The Veterinary Journal 183 (2010) 287–293

Contents lists available at ScienceDirect

The Veterinary Journal

journal homepage: www.elsevier.com/locate/tvjl

Quantitative real-time PCR detection of insulin signalling-related genes in pancreatic islets isolated from healthy cats

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ARTICLE INFO

Article history:
Accepted 23 November 2008

Keywords:
Feline
Diabetes
β-Cell
Transcript
mRNA

ABSTRACT

The cat has recently been proposed as a valuable model for type 2 diabetes mellitus (T2DM), because feline diabetes shares several similarities with the disease in humans. Impaired β-cell function, decreased β-cell mass, insulin resistance that is often related to obesity, and pancreatic amyloid deposition, are among these common features. In this study, and to further develop the cat as a model of T2DM, feline pancreatic islets were isolated and real-time PCR quantification of mRNA transcripts of genes central to β-cell function and survival established. In particular, mRNA quantification systems were determined for insulin, the insulin enhancer pancreatic duodenal homeobox-1 (PDX-1), the insulin suppressor CCAAT/enhancer binding protein-beta (C/EBPβ), glucose transporter isoform 2 (GLUT2), Fas receptor, the caspase-8 inhibitor FLIP (FLICE [caspase-8]-inhibitory protein) and two chemokines, interleukin (IL)-8 and monocyte chemoattractant protein-1 (MCP-1). Pancreatic islets were isolated by collagenase digestion from healthy cat donors. Partial feline mRNA sequences were determined for PDX-1, C/EBPβ, GLUT2 and FLIP using primers identified from conserved regions of human, dog and rat mRNA. These novel and the previously available sequences (insulin, Fas receptor, IL-8 and MCP-1) were used to design feline-specific primers suitable for real-time PCR in isolated pancreatic islets. The adopted protocol of collagenase digestion yielded pancreatic islets that were frequently surrounded by acinar cells. Quantification of mRNA transcripts was simple and reproducible in healthy cats. Characterisation of genes related to insulin signalling in cats will prove useful to better understand the pathogenesis of feline diabetes and possibly of human T2DM.

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Introduction

The domestic cat has been proposed as an attractive model of type 2 diabetes mellitus (T2DM) (Lutz and Rand, 1995; Henson and O'Brien, 2006; Zini et al., in press). In contrast to commonly used rodent models, cats spontaneously develop a form of diabetes that is clinically and pathologically very similar to human T2DM. Among shared features, diabetic cats are often middle aged, genetics play a role in diabetes risk, obesity leads to insulin resistance, and impaired β-cell function can be recognized in healthy and diabetic cats. The most striking histological similarity between human T2DM and diabetes in cats occurs at the pathological level in the pancreatic islets. As in diabetic humans, islet amyloidosis is found in more than 80% of diabetic cats and most of them have around 50% β-cell loss (Lutz and Rand, 1997; Appleton et al., 2001; Butler et al., 2003; Rand et al., 2004; Henson and O'Brien, 2006). Late complications, similar to those occurring in humans, such as diabetic retinopathy and polyneuropathy, are also described in cats (Linsenmeier et al., 1998; Mizisin et al., 2002). The study of feline diabetes is often hampered by the lack of tools to characterize the molecular mechanisms through which glucose metabolism is disturbed in diabetic cats, particularly in pancreatic islets and β-cells.

Based on studies in affected humans and in rodent models, a defect in the β-cell insulin secretory machinery is considered to be one of the most important processes leading to T2DM (Maedler and Donath, 2004). It has been demonstrated that chronic high glucose levels per se can impair the insulin stimulus-secretion coupling. This occurs, at least in part, due to the direct effect of glucose excess on the regulatory elements of the insulin gene, in particular the insulin enhancer pancreatic duodenal homeobox-1 (PDX-1) and the insulin suppressor CCAAT/enhancer binding protein-beta (C/EBPβ) (Seufert et al., 1998; Marshak et al., 1999). PDX-1 is a homeodomain protein that binds to the promoter of the insulin gene and exerts a potent stimulation of insulin tran-

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1090-0233/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tvjl.2008.11.012

scription (Marshak et al., 1999). C/EBPβ belongs to a family of basic leucine zipper transcription factors and serves as a transcriptional repressor of insulin in pancreatic β-cells via interactions with the insulin promoter (Seufert et al., 1998).

In isolated pancreatic islets of humans and in vivo in diabetic rats, it has been shown that chronically elevated glucose levels induce β-cell exhaustion followed by decreased insulin gene expression. This change is associated with decreased expression and protein binding of the transcription enhancer PDX-1 and transcriptional upregulation of the suppressor element C/EBPβ (Seufert et al., 1998; Marshak et al., 1999). In addition to the regulatory activity of PDX-1 on the insulin promoter, the enhancer element also participates in the transcriptional control of the glucose transporter isoform 2 (GLUT2) (Waeber et al., 1996). GLUT2 is a membrane protein that facilitates glucose diffusion through cell membranes in pancreatic β-cells, liver, small intestine and kidney. In β-cells, PDX-1 binds to the GLUT2 promoter and transactivates transcription of the GLUT2 gene (Waeber et al., 1996). Similar to PDX-1, GLUT2 expression is decreased in rat and mouse models of T2DM (Johnson et al., 1990; Chankiewitz et al., 2006).

Several authors have recently highlighted the role of a reduced β-cell mass in T2DM (Butler et al., 2003; Yoon et al., 2003). In cultured islets from humans and rodents, sustained elevation of glucose concentration directly initiated β-cell apoptosis through Fas receptor upregulation and activation (Donath et al., 1999; Maedler et al., 2001). The apoptosis-inducing receptor Fas is a sub-member of the tumour necrosis factor receptor family. Engagement of Fas receptors by Fas ligand results in β-cell apoptosis through activation of caspase-8 (Maedler et al.,2001, 2002a). However, Fas receptor signalling may also be implicated in proliferative signals. An endogenous inhibitor of caspase-8, FLIP (FLICE [caspase-8]-inhibitory protein) seems to switch Fas signalling from apoptosis to survival/proliferation (Maedler et al., 2002b). In human β-cells, FLIP is constitutively expressed but downregulated by high glucose (Maedler and Donath, 2004). Finally, increased numbers of islet-associated macrophages have been observed in rodent models and in humans with T2DM (Ehses et al., 2007). In the same study, an increased amount of chemokines, especially interleukin (IL)-8, was secreted by cultured human and mouse islets exposed to high glucose levels. Inflammatory cells migrated into pancreatic islets because of a combination of upregulated chemotactic factors are suspected to contribute to β-cell death in T2DM (Ehses et al., 2007).

The aim of the present study was to establish in cats real-time polymerase chain reaction (PCR) detection methods to quantify the expression of mRNAs of crucial genes involved in the processes of diabetic islet pathology. The methods were established using pancreatic islets isolated from healthy cats. In particular, real-time PCR systems were established to quantify transcripts of insulin, PDX-1, C/EBPβ, GLUT2, and the chemokines IL-8 and monocyte chemoattractant protein-1 (MCP-1). Detection tools for mRNA transcripts of two genes related to apoptosis, specifically the Fas receptor and FLIP were also set-up in the cat.

Materials and methods

Animals and isolation of pancreatic islets

A group of five healthy neutered male domestic short-hair cats, 18 months-old and weighing 3.3–4.1 kg (median 3.7 kg), was maintained at the animal care facility of the Clinic for Small Animal Internal Medicine, Vetsuisse Faculty, University of Zürich, Switzerland. Animal studies were approved by the Cantonal Veterinary Office of Zürich. Cats were determined to be healthy on the basis of physical examination and clinical laboratory data.

After fasting for 12 h, cats were sedated with tiletamine/zolazepam (Zoletil 100, Virbac) and anaesthesia was induced with propofol (Propofol 1%, Fresenius-Kabi). An IV overdose of sodium pentobarbital (Esconarkon, Streuli Pharma) was injected to euthanase the cats. Immediately thereafter, the whole pancreas was surgically excised under sterile conditions from each donor.

The isolated tissue was immersed in Hank's balanced salt solution (HBSS) and care was taken to remove fat tissue surrounding the pancreas with sterile surgical scissors. The pancreas was weighed and injected with a chilled HBSS solution containing 1 unit/mL of collagenase NB8 (Serva Electrophoresis), using a 27G needle connected to a 10 mL syringe. The injected solution volume (mL) to pancreas weight (g) ratio was approximately 5:1 and the solution was injected in equal aliquots through 5–10 different punctures. The inflated pancreas was placed in a Falcon tube containing the same amount of injected solution and was incubated at 37 °C for 35–40 min.

The digested tissue was washed with an ice-cold solution of HBSS and filtered through a stainless steel screen with a 1 mm mesh. The residual non-filtered tissue remaining on the steel screen was placed in an additional tube containing 30 mL solution of HBSS and collagenase for a second digestion, incubated for 5–10 min and then filtrated again. Immediately after filtration, the two filtrates were placed in separate Falcon tubes and filled up with HBSS containing 10% fetal calf serum (FCS) in a 1 to 1 volume ratio. The tubes were left on ice for 5 min allowing sedimentation of the digest. Thereafter the supernatants were aspirated and the pellets washed 2–3 times at 5 min intervals with HBSS. Rinsed pellets were then transferred into a single tube.

After collagenase digestion, the isolated tissue was rapidly frozen in liquid nitrogen and stored at −80 °C until further use. In addition, from each isolate an aliquot was placed in vials containing 4% buffered formaldehyde for 24 h and embedded into paraffin using standard methods.

To verify whether collagenase treatment yielded free pancreatic islets, sections of formalin-fixed, paraffin-embedded digests were prepared and subsequently stained immunohistochemically for insulin and cytokeratin using a polyclonal guinea pig anti-swine insulin antibody (Dako Cytomation) and a monoclonal mouse anti-human cytokeratin clone MNF116 antibody (Dako). Paraffin sections (3 μm) were deparaffinised in xylene and rehydrated through graded ethanol to water. Antigen retrieval consisted of incubation with protease (REAL Proteinase K, Dako) diluted in Tris buffer (pH. 7.5) for 5 min (for insulin) and 10 min (for cytokeratin), as indicated by the manufacturer. The immunohistochemical reactions were performed using a commercially available detection kit (Dako REAL Detection system, peroxidase/AEC rabbit/mouse) according to the manufacturer's instructions. All steps were performed at room temperature in an automated device (Dako Autostainer). Primary antibody incubation conditions were 1:200 for 1 h for insulin and 1:50 for 30 min for cytokeratin. The sections were counterstained with Mayer's haematoxylin.

RNA isolation and reverse transcription

Total RNA from pancreatic islets was extracted using RNeasy Mini Kit (Qiagen). Pancreatic pellets (30 mg) were homogenised using the Mixer Mill MM 300 (Qiagen) for 1 min at 30 Hz. Possible genomic DNA contamination in islet-derived RNA samples was eliminated by including DNase-treatments (DNase-Free DNase Set, Qiagen). RNA was quantified spectrophotometrically (ND-1000 Spectrophotometer, NanoDrop) and the quality was assessed by identifying 18S and 28S rRNA bands on gel electrophoresis. cDNA was obtained from 1 μg samples of islet-derived RNA (Omniscript RT Kit, Qiagen) in the presence of 13 U of RNasin (Promega). cDNA was subjected to PCR using PCR Taq core kit (Qiagen) on a conventional thermal cycler (T-personal, Biometra).

Partial sequencing of feline-specific mRNAs

Partial or complete mRNA sequences of feline insulin (NM_001009272), Fas receptor (NM_001009314), IL-8 (AF158598) and MCP-1 (DQ835566) have been previously deposited in GenBank. For PDX-1, C/EBPβ, GLUT2, and FLIP whose mRNA sequences are not available in cats, conserved regions were identified from human, canine and rat sequence alignments. PCR primers were designed with a web-based tool[1] using canine or human sequences. Feline pancreatic islet-derived cDNA was subjected to PCR amplification with canine or human primer pairs located in conserved sections (Table 1). A total volume of 25 μL contained 1 μL cDNA template, 0.2 mM dNTPs, 0.025 units/μL Taq DNA polymerase and 2.5 μL reaction buffer (PCR Taq Core Kit, Qiagen), with specific sense and antisense primers, each at a final concentration of 300 nM. To perform the PCR, for each target, an initial denaturation step of 3 min at 94 °C was followed by 40 cycles of 30 s at 94 °C, an annealing of 45 s at 60 °C and extension of 60 s at 72 °C.

When present, multiple amplicons were eliminated by optimising PCR conditions or by replacing the primers until single products of the expected size were obtained. Amplicons were purified with QIAquick PCR purification kit (Qiagen) and both strands were sequenced using the same primers (Microsynth).

For PDX-1 mRNA, single amplicons were not achieved (see Supplementary File 1). Therefore, the expected PDX-1 band was extracted from a 2% agarose gel using the QIAex gel extraction kit II (Qiagen) followed by cloning. To clone the PCR fragments the TOPO TA cloning kit (Invitrogen) was used. Briefly, 4 mL of cleaned PCR product, 1 mL of salt solution and 1 mL of vector were mixed and processed further

[1] See: http://bibiserv.techfak.uni-bielefeld.de/genefisher/

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