

Dynamics of glucagon secretion in mice and rats revealed using a validated sandwich ELISA for small sample volumes

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Wewer Albrechtsen NJ, Kuhre RE, Windeløv JA, Ørgaard A, Deacon CF, Kissow H, Hartmann B, Holst JJ. Dynamics of glucagon secretion in mice and rats revealed using a validated sandwich ELISA for small sample volumes. *Am J Physiol Endocrinol Metab* 311: E302–E309, 2016. First published May 31, 2016; doi:10.1152/ajpendo.00119.2016.—Glucagon is a metabolically important hormone, but many aspects of its physiology remain obscure, because glucagon secretion is difficult to measure in mice and rats due to methodological inadequacies. Here, we introduce and validate a low-volume, enzyme-linked immunosorbent glucagon assay according to current analytical guidelines, including tests of sensitivity, specificity, and accuracy, and compare it, using the Bland-Altman algorithm and size-exclusion chromatography, with three other widely cited assays. After demonstrating adequate performance of the assay, we measured glucagon secretion in response to intravenous glucose and arginine in anesthetized mice (isoflurane) and rats (Hypnorm/midazolam). Glucose caused a long-lasting suppression to very low values (1–2 pmol/l) within 2 min in both species. Arginine stimulated secretion 8- to 10-fold in both species, peaking at 1–2 min and returning to basal levels at 6 min (mice) and 12 min (rats). D-Mannitol (osmotic control) was without effect. Ketamine/xylazine anesthesia in mice strongly attenuated ($P < 0.01$) α -cell responses. Chromatography of pooled plasma samples confirmed the accuracy of the assay. In conclusion, dynamic analysis of glucagon secretion in rats and mice with the novel accurate sandwich enzyme-linked immunosorbent assay revealed extremely rapid and short-lived responses to arginine and rapid and profound suppression by glucose.

α -cell; islet biology; immunoassay; enzyme-linked immunosorbent assay

GLUCAGON REGULATES BLOOD GLUCOSE by stimulating hepatic glucose production (3). Importantly, elevated fasting and postprandial glucagon levels in patients with type 2 diabetes contribute to the hyperglycemia of these patients as demonstrated clearly using specific antagonists of the glucagon receptor (13). However, regulation of glucagon secretion has only been incompletely characterized, due partly to technical limitations with regard to its quantification (volume requirements, inadequate specificity, and accuracy) using current immune-based detection methods: radioimmunoassay (RIA) and enzyme-linked immunosorbent assays (ELISA) (7, 8, 16, 18, 22).

Here, we introduce a novel low-volume sandwich ELISA that exclusively detects fully processed pancreatic glucagon, corresponding to proglucagon-(33–61) (see Fig. 1), and use it to characterize the dynamics of glucagon secretion in mice and

rats in response to intravenous injections of L-arginine, D-glucose, and D-mannitol. By extensive assay validation, we conclude that the applied assay is reliable for measurements of glucagon in small plasma volumes. Because of this technical advance, we were able to make reliable real-time estimates of glucagon secretion (in vivo) in mice and rats. Our study thus provides a platform for future studies addressing the molecular mechanisms underlying glucagon secretion.

MATERIALS AND METHODS

Animal Studies

Studies were conducted with permission from the Danish Animal Experiments Inspectorate (2013-15-2934-00833) and the local ethical committee (EMED: P-15-335, P15-369 and P-14-213).

Glucagon secretion in rats. Male Wistar rats (~250 g, from Taconic, Ejby, Denmark) were housed two per cage under standard conditions for at least 1 wk before experiments. Experiments were carried out on nonfasted rats immediately before their nocturnal feeding period (1700). Rats were divided into weight-matched groups (L-arginine: 289 ± 7 g; D-glucose: 290 ± 4 g; and D-mannitol: 291 ± 8 g, $n = 9$), receiving intravenous infusions of either L-arginine (0.25 mg/kg; catalog no. W381918; Sigma Aldrich, Brøndby, Denmark), D-glucose (1 g/kg; catalog no. G8270; Sigma Aldrich), or D-mannitol (1 g/kg; negative control/osmolality control; catalog no. M4125; Sigma Aldrich), all dissolved in 0.9% NaCl so that the injection volume was 1.5 ml/300 g (wt/vol: L-arginine 5%, D-glucose 20%, D-mannitol 20.2%). Rats from the same cage received different treatments. The rats were anesthetized with Hypnorm/midazolam (1.25 mg/ml midazolam, 2.5 mg/ml fluanisone, and 0.079 mg/ml fentanyl citrate); the abdominal cavity was opened, and a needle was inserted in the inferior caval vein. Basal samples were collected 5 min after insertion (–5 min) and immediately before administration of test substance (0 min). At time 0, L-arginine, D-glucose, or D-mannitol was administered through the needle in the vena cava, followed by 100 μ l saline to flush the needle. Blood (200 μ l/time point) for hormone and glucose analysis was then collected at 1, 2, 4, 6, 8, 12, and 20 min, through the same needle, and transferred into EDTA-coated tubes (catalog no. 200 K3E, Microvette; Sarstedt, Nümbrecht, Germany). Blood glucose was measured immediately after collection by a handheld glucometer (Accu-chek Compact plus device; Roche, Mannheim, Germany). The remaining sample was centrifuged (1,650 g, 4°C, 10 min), and plasma was transferred to fresh Eppendorf tubes and immediately frozen. Samples were stored at –20°C. Following collection of the 20-min sample, rats were totally bled (from the vena cava), and blood was divided equally into EDTA-coated tubes (catalog no. 367841; Becton-Dickinson, Albertslund, Denmark) and metabolic stabilizing tubes (26) (P800) (catalog no. 366420; Becton-Dickinson), both prechilled on ice. In a separate set of studies, conscious male Wistar rats (~300 g) were given a gavage bolus of glucose (50% wt/vol; 2 g/kg body wt) or water (same volume; negative control). Blood (~100 μ l/time point) was drawn at 0, 15, 30, and 60 min by sublingual vein puncture as described previously (14).

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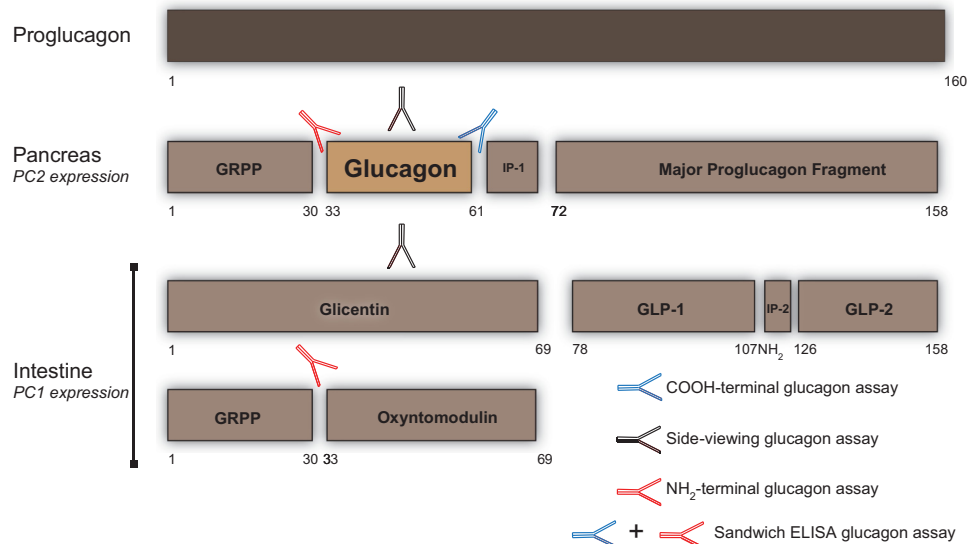


Fig. 1. Measurement of pancreatic glucagon. Molecular processing of proglucagon to glucagon in humans and immunobased methods for detection. Glucagon [proglucagon-(33–61)] results from prohormone convertase-dependent processing of proglucagon-(1–160). In the intestine, proglucagon is processed by prohormone convertase 1/3 activity to glicentin-(1–69), which may be further cleaved into glucagon-reactive polypeptide (GRPP) and oxyntomodulin-(33–69). Complete specificity of an immune-based assay requires a “sandwich” approach, involving a combination of NH₂ (red)- and COOH (blue)-terminally directed antibodies to avoid cross-reactivity with other moieties sharing the same antigenic determinant. GLP, glucagon-like peptide; ELISA, enzyme-linked immunosorbent assay; IP-1, intervening peptide-1.

Glucagon secretion in mice. Female C57bl/6Jrj mice (~20 g) were obtained from Janvier Labs (Saint-Berthevin Cedex, Cedex, France) and housed eight per cage under standard conditions for at least 1 wk before experiments. Experiments were carried out on 5-h-fasted (9:00 AM to 2:00 PM) mice. Mice were divided into weight-matched groups (each 20 ± 1 g) receiving intravenous L-arginine, D-glucose, or D-mannitol (same doses as for the rat study, see above). In *study A*, mice ($n = 80$) were anesthetized with isoflurane [catalog no. KDG9623 (Baxter, Lillerød, Denmark), dose 1.5%], and, in *study B*, mice ($n = 32$) were given an intraperitoneal injection of ketamine/xylazine [ketamine (catalog no. 511485, Ketaminol, Intervet; Merck), dose 100 mg/kg and xylazine (catalog no. 148999 Rompunvet; Bayer, Leverkusen, Germany), dose 10 mg/kg]. In both studies, the abdomen was opened, and a needle was inserted in the inferior caval vein. At *time 0* (0 min) L-arginine, D-glucose, or D-mannitol was administered through the vena cava. In *study A*, blood was collected at *time 0*, 1, 2, 4, 6, 8, 12, and 20 min through the same needle (300 μ l/time point; 1 mouse/time point) in prechilled EDTA-coated tubes (catalog no. 200 K3E, Microvette; Sarstedt), whereas, in *study B*, blood (100 μ l/time point) was collected from the orbital vein at *times* –5, 2, 4, 10, and 20 min in prechilled EDTA-coated capillary tubes (catalog no. 164213; Vitrex Medical, Herlev, Denmark). For both studies, blood glucose was measured immediately after blood collection using the same glucometer as for the rat study. The remaining sample was centrifuged (1,650 g, 4°C, 10 min), and plasma was transferred to fresh Eppendorf tubes and immediately frozen. Samples were stored at –20°C.

Biochemical Measurements

Glucagon levels were measured using a novel sandwich ELISA employing an NH₂-terminal antiglucagon antibody (code name: M4F9S) as capture antibody and a COOH-terminal specific antiglucagon antibody (code name: E6A11K) as detection antibody, from Mercodia (catalog no. 10-1281-01, Uppsala, Sweden). A lab protocol (lot no. 24238) provided by Mercodia was followed closely: 10 μ l of calibrators or samples were pipetted in a 96-well plate coated with NH₂-terminal antiglucagon antibody (diluted 1:70,000). Enzyme conjugate (50 μ l containing the COOH-terminal detection antibody, dilution 1:30,000) was added to each well, a plate sealer was attached to the plate, and the plate was incubated using a plate shaker [600 revolutions/min (rpm)] for 24 h at 5°C. Plates were then washed with 300 μ l wash buffer/well, five times in total. 3,3',5,5'-Tetramethylbenzidine (200 μ l) was added followed by incubation on a plate shaker (50 rpm) for 30 min at room temperature. H₂SO₄ (50 μ l, stop solution) was added to each well, and, after 1 min on the plate shaker,

optical density was read at 450 nm. Sample concentrations were calculated using a four-parametric logarithm regression model on the basis of absorbance readings from the five calibrators. Insulin levels were measured using a sandwich ELISA from CrystalChem (catalog nos. 90080 and 90060; Zaandam, Netherlands). For chromatography, 4 ml of a single pool of plasma from mice ($n = 20$) and rats ($n = 27$) were centrifuged (1,650 g, 4°C, 10 min), and the supernatant was fractionated by gel filtration as described previously (4, 11, 15).

Assay Evaluation

Assay evaluation was performed according to the standards of the Clinical & Laboratory Standards Institute [CLSI Guidelines: EP05-A2(5)] and by the Food and Drug Administration as previously described in detail (1, 2).

Peptides. The amounts of synthetic glucagon-(1–29) (catalog no. H-6790; Bachem, Bubendorf, Switzerland), oxyntomodulin (catalog no. H-6058; Bachem), and glicentin (custom made service no. SC1208; GenScript, Piscataway, NJ) used for calibration were verified by quantitative amino acid analysis (QAAA; duplicate determination) at the Department of Systems Biology, Enzyme and Protein Chemistry (Søltofts Plads, Danish Technical University, Lyngby, Denmark), as well as by analysis using an in-house midregion specific glucagon RIA, employing antiserum code no. 4304. Peptides were dissolved in phosphate buffer containing 1% human serum albumin (catalog no. 12666; Calbiochem, affiliate of Merck, Darmstadt, Ger-

Table 1. Specificity of the glucagon assay

Peptide (1,000 pmol/l)	Raw (450 nm)	Calculated, pmol/l
Glucagon-(1-61)	0.080 $\pm$ 0.01	0 $\pm$ 0.1
Glucagon-(19-29)	0.104 $\pm$ 0.04	1 $\pm$ 0.2
Glicentin	0.121 $\pm$ 0.07	3 $\pm$ 1
Oxyntomodulin	0.102 $\pm$ 0.05	1 $\pm$ 0.3
NH ₂ GLP-1-(7-36)	0.070 $\pm$ 0.03	0 $\pm$ 0.2
GLP-2	0.068 $\pm$ 0.06	0 $\pm$ 0.4
GIP-(1-30)	0.062 $\pm$ 0.02	0 $\pm$ 0.2
NH ₂ PYY-(1-36)	0.097 $\pm$ 0.10	1 $\pm$ 1
Neurotensin	0.050 $\pm$ 0.06	0 $\pm$ 0.4
Insulin	0.099 $\pm$ 0.04	1 $\pm$ 0.2
Mouse IgG (400 μ g/ml)	0.0150 $\pm$ 0.12	4 $\pm$ 2
Rat IgG (400 μ g/ml)	0.0102 $\pm$ 0.09	2 $\pm$ 1

Values are means $\pm$ SD. Cross-reactivity with various peptide hormones spiked in assay buffer at 1,000 pmol/l concentration. Absorbance and calculated levels are shown below.

Table 2. Precision and sensitivity analysis of the glucagon ELISA

	Added Amount of Glucagon, pmol/l					
	0	1	2	5	10	20
Assay buffer						
Measured glucagon, pmol/l	0 ± 1	2 ± 1*	3 ± 1*	7 ± 2*	11 ± 4*	25 ± 5*
Recovery ± SD, %	NA	200 ± 60*	150 ± 45*	140 ± 35*	110 ± 45*	125 ± 30*
Pooled mouse plasma						
Measured glucagon, pmol/l	2 ± 2	2 ± 2	4 ± 1*	8 ± 3*	12 ± 2*	28 ± 4*
Recovery ± SD, %	NA	NA	100 ± 34*	120 ± 40*	100 ± 25*	130 ± 20*
Pooled rat plasma						
Measured glucagon, pmol/l	3 ± 2	4 ± 3	6 ± 2*	10 ± 2*	17 ± 6*	31 ± 5*
Recovery ± SD, %	NA	100 ± 80	150 ± 40*	140 ± 30*	140 ± 40*	140 ± 25*

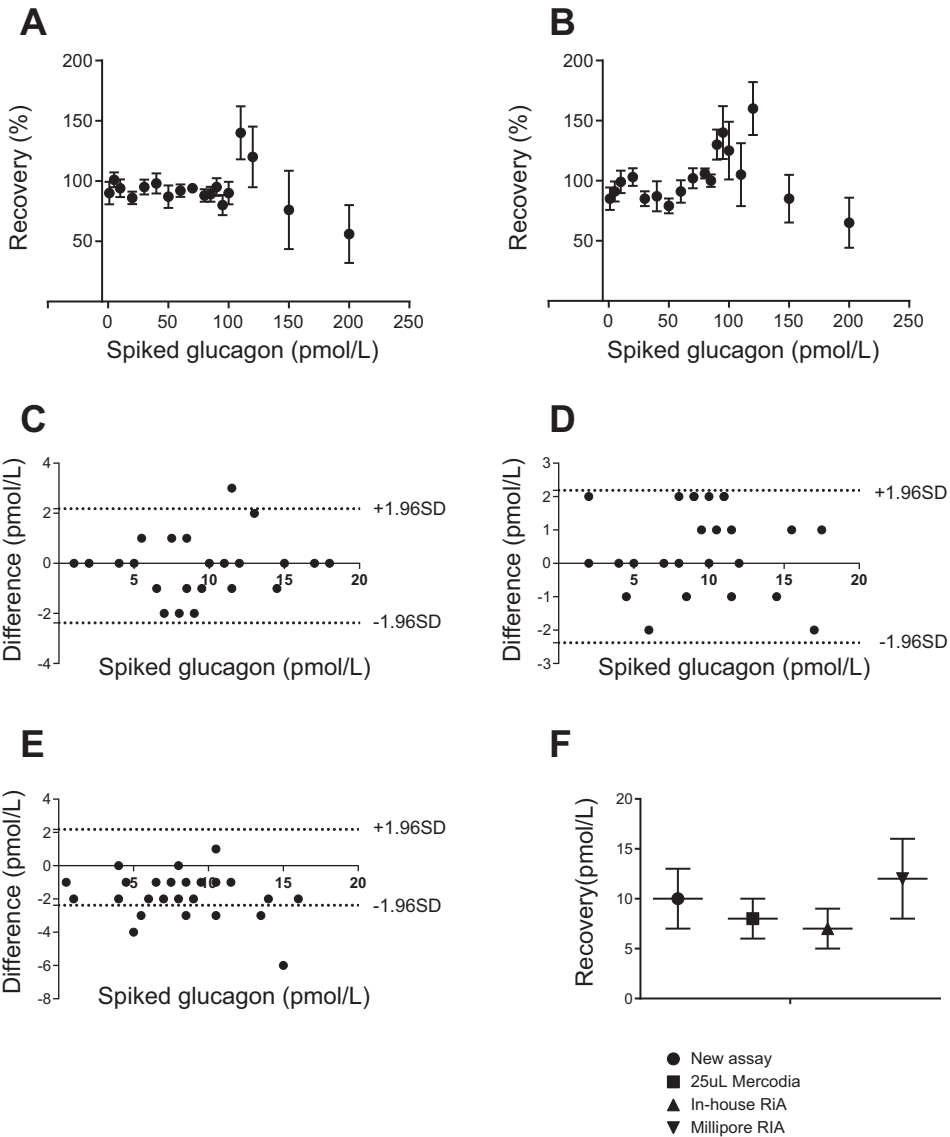
Measured glucagon is shown as mean ± SE. Units are pmol/l, and mean recovery is shown as % ± SD of 5–7 replicated determinations of glucagon concentrations in assay buffer, charcoal-stripped pooled mouse plasma, and charcoal-stripped pooled rat plasma without and with the addition of known amounts of synthetic glucagon. Average coefficient of variation for standard curve ~8%. *Significant ($P < 0.05$) changes by 1-way ANOVA for repeated measurements followed by Bonferroni post hoc analysis. NA, not applicable.

many), and 1 $\mu\text{mol/l}$ stock solutions were prepared for each peptide. The peptide concentrations measured by RIA were used for determination of the amounts of peptides added in recovery studies, since these assays (unlike the QAAA) could be carried out on the albumin-

containing stock solutions of the peptides. Methods are described in more detail elsewhere (1).

Specificity, sensitivity, and precision analysis. Specificity was analyzed by recovery experiments. Known amounts of each peptide (5–200

Fig. 2. Determination of working ranges of the new 10- μl assay from glucagon recovery experiments and comparison of the novel enzyme-linked immunosorbent assay (ELISA) with three other glucagon assays. *A*: recoveries of exogenous glucagon from spiked pooled mouse plasma. *B*: recoveries of exogenous glucagon from spiked pooled rat plasma (means with a 95% confidence interval). *C–E*: Bland-Altman plots of concentrations measured in plasma spiked with glucagon obtained with the new sandwich ELISA vs. 3 other glucagon immunoassays. *C*: 10 μl kit compared with the Mercodia 25 μl kit. *D*: 10 μl Mercodia kit vs. Millipore RIA assay. *E*: 10 μl Mercodia kits vs. in-house COOH-terminally directed RIA code name 4305 without correction for losses incurred in the solvent extraction in the RIA (recovery $70 \pm 8\%$). *F*: recovery of endogenous glucagon in overnight-fasted rats ($n = 8$) (same rats as used in *B*) measured using the new glucagon assay, Mercodia (25 μl), in-house RIA, and Millipore RIA. Plots in *C–E* show for each measured sample the difference between the results obtained with the two assays (including 95% limits) and the average of the measured concentration (pmol/l); $n = 6$ –10 biological replicates and $n = 4$ –6 technical replicates.



pmol/l) were added to separate aliquots of the matrix (assay buffer) or to plasma from rats or mice. One aliquot from each spiked buffer or plasma sample was measured in duplicate, using three separate kits for each assay. Precision was determined using a charcoal-stripped plasma pool spiked with 0, 1, 2, 5, 10, and 20 pmol/l of glucagon. Each concentration was analyzed five to seven times in each assay run. This procedure was replicated with an additional (different) pool of plasma and analyzed with an interval of >6 mo by different researchers/highly skilled technicians using kits with different lot numbers. Sensitivity was estimated by determination of the lowest concentrations of added peptide that could be measured as being significantly different from zero addition (see Table 2). The manufacturers' instructions were followed closely, including recommendations for sample preparation.

Bland-Altman analysis. For comparison of the new ELISA with existing assays, we used pooled plasma spiked with exogenous glucagon as described elsewhere (23) and plotted the differences in glucagon concentrations vs. the average of the measurements obtained with the new glucagon assay and each of three comparator assays: the human ELISA from Mercodia (25 μ l volume requirement for a single determination, catalog no. 10-1271-01), an in-house thoroughly characterized RIA (350 μ l volume requirement, COOH-terminally directed antibody, codename 4305) (12), and the Merck Millipore RIA (100 μ l volume requirement, catalog no. GL-32K) (1).

Statistics

For the sensitivity study, a one-way ANOVA for repeated measurements followed by Bonferroni post hoc analysis was performed comparing the spiked samples and the baseline (nonspiked plasma pool). For testing of normality and homoscedasticity in datasets, we applied the Shapiro-Wilk test (swilk command) and drafted residual plots. Net area under the curve (nAUC_{0–20 min}) was calculated using the trapezoidal rule, adjusting for baseline values. Significance was assessed by one-way ANOVA corrected for multiple testing using the Sidak-Holm algorithm. In all tests, $P < 0.05$ was considered significant. Data are shown as means $\pm$ SE unless stated otherwise. Calculations were made using GraphPad Prism (version 6.04 for Windows; GraphPad Software, La Jolla, CA) and STAT14 (SE) (StataCorp, College Station, TX). For Figs. 1–5, we used the Adobe CS6 software suite (Adobe, San Jose, CA).

RESULTS

Assay Evaluation

The ELISA did not cross-react with any of the other proglucagon-derived peptides (see Fig. 1), including elongated glucagon-(1–61), truncated glucagon [miniglucagon-(19–29)], glicentin, oxyntomodulin, glucagon-like peptide (GLP)-1,

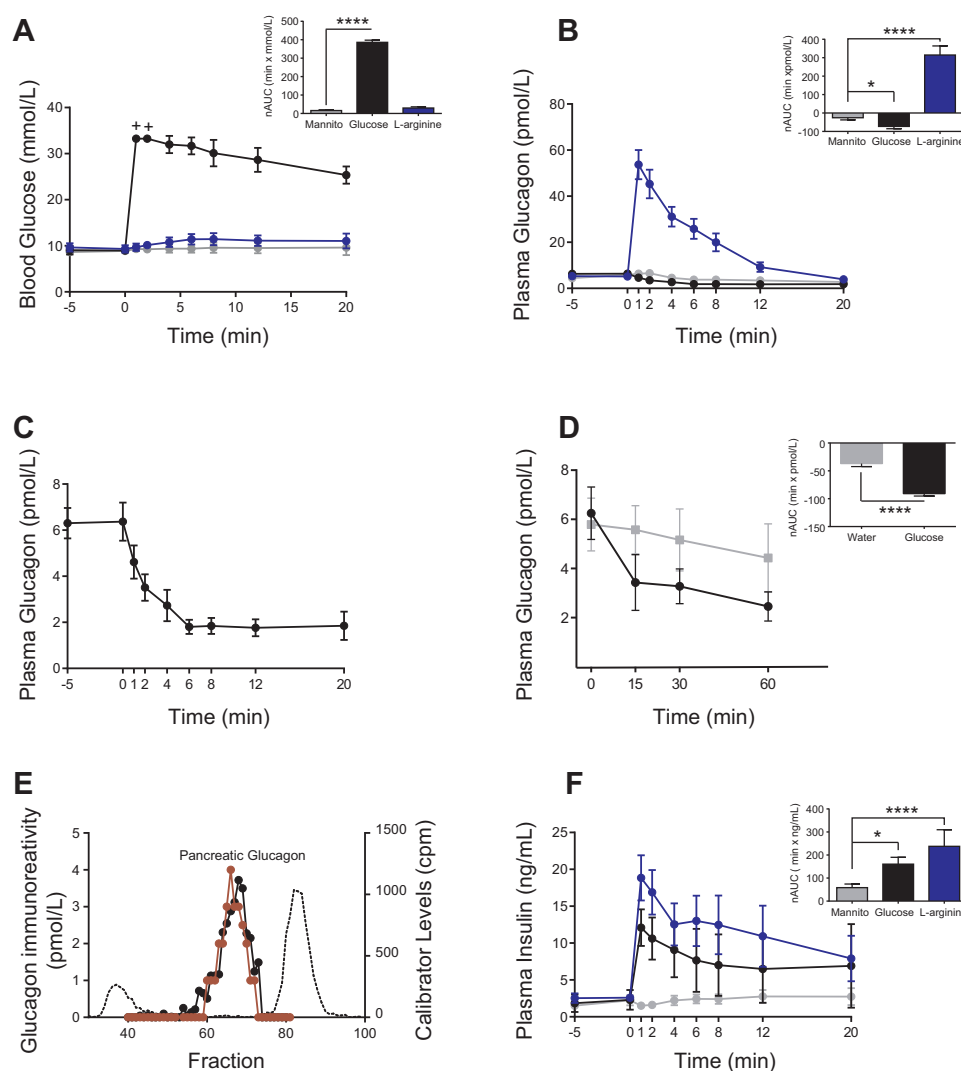


Fig. 3. Glucagon dynamics in rats. A: blood glucose concentrations (mmol/l) and net area under the curve (nAUC) values (min $\times$ mmol/l) (inset), $n = 9$. B: plasma glucagon (pmol/l) and nAUC values (min $\times$ pmol/l) (inset), $n = 9$. C: plasma glucagon after glucose treatment as shown in B, $n = 9$. D: plasma glucagon (pmol/l) and nAUC values (min $\times$ pmol/l) (inset) for oral administration of glucose or saline, $n = 4$. E: gel-chromatography data from pooled plasma from 27 rats; red circles are data from the ELISA, and black circles are data from the in-house COOH-terminally directed RIA. Broken lines show calibrator curves for ^{22}Na - and ^{125}I -labeled albumin. F: plasma insulin (ng/ml) and nAUC values (min $\times$ ng/ml) (inset), $n = 9$. Treatment groups are as follows: mannitol (gray), glucose (black), and L-arginine (blue). * $P < 0.05$ and **** $P < 0.0001$ using 1-way ANOVA corrected for multiple testing using the Sidak-Holm algorithm. †Over detection limit.

GLP-2, glucose-dependent insulinotropic polypeptide, peptide YY, or with neurotensin and insulin [see Table 1, which shows differences (adjusted for background absorbance) between assay buffer/plasma with or without addition of 1,000 pmol/l of peptide]. Precision and sensitivity analysis of the ELISA using assay buffer (Table 2), pooled mouse plasma (Table 2), and pooled rat plasma (Table 2) showed an experimental lower limit of quantification (LoQ_E) of 1–2 pmol/l. The limit of blank (LoB) (defined as the highest apparent concentration expected to be found when blank samples are measured, $LoB = \text{mean}_{\text{blank}} + 1.645SD$) was estimated to be 0.2 pmol/l, and the lower limit of detection ($LoD = LoB + 1.645SD$) was estimated to be ~ 1 pmol/l. By analyzing recoveries of glucagon across a wide concentration range from 5 to 200 pmol/l (Fig. 2, A and B), the upper limit of detection was estimated to be 100 pmol/l with a midrange point of 40 pmol/l [defined as the numerical median value of the dynamic range of the assay (6)]. In addition, the recoveries were linear and stable across the concentration ranges from 1 to $\sim 90 \pm 7$ pmol/l in mouse plasma (Fig. 2A) and 1 to $\sim 85 \pm 4$ pmol/l in rat plasma (Fig. 2B). Interassay variation was $9 \pm 3\%$, and intra-assay variation was $4 \pm 2\%$ (estimated from pooled plasma from rats and mice across 8 separate assay runs). The performance of the ELISA was comparable, upon a Bland-Altman analysis, to three other glucagon assays (Millipore, Mercodia, and COOH-terminal in-house RIA) (Fig. 2, C, D, and E). Finally, to compare its ability to measure endogenous glucagon, plasma from eight overnight-fasted rats was measured using the four glucagon assays. Values for endogenous glucagon were essentially similar across the four assays (Fig. 2F) although the in-house RIA gave about 30% lower values; however, this assay includes an ethanol extraction step, resulting in a loss of about 30% (23) for which no correction was made.

Blood Glucose, Glucagon, and Insulin Responses in Rats

In the mannitol group (control group), blood glucose levels (Fig. 3A) did not change at any time from baseline ($P = 0.68$). L-Arginine administration tended to increase overall blood glucose levels ($P = 0.08$ for the AUC analysis), and glucose administration markedly elevated blood glucose levels ($P < 0.0001$).

Mannitol had no effect ($P = 0.34$) on plasma glucagon levels compared with baseline values (Fig. 3B). L-Arginine robustly and significantly elevated plasma glucagon, increasing the concentration >10 -fold compared with baseline (5.1 ± 2 to 54 ± 19 pmol/l, $P < 0.0001$), with a peak 1 min after injection (Fig. 3B). Intravenous injection of glucose lowered plasma glucagon (Fig. 3C) levels by 80% ($P = 0.009$), and a value of 1.5 pmol/l was reached after 6 min. In a separate study, glucose was administered orally to investigate whether the ELISA was capable of detecting changes of plasma glucagon during hyperglycemia in a more physiological setting. Oral glucose administration attenuated glucagon secretion 15, 30, and 60 min after injection, whereas water had no effect at any time point (Fig. 3D) (glucose: $nAUC_{0-60\text{min}} = -91 \pm 12 \text{ min} \times \text{pmol/l}$ vs. water: $-31 \pm 15 \text{ min} \times \text{pmol/l}$, $P < 0.001$). To ensure accuracy (or trueness) of the ELISA-based measurements, we subjected pooled plasma from the rats receiving intravenous administration of glucose, L-arginine, or mannitol ($n = 27$) to size-exclusion chromatography. As shown in Fig.

3E, a coefficient of distribution (~ 0.8) similar to that of a recombinant glucagon calibrator was obtained, with no differences between the analyses using the novel ELISA or the reference in-house COOH-terminal specific RIA (23). Plasma levels of insulin (Fig. 3F) increased during glucose ($P < 0.05$) and L-arginine ($P < 0.01$) injection. Plasma glucagon levels were also comparable in pooled samples collected in EDTA- or metabolic-stabilizing (P800) tubes ($P = 0.29$). Distribution of the plotted residuals was scattered equally in positive and negative directions (Fig. 4), indicating that the P800 tubes were not superior for sample handling when measuring glucagon.

Blood Glucose, Glucagon, and Insulin Responses in Mice

In *experiment A* (using isoflurane for anesthesia), blood glucose levels in the mannitol group (control group) (Fig. 5A) were not different at any time compared with baseline ($P = 0.56$). L-Arginine administration modestly increased blood glucose levels ($P = 0.03$), whereas glucose administration markedly elevated blood glucose levels ($P < 0.0001$ for AUC analysis). Glucagon increased (Fig. 5B) about eightfold in response to L-arginine (8 ± 3 to 63 ± 11 pmol/l, $P < 0.0001$), peaking at 2 min, but basal levels were reached again at 6 min. After glucose, glucagon concentrations fell markedly already

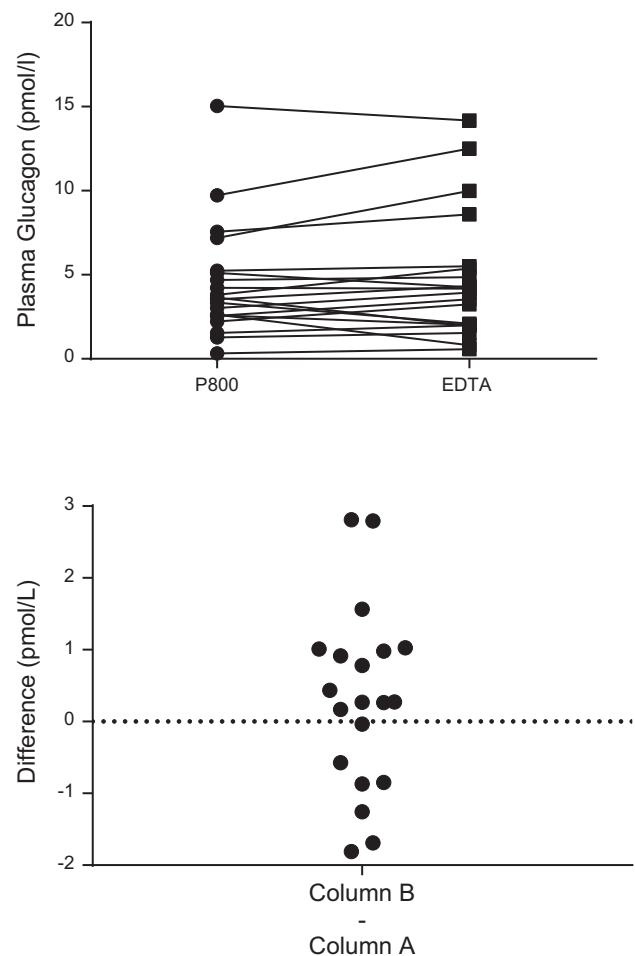


Fig. 4. Stability of glucagon in vitro. Comparison of plasma glucagon levels from blood either collected in EDTA and P800 tubes. Data are shown for each animal for blood obtained in EDTA-coated or P800-coated tube. The corresponding residuals for these data are shown below.

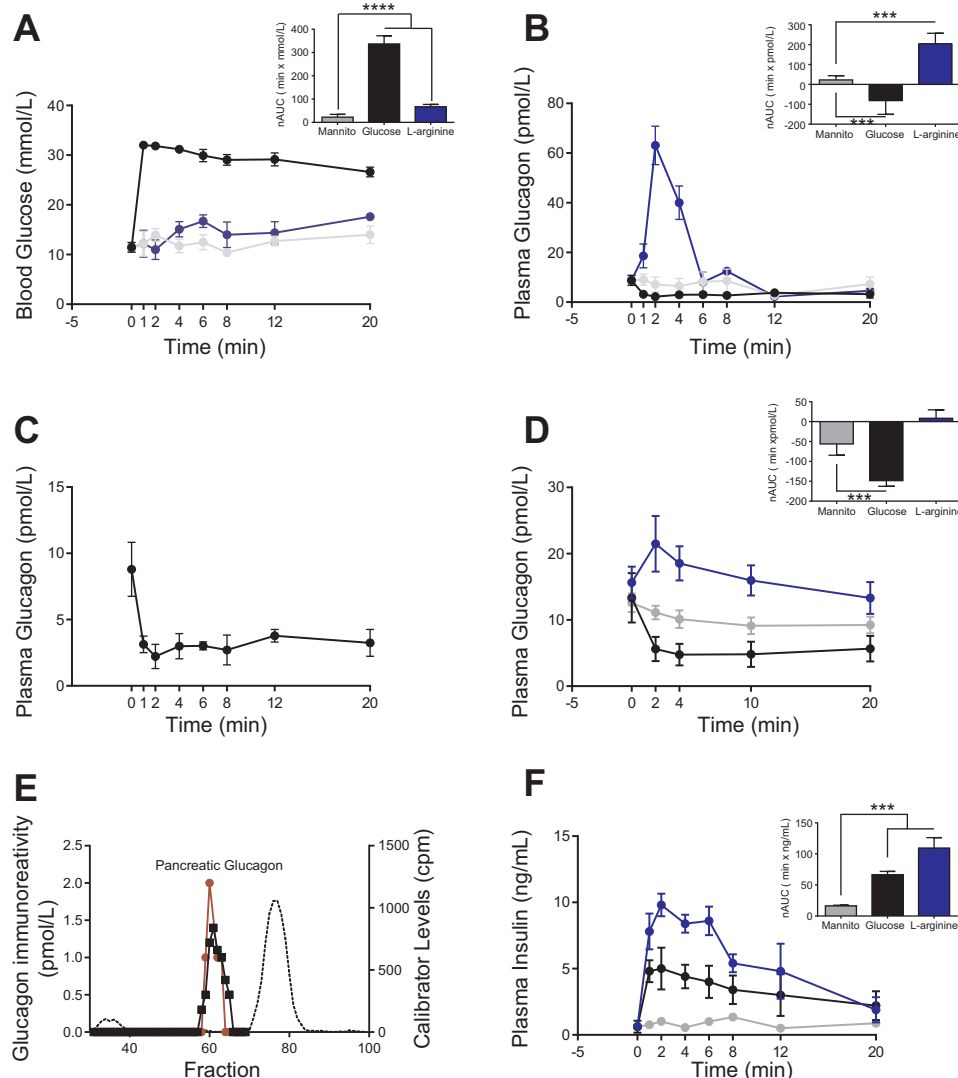


Fig. 5. Glucagon dynamics in mice. *A*: blood glucose concentrations (mmol/L) and nAUC values (min × mmol/L) (inset) from mice anaesthetized with isoflurane, $n = 4-6$ /time point. *B*: plasma glucagon (pmol/L) and nAUC values (min × pmol/L) (inset) from mice anaesthetized with isoflurane, $n = 4-6$ /time point. *C*: plasma glucagon after glucose treatment from mice anaesthetized with isoflurane as shown in *B*, $n = 4-6$ /time point. *D*: plasma glucagon levels from mice anaesthetized with ketamine/xylazine and nAUC values (min × pmol/L) (inset), $n = 10-12$. *E*: gel chromatography data from 20 mice; red circles are data from the ELISA, and black circles are data from the in-house COOH-terminally directed RIA. Broken lines show calibrator curves for ^{22}Na - and ^{125}I -albumin. *F*: plasma insulin (ng/mL) and nAUC values (min × ng/mL) (inset) from mice anaesthetized with isoflurane (same mice as in *A*). Treatment groups are as follows: mannitol (gray), glucose (black), and L-arginine (blue). *** $P < 0.001$ and **** $P < 0.0001$ using 1-way ANOVA corrected for multiple testing using the Sidak-Holm algorithm.

after 1 min (Fig. 5C). A nadir at 2 pmol/L was reached after 2 min, and suppression was maintained throughout the remainder of the experiment.

Figure 5D shows levels of glucagon in *experiment B*, where mice were anaesthetized with ketamine/xylazine. L-Arginine significantly elevated plasma glucagon (15 ± 3 to 22 ± 4 pmol/L, $P = 0.02$) 2 min after injection (Fig. 5D). This response was significantly lower than that observed in *study A* ($P = 0.001$). Mannitol had no significant effect on plasma glucagon levels ($n\text{AUC}_{0-20 \text{ min}} = -56 \pm 85 \text{ min} \times \text{pmol/L}$, $P = 0.37$). Intravenous injection of glucose lowered plasma glucagon levels ($P = 0.008$) compared with mannitol, reaching a minimum of 5 pmol/L at 2–4 min.

The size-exclusion chromatography profile of pooled mouse plasma ($n = 20$) was comparable to that obtained for the rat plasma (Fig. 5E). Plasma levels of insulin (Fig. 5F) increased significantly during glucose and L-arginine injection compared with mannitol ($P < 0.001$).

DISCUSSION

Studies on the dynamics of glucagon secretion in rodents have been hampered by the volume requirements (100–700 μL)

of the available assays (RIAs and ELISAs) compared with what can be meaningfully obtained from these animals. Furthermore, some of the available assays may be inaccurate, i.e., exhibit binding to unknown plasma moieties (matrix effect) and/or show cross-reactivity with the glucagon-related molecules proglucagon-(1–61), glicentin, and oxyntomodulin (1; see Fig. 1). Moreover, the sensitivity of the available assays is, in general, inadequate for quantification of suppression of secretion (25). As a result, the dynamics of glucagon secretion in rodents and mechanisms causing suppression of secretion are still under investigation. While responses to stimuli like arginine may provide information about α -cell secretory capacity, it is glucose-induced suppression that is the relevant parameter in studies of the pathogenesis of glucose intolerance and diabetes. Here, we validated and applied a novel glucagon ELISA that only requires 10 μL plasma.

First, we evaluated the assay performance by standard procedures according to the Clinical and Laboratory Standards Institute and the Federal Drug Agency. Subsequently, we carried out a series of proof-of-concept experiments in mice and rats, where glucagon secretion was, respectively, either stimulated (L-arginine) or inhibited (glucose) to evaluate how

well the assay would pick up dynamic changes in glucagon secretion. In isolated perfused pancreases (21) from both species, arginine invariably and rapidly stimulates (9) while glucose powerfully inhibits glucagon secretion (10), and, in the present study in rats, similar responses were observed. Importantly, the kinetic analysis of these changes showed extremely rapid responses, with peak values after arginine stimulation occurring as early as 1 min and reaching basal levels again after ~6 min in mice and 12 min in rats. For glucose, the nadir was reached within 6 min, and suppression lasted for the duration of the experiment. It is also important to note that the suppression was profound, reaching levels close to 1 pmol/l, which is extremely demanding in terms of assay sensitivity and accuracy. Similar responses were seen in mice anesthetized with isoflurane, but, interestingly, the pattern was clearly influenced by the anesthesia; thus, during ketamine/xylazine anesthesia, although the responses were equally rapid, arginine increased glucagon levels by only 25% (rather than 10-fold), whereas the suppression by glucose was less pronounced, reaching a nadir of around 50% of basal levels. Our experiments cannot provide clear explanations for these differences. Ketamine/xylazine anesthesia is known to interfere strongly with insulin secretion (19), and our data suggest that also glucagon secretion may be compromised. It has been reported that ketamine affects voltage-gated sodium channels, and, since sodium channels may be implicated in glucagon secretion (27), this may explain our observation of an attenuated pancreatic glucagon response in mice anesthetized by ketamine. It is of course a limitation that the current study was carried out in anaesthetized animals, but it was considered impossible to implement a similar protocol in conscious animals, particularly in mice (because of ethical concerns). In addition, the handling of conscious animals is associated with considerable stress (17), which was avoided by our protocol. On the other hand, the anesthesia itself may influence the autonomic nervous system. It is noteworthy that similar responses were obtained in the rat studies and in the mouse isoflurane studies, suggesting that these may represent relatively uninhibited responses, similar to those observed in isolated perfused pancreas preparations from both mice (20) and rats (10), where extraneous influences from stress and anesthesia are probably minimized.

In conclusion, we have extensively validated a novel ELISA for measurement of pancreatic glucagon in rodents, and furthermore used this assay to characterize the secretion of glucagon in rats and mice in response to intravenous injection of L-arginine and glucose. We find that this novel ELISA is suitable for rodent glucagon studies, thus representing a significant technical advancement in this field of endocrinology. The new assay allowed us to study the secretion kinetics of glucagon in the rodents, which are characterized by extremely rapid changes occurring within very few minutes. In addition, the high sensitivity of the assay allowed a reliable determination of the profound glucose-induced suppression of secretion, which would have been missed with previously available assays. Thus, the way is paved for future rodent studies investigating the regulation of glucagon secretion.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

N.J.W.A., R.E.K., J.A.W., A.Ø., C.F.D., H.K., B.H., and J.J.H. conception and design of research; N.J.W.A., R.E.K., J.A.W., A.Ø., H.K., and B.H. performed experiments; N.J.W.A. and R.E.K. analyzed data; N.J.W.A., R.E.K., A.Ø., C.F.D., H.K., B.H., and J.J.H. interpreted results of experiments; N.J.W.A. prepared figures; N.J.W.A., R.E.K., and J.J.H. drafted manuscript; N.J.W.A., R.E.K., J.A.W., A.Ø., C.F.D., H.K., B.H., and J.J.H. edited and revised manuscript; N.J.W.A., R.E.K., J.A.W., A.Ø., C.F.D., H.K., B.H., and J.J.H. approved final version of manuscript.

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