suc-Ala-Ala-Phe-4-nitroanilide) on the amino side of phenylalanine (reaction 1). The Phe-4-nitroanilide formed is subsequently degraded by aminopeptidase M to phenylalanine and 4-nitroaniline (reaction 2), resulting in an increase in absorbance. Briefly, plasma was diluted ninefold in 0.9% NaCl, and 50 μl were added to a quartz cuvette (1-cm path), followed by 20 μl of aminopeptidase M containing 4 μg of enzyme protein (0.096 U Sigma). The reaction was initiated by addition of 280 μl of substrate (0.262 μmol, diluted in 0.05 mol/l Tris buffer, pH 7.5; Bachem). Samples were incubated at 26°C for 90 min, and the increase in absorbance (405 nm) was read in a spectrophotometer every 15 min. Because it has been reported that commercially available aminopeptidase M preparations may be contaminated with NEP 24.11 (39), a control incubation (50 μl of 0.9% NaCl, 20 μl of aminopeptidase M, and 280 μl of substrate) was made. As a positive control, porcine kidney NEP 24.11 (Calbiochem) was added in place of plasma. The specificity of the reaction for NEP 24.11 was tested by the addition of the active NEP 24.11 inhibitor candoxatril (3.5 μmol/l final dilution; gift from Dr. Jane Lundbeck, Novo Nordisk).

Calculations and statistical analysis. For each animal, the net extraction of glucagon across the kidney and hindlimb was calculated as a ratio, defined as

\[
\frac{\left[\text{glucagon-IR}_{\text{carotid artery}} - \text{glucagon-IR}_{\text{vein}}\right]}{\text{glucagon-IR}_{\text{carotid artery}}} \quad \text{where } \left[\text{glucagon-IR}_{\text{carotid artery}} \right] \text{ is the concentration of glucagon immunoreactivity in the carotid artery and } \left[\text{glucagon-IR}_{\text{vein}}\right] \text{ is the concentration of glucagon immunoreactivity in the vein.}
\]

During the glucagon infusions, a stable plateau in arterial glucagon levels was achieved after 20 min. The plateau concentration was therefore defined as the mean of the last four measurements during the glucagon infusion. The plasma t1/2 was calculated by log-linear regression analysis of peptide concentrations in samples collected after termination of the infusion and after subtraction of endogenous arterial glucagon concentrations, and the metabolic clearance rate (MCR) was calculated using the formula

\[
\text{MCR} = \frac{\text{infusion rate}}{\left[\text{glucagon-IR}_{\text{plasma}} - \text{glucagon-IR}_{\text{basal}}\right]} \quad \text{where } \left[\text{glucagon-IR}_{\text{plasma}}\right] \text{ is the plateau concentration of glucagon immunoreactivity in the carotid artery during the infusion and } \left[\text{glucagon-IR}_{\text{basal}}\right] \text{ is the concentration of endogenous glucagon immunoreactivity in the carotid artery.}
\]

The incremental area under the glucagon curves (AUC) was calculated using the trapezoidal method after subtraction of endogenous
glucagon concentrations measured in the sample preceding the start of each infusion.

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 $t_{1/2}$, arterial concentration differences and MCR were analyzed using ANOVA (where appropriate) and two-tailed $t$-tests for paired and nonpaired data. Single-sample two-tailed tests were used to evaluate organ extractions. $P < 0.05$ was considered significant.

RESULTS

NEP 24.11 activity. Pilot studies showed that, after initiation of the reaction by addition of substrate, the absorbance increased linearly with time. Therefore, after the baseline absorbance was measured, the subsequent increase in absorbance was determined after 60 min of incubation. Control incubations with aminopeptidase M alone revealed a time-dependent increase in absorbance, which was inhibited by addition of candoxatril (data not shown). Similarly, incubations with porcine kidney NEP 24.11 time-dependently increased absorbance, which was again inhibitable by candoxatril (data not shown).

Plasma samples collected before and after in vivo administration of candoxatril were tested for NEP 24.11 activity. The results were expressed as a percentage of the NEP 24.11 activity (absorbance) in the basal samples, taken before candoxatril administration in vivo, using the assumption that the inhibitor in the plasma samples would inhibit both the endogenous plasma NEP 24.11 and the contaminant in the aminopeptidase M preparation to the same extent (they are both porcine). This showed that plasma samples collected 20 min after in vivo administration of candoxatril contained enough inhibitor to inhibit NEP 24.11 activity in vitro by $78.0 ± 2.0\%$, falling gradually to $53.5 ± 2.2\%$ inhibition in the samples collected at the end of the experiment (110 min after in vivo candoxatril administration; Fig. 1).

Comparison of two successive glucagon infusions in the absence and presence of candoxatril. Endogenous glucagon immunoreactivity varied significantly (ANOVA, $P < 0.001$) according to which assay was used for determination, with highest concentrations being measured by the M-RIA (46.8 ± 8.9 pmol/l) and lowest with the C-RIA (6.3 ± 2.5 pmol/l). Candoxatril administration increased basal (endogenous) glucagon immunoreactivity threefold (from $6.3 ± 2.5$ to $20.7 ± 6.3$ pmol/l, C-RIA, $P < 0.05$), although endogenous glucagon immunoreactivity determined with M- and N-RIAs was not significantly affected (from 46.8 ± 8.9 to 58.3 ± 9.5 and 24.7 ± 4.3 to 26.8 ± 4.8 pmol/l, M- and N-RIAs, respectively). During infusion of glucagon alone (Fig. 2), plasma glucagon concentrations also varied according to the assay used (ANOVA, $P < 0.01$). Absolute values determined by C- and N-RIAs were similar but were significantly ($P < 0.01$) lower than those determined by the M-RIA (Table 1 and Fig. 2A). After candoxatril administration, the absolute plateau values determined by M- and C-RIAs were both significantly increased compared with glucagon infusion alone, whereas NH$_2$-terminal immunoreactivity was similar during the infusions (Fig. 2A and Table 1). When expressed as the increase relative to basal immunoreactivity (i.e., after subtraction of endogenous immunoreactivity in samples before the first glucagon infusion), concentrations determined by M- and C-RIAs were similar throughout the experiment, and both were further increased to the same extent by candoxatril administration (Fig. 2B). The corresponding values for NH$_2$-terminal glucagon concentrations were lower and were not affected by candoxatril. Compared with infusion of glucagon alone, the incremental AUCs during the infusion were significantly increased by candoxatril when determined by C- and M-RIAs (from 1,320 ± 349 to 3,752 ± 972 pmol·l$^{-1}$·min$^{-1}$, $P < 0.03$, C-RIA and from 1,384 ± 470 to 3,899 ± 1,165 pmol·l$^{-1}$·min$^{-1}$, $P < 0.03$, M-RIA). However, although there was a tendency for NH$_2$-terminal immunoreactivity to increase (incremental AUC, from 760 ± 256 to 1,131 ± 415 pmol·l$^{-1}$·min$^{-1}$), this was not significant. In accord with this, the plasma $t_{1/2}$ determined by C- and M-RIAs increased more than fivefold (from 3.0 ± 0.5 to 17.0 ± 2.5 min, $P < 0.005$, C-RIA; 2.8 ± 0.5 to 17.0 ± 3.0 min, $P < 0.01$, M-RIA) and the metabolic clearance rates fell (19.1 ± 3.2 to 9.4 ± 2.0 ml·kg$^{-1}$·min$^{-1}$, $P < 0.02$, C-RIA; 19.2 ± 4.8 to 9.0 ± 2.3 ml·kg$^{-1}$·min$^{-1}$, $P < 0.05$, M-RIA). However, neither $t_{1/2}$ nor MCR rates determined by N-RIA were significantly affected by candoxatril ($t_{1/2}$ 2.7 ± 0.4 to 4.5 ± 1.6 min; MCR, 30.3 ± 6.4 to 28.5 ± 9.0 ml·kg$^{-1}$·min$^{-1}$). When individual organ arteriovenous glucagon concentration differences were determined, significant ($P < 0.002$) extraction of glucagon by the kidneys was detected. Inclusion of candoxatril reduced, but did not eliminate, this renal extraction (from 40.4 ± 3.8 to 18.6 ± 4.1%, $P < 0.02$, C-RIA; 29.2 ± 3.1 to 14.7 ± 2.2%, $P < 0.02$, M-RIA; 26.5 ± 4.0 to 19.7 ± 3.5%, $P < 0.06$, N-RIA). Femoral extraction (representing degradation by connective, supportive, and muscular tissues) was also detected, particularly by the C-RIA ($P < 0.005$) but also with the other two assays ($P < 0.02$). This was reduced by candoxatril when determined using the C-RIA (from 22.7 ± 2.4 to 8.0 ± 5.1%, $P < 0.05$) but was not changed significantly when determined using M- or N-RIAs (from 10.0 ± 2.8 to 4.7 ± 3.7%, M-RIA; 10.5 ± 2.5 to 7.8 ± 4.2%, N-RIA).
Fig. 2. Plasma glucagon immunoreactivity in blood sampled from the carotid artery, expressed as absolute concentrations (A) and as relative increase \( \Delta \) after subtraction of endogenous concentrations measured in samples before start of the 1st glucagon infusion (B). Animals received 30-min infusions of glucagon (1 pmol\( \cdot \)kg\(^{-1} \cdot \)min\(^{-1} \)), given alone and during NEP 24.11 inhibition. Candoxatril (5 mg/kg) was given 30 min after end of the 1st glucagon infusion, and after a further 30 min, the 2nd glucagon infusion was initiated. Samples were measured with midregion (○), COOH-terminally-directed (▲), and NH\(_{2}\)-terminally-directed (■) RIAs. Data are means ± SE; \( n = 6 \). Horizontal arrows indicate periods of infusion.

Blood glucose increased in response to both glucagon infusions but to a lesser extent during the second infusion in the presence of candoxatril (Fig. 3A), with the AUC\(_{0–60 \text{ min}} \) during the glucagon infusion with candoxatril corresponding to 85.2 ± 2.2% of the corresponding area during infusion of glucagon alone. A similar pattern was seen for plasma insulin concentrations (Fig. 3B).

Comparison of two successive glucagon infusions. In the control experiment, when glucagon was infused twice in the absence of candoxatril, glucagon pharmacokinetics remained stable (Fig. 4A). Thus there was no significant difference in the \( t_{1/2} \) (2.8 ± 0.1 to 3.1 ± 0.2 min, C-RIA; 2.9 ± 0.3 to 3.1 ± 0.2 min, M-RIA; 2.7 ± 0.4 to 2.8 ± 0.5 min, N-RIA; first infusion compared with second) or MCR (19.4 ± 1.2 to 21.4 ± 0.9 ml\( \cdot \)kg\(^{-1} \cdot \)min\(^{-1} \), C-RIA; 11.1 ± 1.5 to 12.5 ± 1.4 ml\( \cdot \)kg\(^{-1} \cdot \)min\(^{-1} \), M-RIA; 25.7 ± 3.4 to 26.1 ± 1.9 ml\( \cdot \)kg\(^{-1} \cdot \)min\(^{-1} \), N-RIA; first infusion compared with second). Calculation of the renal and femoral extractions also revealed no significant differences between the two infusions (renal extraction, 49.7 ± 5.4 to 46.9 ± 6.0%, C-RIA; 39.5 ± 5.3 to 36.3 ± 2.7%, M-RIA; 40.2 ± 4.0 to 37.4 ± 4.6%, N-RIA; femoral extraction, 28.6 ± 5.5 to 18.4 ± 2.5%, C-RIA; 23.1 ± 4.4 to 10.9 ± 5.3%, M-RIA; 28.5 ± 6.7 to 19.7 ± 4.1%, N-RIA; first infusion compared with second).

Table 1. Plasma concentrations of glucagon during infusion of glucagon alone and with NEP 24.11 inhibition

<table>
<thead>
<tr>
<th>Glucagon Concentrations, pmol/l</th>
<th>Midregion</th>
<th>COOH Terminal</th>
<th>NH(_{2}) Terminal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucagon alone</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carotid artery</td>
<td>92 ± 22</td>
<td>47 ± 12</td>
<td>48 ± 11</td>
</tr>
<tr>
<td>Renal vein</td>
<td>67 ± 7(^{†})</td>
<td>28 ± 7(^{†})</td>
<td>35 ± 8(^{*})</td>
</tr>
<tr>
<td>Femoral vein</td>
<td>80 ± 20(^{*})</td>
<td>36 ± 9(^{*})</td>
<td>42 ± 9(^{NS})</td>
</tr>
<tr>
<td>Glucagon + candoxatril</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carotid artery</td>
<td>139 ± 30</td>
<td>96 ± 23</td>
<td>59 ± 13</td>
</tr>
<tr>
<td>Renal vein</td>
<td>121 ± 29(^{†})</td>
<td>82 ± 23(^{†})</td>
<td>49 ± 13(^{†})</td>
</tr>
<tr>
<td>Femoral vein</td>
<td>127 ± 25(^{NS})</td>
<td>87 ± 20(^{NS})</td>
<td>52 ± 11(^{NS})</td>
</tr>
</tbody>
</table>

Data are means ± SE of individual plasma concentrations in samples taken during minutes 20–25 of each infusion; \( n = 6 \) pigs. Animals received two 30-min intravenous glucagon infusions (1 pmol\( \cdot \)kg\(^{-1} \cdot \)min\(^{-1} \)). During the 2nd infusion, neutral endopeptidase (NEP) 24.11 activity was inhibited by candoxatril (5 mg/kg). Plasma samples were measured with midregion (4304), COOH-terminal (4305), and NH\(_{2}\)-terminal (4830) RIAs for glucagon. NS, not significant. * \( P < 0.05 \), † \( P < 0.01 \), difference from carotid artery.
Sponding to activity in vitro by 80% of samples contained enough active inhibitor to inhibit NEP 24.11. In the present study, plasma glucagon immunoreactivity was determined with midregion ( ), COOH-terminally directed ( ), and NH2-terminally directed ( ) RIA. Data are means ± SE; n = 3. Horizontal arrow indicates period of glucagon infusions.

Fig. 4. Plasma glucagon immunoreactivity (A) and blood glucose concentrations (B) during and after 2 successive glucagon infusions (1 pmol·kg−1·min−1). Glucagon immunoreactivity was determined with midregion ( ), COOH-terminally directed ( ), and NH2-terminally directed ( ) RIA. Data are means ± SE; n = 3. Horizontal arrow indicates period of glucagon infusions.

In this study, the role of NEP 24.11 in glucagon metabolism in vivo was examined using the selective inhibitor candoxatril (28, 37). The study was carried out in anesthetized pigs, which ensured that vascular catheters remained accurately placed and possibly also reduced any stress associated with their presence and the ligation of catheterized vessels. However, it cannot be excluded that the anesthesia itself may have had an effect on glucagon metabolism compared with the conscious animal. Although the effective dose of candoxatril in pigs is unknown, the chosen dose (5 mg/kg) inhibits renal NEP in rats by 80–90% for ≥1 h (48), whereas 100 mg twice daily (corresponding to ~1.5 mg/kg twice daily) in humans significantly reduces plasma NEP activity (47). In the present study, plasma samples contained enough active inhibitor to inhibit NEP 24.11 activity in vitro by 80–50% (depending on the time elapsing after candoxatril administration). However, this is likely to be an underestimate of the true inhibition that occurred in vivo. In the activity assay, 50 µl of diluted plasma were incubated in a total volume of 350 µl, meaning that the inhibitor was diluted 70-fold in the assay compared with its concentration in the plasma. Furthermore, there is also the assumption that the endogenous NEP 24.11 activity in the plasma and that contaminating the aminopeptidase M preparation (39) were inhibited to the same extent, which may not necessarily be the case. However, the aminopeptidase M was derived from pig kidney, meaning that both endogenous and “exogenous” NEP 24.11 are porcine enzymes. Nevertheless, the data provide strong evidence that the dose of candoxatril used in vivo was sufficient to have had a significant inhibitory effect on NEP 24.11 activity.

Glucagon metabolism was studied by determining changes in immunoreactivity with three well-characterized RIAs with strict specificities for the terminal and midregions of glucagon, respectively, allowing qualitative identification of the degradation. Decreases in concentration determined with these assays thus accurately reflect decreased concentrations of peptide moieties containing these regions. Increases likewise reflect accumulation of such moieties. The midregion assay detects glucagon fragments with limited terminal truncations; a reduction in immunoreactivity, therefore, suggests extensive degradation to disrupt the peptide’s central sequence (residues 6–15). A reduction in COOH-terminal immunoreactivity indicates removal of COOH-terminal residues, since the assay depends on an intact COOH terminus, whereas reduced NH2-terminal immunoreactivity is indicative of loss of NH2-terminal residues since NH2-terminally truncated fragments are not recognized (7). As previously reported (7), endogenous glucagon immunoreactivity varied according to assay and is, at least partly, explained by assay specificities. Thus, although the COOH-terminal assay is specific for pancreatic glucagon, the NH2-terminal assay also detects oxyntomodulin, whereas the midregion assay cross-reacts with both glicentin and oxyntomodulin in addition to glucagon itself. Exogenous glucagon concentrations (expressed as incremental AUC, assuming that the infusion itself does not affect endogenous glucagon immunoreactivity), determined by COOH-terminal and midregion assays, were similar but exceeded those determined by the NH2-terminal assay, suggesting that some NH2-terminal degradation does occur in vivo. A candidate enzyme could be dipeptidyl peptidase IV (DPP IV), since kinetic studies have indicated that glucagon can be metabolized by DPP IV in vitro, generating an NH2-terminally truncated metabolite (14, 34). Glucagon is also NH2-terminally degraded by 20% human serum in vitro (14, 34) but with a relatively slow t1/2 (~330 min (14)). In a recent study (7), we have shown that plasma also can NH2-terminally degrade glucagon in vitro (t1/2 of 455 min), albeit more slowly than for the related peptides GLP-1 [20 min (9)] and GIP [75 min (8)], measured under the same assay conditions. In that study, we could not prevent the in vitro degradation by using valine-pyrrolidide [a selective DPP IV inhibitor (26)], nor could we demonstrate any significant effect of valine-pyrrolidide on metabolism of exogenous glucagon in vivo. Taken together, these observations seem to suggest that, although DPP IV can cleave glucagon, it may be unlikely to contribute in a major way to glucagon metabolism in vivo and that some other NH2-terminally cleaving enzyme could be involved. This latter suggestion is supported by the finding that valine-pyrrolidide, at a dose that completely prevents NH2-terminal degradation of exogenous GLP-1 in vivo (5), is unable to do the same for the related hormone GIP (4).

In vitro studies have indicated that members of the glucagon-VIP family of peptides can be degraded by NEP 24.11 (18). Six cleavage sites in the central and COOH-terminal regions of GLP-1 were identified, but the physiological rele-
vance was not examined. The amino acid sequence of glucagon reveals seven potential target bonds, and Hupe-Sodmann et al. (18) found that glucagon was hydrolyzed in vitro by NEP 24.11 just as readily as GLP-1. Three of these target bonds are identical in both peptides. Cleavage at two of them [which were suggested to be primary targets in GLP-1 (18) and correspond to Tyr\(^{13}\)-Leu\(^{14}\) and Trp\(^{25}\)-Leu\(^{26}\) in glucagon] would generate fragments that are undetectable by the midregion [glucagon-(6–15)] and COOH-terminal assays but that would still be recognized by the NH\(_2\)-terminal assay. However, the third potential target bond that glucagon has in common with GLP-1 (corresponding to Thr\(^{3}\)-Phe\(^{6}\) in glucagon) was not cleaved in GLP-1. Other potential cleavage targets in glucagon are Asp\(^{6}\)-Tyr\(^{10}\), Lys\(^{12}\)-Tyr\(^{13}\), Asp\(^{21}\)-Phe\(^{22}\), and Phe\(^{22}\)-Val\(^{23}\), which, if cleaved, would also reduce immunoreactivity detected with the midregion or COOH-terminal assays. In the present study, candoxatril increased endogenous glucagon immunoreactivity determined by COOH-terminal RIA, and although a trend toward an increase was seen with the midregion assay, it might be that cross-reactivity with other endogenous PG products masked any significant change or that the midregion of the peptide was still susceptible to cleavage by another enzyme. However, during the glucagon infusion, candoxatril increased the plasma half-life and reduced the MCR similarly, as determined by both COOH-terminal and midregion assays.

There was no significant effect on these parameters determined by NH\(_2\)-terminal RIA. Taken together, these findings lead us to speculate that, during the first glucagon infusion in the absence of candoxatril, levels of intact, biologically active glucagon-(1–29) increase. This increase is detected equally with all three assays. However, in addition, an as yet unidentified aminopeptidase or aminopeptidase/Metalloprotease cleaves the NH\(_2\) terminus of glucagon to generate a truncated metabolite [possibly glucagon-(2–29) or -(3–29)], undetectable by the NH\(_2\)-terminal assay but detectable by the midregion and COOH-terminal assays, thereby explaining the higher immunoreactive levels determined with these assays. In addition, cleavage by NEP could generate fragments [e.g., glucagon-(1–13)], which would be detectable only with the NH\(_2\)-terminal assay, and others [e.g., glucagon-(2–13) or -(3–13), and glucagon-(13–25), as well as other COOH-terminal fragments such as glucagon-(26–29)], which would be undetectable with any of the assays used in the present study. In the presence of candoxatril, cleavage at the Tyr\(^{13}\)-Leu\(^{14}\) and Trp\(^{25}\)-Leu\(^{26}\) bonds would be prevented, leading to an accumulation of glucagon-(2–29) or glucagon-(3–29) in addition to glucagon-(1–29) and explaining the increase in immunoreactivity detected by the midregion and COOH-terminal assays but not with the NH\(_2\)-terminal assay. Surprisingly, in the last half-hour (30 min after cessation of the second infusion), we observed an increase in arterial glucagon concentrations in four of the six animals, detected with all three assays, which was not seen in the control study. The reason for this is unclear but does not appear to be a counterregulatory response to low blood glucose levels, as these were similar by 30 min after each glucagon infusion. Possibly, candoxatril enhances an endogenous glucagon secretagogue (or reduces formation of an endogenous inhibitor), which, together with the reduction in MCR, raises glucagon concentrations.

The plasma clearance of the active inhibitor candoxatrilat is relatively slow, ranging from 1.9 \(\text{ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}\) in humans to 15 \(\text{ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}\) in rats (19). Therefore, it was not possible to use a conventional cross-over experimental design, whereby one-half of the animals received the inhibitor with the first glucagon infusion. This raises the possibility that the reduced glucagon clearance during the second infusion after candoxatril could be due to factors other than NEP inhibition. Blood flow and urine production were not measured in the present study, but the fluid loss was replaced, and heart rate and blood pressure remained constant. In addition, there were no significant differences in glucagon pharmacokinetics or pharmacodynamics when glucagon was given as two successive infusions in the absence of candoxatril, further supporting that the reduced clearance of glucagon detected with the midregion and COOH-terminal assays in the presence of the inhibitor is due to candoxatril.

Although the experiment was not designed to examine glucagon pharmacodynamics, we did monitor glucose and insulin concentrations. Surprisingly, blood glucose tended to be lower during the second compared with the first glucagon infusion both in the control study and in the presence of candoxatril, with the relative change being similar in the two experiments. This could be due to the “evanescent effect of glucagon,” whereby blood glucose only transiently increases and then falls despite ongoing hyperglucagonemia (12, 40), although other studies have shown that blood glucose can increase again if the glucagon infusion is stopped and then restarted (36). In the present study, the animals were fasted, meaning that their glycogen stores may have become depleted during the first glucagon infusion, thereby preventing blood glucose from increasing to the same extent in response to the second infusion. As noted above, there was only a small increase in NH\(_2\)-terminal glucagon concentrations after candoxatril, suggesting that much of the increase detected with the COOH-terminal and midregion assays reflects accumulation of NH\(_2\)-terminally truncated metabolites. Because the NH\(_2\) terminus of glucagon is important for receptor activation (21), this may explain the failure to see enhanced glucose concentrations during NEP inhibition. Insulin levels increased during the first glucagon infusion in response to the glucagon-stimulated increase in blood glucose. Glucagon itself can directly stimulate insulin secretion but is unlikely to have had a large effect in the present study because of the low (5 mmol/l) glucose concentrations (49). The modest rise in insulin concentrations during the second infusion after candoxatril likely reflects the smaller glucose response and, hence, the reduced stimulus to the \(\beta\)-cell. Further studies, using an experimental protocol designed specifically to examine endogenous glucose production, are needed to reveal whether changes in glucagon degradation resulting from candoxatril administration are associated with a change in its pharmacodynamic parameters.

Our recent study (7) revealed that the kidney and extremities are major sites of glucagon elimination in vivo, with the liver playing only a minor role. Therefore, in the present study, the effect of candoxatril on renal and femoral extraction was examined. In agreement with previous studies (10, 11, 17, 20, 31), we detected substantial renal extraction with all assays, suggesting that glucagon is degraded to small or undetectable fragments. Detailed studies have suggested that this involves glomerular filtration with proximal tubular catabolism and uptake as well as peritubular uptake (2, 30, 42). This is consistent with the action of NEP 24.11, which is found in high
concentration in the kidney (13), and is supported by the present observation that renal clearance was reduced by candoxatril when determined with the assays (midregion and COOH-terminal) that are sensitive to such cleavages. However, it is noteworthy that renal clearance was not eliminated by candoxatril. Thus, although NEP inhibition may reduce glucagon degradation throughout the kidney, glucagon will still be lost by glomerular filtration. This speculation is supported by a rough calculation of peptide losses ascribable to glomerular filtration compared with the total loss during passage across the kidney. Renal plasma flow (RPF) and glomerular filtration rate (GFR) were not directly assessed, but using standard values for the pig [RPF, 19.5 ml·kg⁻¹·min⁻¹; GFR, 5 ml·kg⁻¹·min⁻¹ (25)], 61 ± 6% of the total renal extraction of COOH-terminal glucagon immunoreactivity can be accounted for by glomerular filtration during infusion of glucagon alone. This proportion increases to 115 ± 21% after candoxatril, suggesting that NEP 24.11 is the major contributor to the glomerular filtration-independent renal degradation of glucagon. These calculations must be interpreted with some caution, however, because they assume that kidney function remains constant during both infusions. NEP inhibition increases endothelin-1 and atrial natriuretic peptide (ANP) concentrations (32), which might be expected to reduce renal function (endothelin) or to increase RPF and GFR (ANP); indeed, one study in healthy subjects has reported modest (10%) increases in GFR and reductions in RPF (38). However, other studies indicate that NEP inhibition does not affect renal hemodynamics, since neither RPF nor GFR was altered in healthy humans (24), in human heart transplant recipients (32), or in dogs with chronic heart failure (41). NEP inhibition is associated with increased diuresis and natriuresis (a consequence of increased ANP), but it was concluded that this occurs via a renal tubular rather than a glomerular filtration mechanism (24, 32, 41). The rising arterial glucagon concentrations during the second infusion with candoxatril raise the possibility of direct effects of glucagon on renal hemodynamics, although this is controversial. Thus glucagon has been reported to increase [35 (dog); 45 (dog); 44 (type 1 diabetic patients)] or have no effect [1 (dog); 44 (healthy human)] on RPF and GFR. However, as discussed above, it is likely that much of the increase in glucagon immunoreactivity occurring after candoxatril administration is due to the accumulation of inactive glucagon metabolites, such as glucagon-(2–29) or -(3–29). Further studies designed specifically to address these issues should be able to resolve the precise role of NEP 24.11 in glucagon’s renal metabolism.

During infusion of glucagon alone, degradation across the hindlimb, representing metabolism by adipose, connective, supportive, and muscular tissues, was evident, particularly with the COOH-terminal assay (>20%) and to a lesser extent with the other assays. This was reduced by candoxatril when determined by COOH-terminal RIA but was largely unaffected when determined with the other two assays, suggesting a minor role of NEP 24.11 in peripheral glucagon metabolism.

In conclusion, these studies have demonstrated that candoxatril reduces degradation of glucagon. Candoxatril was developed as a selective NEP 24.11 inhibitor and does not inhibit the unrelated enzymes trypsin and chymotrypsin (serine proteases) or renin (aspartyl protease). The zinc metalloprotease superfamily of enzymes, of which NEP 24.11 is a member, all have similarities in their active sites and in their respective mechanisms of action (22), but candoxatril has been shown not to affect the activity of carboxypeptidase A, leucine aminopeptidase (aminopeptidase M), or angiotensin-converting enzyme (28), which all belong to this superfamily. It is now known that a family of NEP 24.11-related enzymes exists within the superfamily [reviewed by Turner et al. (43)]. Candoxatril has been shown not to cross-react with one of these, endothelin-converting enzyme-1 (3), but whether the activity of other members of this family is affected by candoxatril is still unknown. Nevertheless, the results of the present study, taken together with the in vitro demonstration that NEP 24.11 can cleave glucagon (18) and the enzyme’s renal localization (13), strongly suggest that NEP 24.11 is an important mediator of the degradation of both endogenous and exogenous glucagon in vivo.

ACKNOWLEDGMENTS

We thank Dr. Per Franklin Nielsen (Novo Nordisk, Bagsvaerd, Denmark) for the gift of the porcine kidney NEP 24.11, and Dr. L. B. Knudsen (Novo Nordisk A/S, Måløv, Denmark) for the gift of des-His⁵-glucagon.

GRANTS

These studies were supported by the Danish Medical Research Council, the Novo Nordisk Foundation, and the Danish Biotechnology Program.

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