Electronic supplementary material

Validation of the porcine insulin radioimmunoassay for use in cats

The kit was validated by calculating dilutional parallelism and precision. Validation was performed with ten cat plasma samples. Observed-to-expected ratios for serial dilutions (12.5–100%) ranged from 103.2% to 106.4%. The calculated coefficients of variation for within-run and between-run precision were 7.0% and 6.5%, respectively. The sensitivity of the assay was 2 pmol/l.

Insulin secretion in cats

Insulin secretion in cats was quantified with the formula developed by Wareham et al. [30] using glucose and insulin levels measured during the IVGTT.

$$\text{Insulin secretion index} = \frac{[30\text{ min insulin (pmol/l)} - \text{fasting insulin (pmol/l)}]}{[30\text{ min glucose (mmol/l)}]}$$

Histochemistry methods

For immunohistochemistry, paraffin sections were deparaffinised, rehydrated in graded ethanol solutions and immersed in 3% hydrogen peroxide for 5 min. To detect insulin, amylin and glucagon, a polyclonal guinea pig antibody anti-swine insulin, 1:200 (Code A0564, Dako, Glostrup, Denmark), a polyclonal rabbit antibody anti-feline amylin, 1:800 (Code T-4142, Peninsula Laboratories, San Carlos, CA, USA) and a polyclonal rabbit antibody anti-human glucagon, 1:200 (Code A0565, Dako) were used. Antigen retrieval was performed for insulin detection by incubating the sections for 5 min with protease (Dako REAL Proteinase K, Dako) diluted in Tris–HCl buffer (pH 7.5) as indicated by the manufacturer. The immunohistochemical reactions were performed using a commercially available detection kit (Dako REAL

Detection system, peroxidase/AEC rabbit/mouse, Dako) according to the manufacturer's instructions. All steps were performed at room temperature in an automated device (Dako Autostainer, Dako). All primary antibodies were incubated for 1 h. The sections were counterstained with Mayer's haemalum.

To detect NKX6.1 in beta cells, a double immunostaining was performed with a polyclonal rabbit anti-rat NKX6.1, 1:4000 (Hagedorn Research Institute, Gentofte, Denmark) and a polyclonal guinea pig anti-swine insulin, 1:500 (Code A0564, Dako). Sections were microwave-treated in Tris–EDTA buffer (pH 9.0) for 18 min (3 min at 650 W, 15 min at 160 W) and allowed to cool for 30 min. Sections were blocked with avidin, biotin and normal donkey serum and the primary rabbit anti-Nkx6.1 antiserum was applied overnight at room temperature. The next day, sections were rinsed three times for 3 min in Tris-buffered saline with Tween (0.5%) (TBST). Donkey anti-rabbit biotin (Jackson ImmunoResearch Laboratories, West Chester, PA, USA) was added at 1:2000 for 30 min. The sections were rinsed in TBST and horseradish peroxidase–streptavidin conjugate (Zymed/Invitrogen, Taastrup, Denmark) was added for 30 min followed by three rinses in TBST. The diaminobenzidine (DAB)–nickel substrate (Vector Laboratories, Peterborough, UK) was added to the slides for 3–5 min and the reaction was stopped by placing the slides in distilled water. The sections were rinsed in TBST and then blocked with 10% rabbit serum (vol/vol) in TBST for 30 min. The guinea pig anti-insulin (Dako) was added overnight. After the slides were rinsed in TBST, rabbit anti-guinea pig–horseradish peroxidase was added at 1:100 for 30 min and the slides were rinsed in TBST. The NovaRed substrate (Vector Laboratories) was added to the slides for 2–4 min and the reactions was stopped in distilled water. To assess the beta cell specificity of NKX6.1 in pancreatic islets, sections from cats that did not receive infusion and from cats infused with

saline or lipids were used. NKX6.1 staining was detected in insulin-positive cells but not in other islet-cell types.

To detect cleaved caspase-3 and Ki67 in pancreatic islets, a triple immunostaining was performed with a polyclonal rabbit anti-human cleaved caspase-3, 1:100 (Code 9669, Cell Signaling, Boston, MA, USA) or a monoclonal mouse anti-human Ki67, 1:100 (Code 556027, BD Pharmingen, Heidelberg, Germany) mixed with a polyclonal guinea pig anti-swine insulin, 1:500 (Dako) and a polyclonal rabbit anti-rat NKX6.1, 1:4000 (Hagedorn Research Institute, Gentofte, Denmark). Antigen retrieval was performed by microwave oven treatment at 650 W for 4 min and 160 W for 15 min in Tris–EDTA buffer (pH 9.0). Thereafter, sections were blocked in 10% normal donkey serum (vol/vol) for 10 min, followed by TNB blocking buffer (0.1 mol/l Tris–HCl pH 7.5, 0.15 mol/l NaCl, 0.5% blocking reagent) for 30 min (for Tyramide Signal Amplification [TSA] stainings only) and incubated with one of the above mixtures of three primary antibodies overnight at room temperature. The next day, the TSA sections were incubated with donkey anti-rabbit or mouse secondary biotinylated antibody (Jackson ImmunoResearch Laboratories) for 30 min and horseradish peroxidase–streptavidin conjugate (Zymed/Invitrogen) for 15 min and developed with the TSA substrate (Cy3, 1:100) (Perkin Elmer, Hvidovre, Denmark) for 10 min to visualise one of the antibodies. The two other primary antibodies were visualised with Cy2 (1:500) and Cy5 (1:500) highly cross-absorbed secondary fluorescent antibodies for multiple labelling (Jackson ImmunoResearch Laboratories). Between each step the sections were rinsed in PBS three times for 3 min.

To detect proliferating cell nuclear antigen (PCNA) and myeloperoxidase in pancreatic islets, double immunostaining using DAB (brown signal) and alkaline phosphatase (red) was performed. DAB staining was performed first using a

monoclonal mouse antibody anti-human PCNA, 1:3000 (Clone PC10, Code ab29; Abcam, Cambridge, UK) and a polyclonal rabbit antibody anti-human myloperoxidase, 1:100 (Code RB-373-A0, Neomarkers-Lab Vision, Newmarket, UK). Antigen retrieval was performed with CC1 solution (Ventana Medical Systems, Illkirch, France), as indicated by the manufacturer. Staining was performed on an automated staining system (Ventana benchmark; Ventana Medical Systems) using the iVIEW-DAB detection kit (catalogue no. 760-091). Thereafter, a mouse anti-human insulin antibody, 1:80 (Code NCL-Insulin, Novocastra Laboratories, Newcastle upon Tyne, UK) was added without additional pretreatment on the automated staining system (Ventana benchmark, Ventana Medical Systems). This time the enhanced-red detection kit (alkaline phosphatase) was used.