Partial sequencing and expression of genes involved in glucose metabolism in adipose tissues and skeletal muscle of healthy cats

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Abstract

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Abstract

Impaired insulin sensitivity is increasingly recognised in cats, but sequences of genes involved in insulin-signalling are largely undetermined in this species. In this study, extended feline mRNA sequences were determined for the adiponectin, glucose transporter-1 (GLUT1), GLUT4, peroxisome proliferative activated receptor-γ1 (PPARγ1), PPARγ2, plasminogen activator inhibitor-1 (PAI-1), monocyte chemoattractant protein-1 (MCP-1) and insulin receptor genes. Conserved dog-specific primers identified from human-dog mRNA alignments were used to amplify feline cDNA in the polymerase chain reaction (PCR). The feline sequences determined by this method were used to design feline-specific primers suitable for real-time PCR to quantify gene expression in insulin sensitive tissues of healthy cats. Partial sequences of feline mRNAs had 86-95% identity with dog and human genes. Expression of adiponectin, GLUT1, GLUT4, PPARγ1, PPARγ2, PAI-1 and insulin receptor mRNA was detected and quantified in subcutaneous and visceral fat and skeletal muscle, whereas MCP-1 mRNA was detected in adipose tissue but not in skeletal muscle. Further characterisation of genes related to glucose metabolism in cats will provide additional insights into insulin-signalling mechanisms in this species.

Keywords: Feline; Glucose; Metabolism; mRNA; Gene sequences
Introduction

Similar to human type 2 diabetes mellitus, cats can develop an obesity-associated form of diabetes mellitus with similar clinical features, including peripheral insulin resistance and disturbed insulin production (Feldhahn et al., 1999; Appleton et al., 2001). The current dramatic increase in obesity in humans has triggered research directed at understanding the molecular mechanisms linking obesity with the development of insulin resistance, hypertension and atherosclerosis. Low chronic inflammation is increasingly recognised as a possible link, but the precise molecular mechanisms remain poorly understood (Wellen and Hotamisligil, 2005).

It has been hypothesised that increased expression of inflammatory cytokines, such as tumour necrosis factor-α and interleukin-6, dysregulate a number of genes important in lipid metabolism and glucose homeostasis (Wellen and Hotamisligil, 2005). In cats, data on expression of genes involved in glucose metabolism are scarce and several feline-specific insulin signalling-associated genes have yet to be sequenced (Brennan et al., 2004; Hoenig et al., 2007).

Adiponectin is a circulating factor with insulin-sensitising and anti-inflammatory properties that is mainly expressed in adipocytes and liver (Kadowaki et al., 2006). Lower levels of adiponectin expression are detected in skeletal muscle (Yang et al., 2006). A strong inverse association has been observed between circulating levels of adiponectin and obesity in humans and cats (Yang et al., 2001; Brennan et al., 2004).

Glucose transporter-1 (GLUT1) is ubiquitously expressed, whereas GLUT4 is expressed predominantly by insulin-sensitive cells, including myocytes and adipocytes (Wood
and Trayhurn, 2003). GLUT4 expression may be decreased in type 2 diabetes mellitus in humans and in obesity in humans and cats (Brennan et al., 2004; Wellen and Hotamisligil, 2005). Conversely, GLUT1 expression is either increased or unaffected in these conditions (Miele et al., 1997; Brennan et al., 2004).

Peroxisome proliferative activated receptors (PPARs) are nuclear receptors that act as transcriptional regulators of nutrient metabolism and energy homoeostasis (Lagathu et al., 2003). Three major PPAR isoforms (α, β/δ, and γ) have been identified, with splicing variants reported for all three subtypes. PPARγ is a key nuclear receptor during adipocyte differentiation (Hamm et al., 2001). While the splice variant PPARγ1 is found in several cells, PPARγ2 is mainly adipocyte-derived, with lower amounts detected in skeletal muscle (Vidal-Puig et al., 1997; Wagatsuma, 2006). Studies involving knockout mice and synthetic PPARγ ligands (i.e., thiazolidinediones) have demonstrated the crucial role of this nuclear receptor in maintaining insulin sensitivity and reducing cytokine expression (Semple et al., 2006).

Plasminogen activator inhibitor-1 (PAI-1) is a member of the serine protease inhibitor family known for its ability to inhibit fibrin clot breakdown (De Taeye et al., 2005). Monocyte chemoattractant protein-1 (MCP-1) is a member of the chemokine family which plays a crucial role in the recruitment of monocytes in tissues (Herder et al., 2006). Even though many cells express PAI-1 and MCP-1, growing evidence suggests that adipocytes are a primary source of these molecules in the circulation (De Taeye et al., 2005; Herder et al., 2006). In obese rats and humans, PAI-1 and MCP-1 are over-expressed by adipose tissue and have been shown to contribute to the development of insulin resistance and type 2 diabetes mellitus (De Taeye et al., 2005; Herder et al., 2006). Insulin-stimulated activation of the insulin receptor is the first step in the mechanism controlling glucose homeostasis. Defective
receptor signalling in insulin target tissues (e.g., muscle, adipose tissue and liver) impairs insulin sensitivity (Denley et al., 2003).

The aim of this study was to determine new sequences (GLUT1, GLUT4, PPARγ1, PPARγ2, PAI-1, MCP-1 and insulin receptor) or to extend known sequences (adiponectin) of feline-specific genes which have a potential relationship to the generation of inflammation-induced insulin resistance. The sequences identified in this study were used to establish real-time polymerase chain reaction (PCR) to detect and quantify the expression of mRNAs in insulin sensitive tissues of healthy cats, including subcutaneous and visceral adipose tissue and skeletal muscle.

Materials and methods

Animals and tissue biopsies

A group of 19 healthy neutered male domestic short haired cats, 1.5-4 years old (median 2.8 years) and weighing 4.2-5.5 kg (median 4.8 kg), were 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 (95/2006). Cats were determined to be healthy on the basis of physical examination and clinical laboratory data.

Tissue biopsies were collected under general anaesthesia. The cats were fasted for 12 hours and acepromazine (50 µg/kg) and buprenorphine (15 µg/kg) were administered IM for sedation and analgesia. After 30 min, anaesthesia was induced with propofol (6 mg/kg) IV, the cats were intubated with a cuffed endotracheal tube and anaesthesia was maintained with isoflurane administered to effect. Biopsies (100-150 mg) were collected from visceral and
subcutaneous fat and from skeletal muscle, rapidly frozen in liquid nitrogen and stored at −80°C until further use. Visceral fat was collected from near the jejunum, subcutaneous fat was collected from the inguinal area and skeletal muscle was collected from the vastus lateralis muscle. After the procedure, all cats recovered uneventfully. Buprenorphine (20 µg/kg) was administered every 6 h on the first day to obtain analgesia and then according to individual needs. For bacterial prophylaxis, oral amoxicillin was administered at 10 mg/kg BID for 5 days.

RNA isolation and reverse transcription

Total RNA from visceral and subcutaneous fat was extracted using RNeasy Mini Kit (Qiagen). Samples (30 mg) were disrupted and homogenised using the Mixer Mill MM 300 (Qiagen) for 1 min at 30 Hz. Total RNA isolation from feline skeletal muscle was performed using RNeasy Fibrous Tissue Mini Kit (Qiagen) and the Mixer Mill MM 300 (Qiagen) for 2 min at 20 Hz. Possible genomic DNA contamination in tissue-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 tissue-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

Conserved sections of adiponectin, GLUT1, GLUT4, PPARγ1, PPARγ2, PAI-1, MCP-1 and insulin receptor mRNAs were identified from human and canine sequence alignments. Using canine sequences, PCR primers were designed with a web-based tool
Feline adipose tissue and skeletal muscle-derived cDNAs were subjected to PCR amplification with canine 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 1x 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, an initial denaturation step of 30 s at 94 °C was followed by an annealing of 45 s at 60 °C and extension of 60 s at 72 °C. Forty cycles of PCR were used for each target.

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). Corresponding protein sequences were deduced from mRNA using on-line software (http://www.expasy.org/).

Quantitative analysis of mRNA

cDNA obtained from feline tissues was subjected to quantitative real-time PCR analysis using feline-specific intron spanning oligonucleotides (Table 2) and the iCycler iQ sequence detection system (BioRad). Primers for adiponectin, GLUT1, GLUT4, PPARγ1, PPARγ2 PAI-1, MCP-1 and insulin receptor gene sequences were designed within the newly determined feline-specific partial mRNA sequences using Primer Express 3.0 software (Applied Biosystems). Primer pair selection criteria were set to generate short amplicons (85-162 bp) with annealing temperature, as well as secondary structure, suitable for SYBRGreen real-time PCR detection. For detection of feline-specific glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, published oligonucleotides were used (Kipar et al., 2001).
PCR reactions were prepared with 10 μL Power SYBR-Green Master Mix (Applied Biosystems), a final concentration of 500 nM of each primer and 5 μL of template diluted 1:5 in a total reaction volume of 20 μL.

An initial denaturing step was performed for 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min, then 95 °C and 60 °C for 1 min each and 80 cycles of 10 s, starting from 60 °C with a 0.5 °C increase after each cycle. At the end of the programme, PCR product identity was confirmed by melting curve analysis and DNA sequencing (Microsynth).

Parallel reactions were performed in duplicate for every feline cDNA sample. Two template-free controls and duplicates without reverse transcriptase were included in every amplification run. For each reaction, duplicate standard curves were generated with serial dilutions of the purified specific PCR product previously obtained by conventional PCR with the same primers. Target gene mRNA was quantified using the relative standard curve method (Perkin-Elmer Cetus User Bulletin no. 2, 1997). Gene expression was normalised to the respective quantities of GAPDH.

Transcripts of adiponectin, GLUT1, GLUT4, PPARγ1, PPARγ2, PAI-1, MCP-1 and insulin receptor were studied in each tissue by repeated measures ANOVA with post-hoc Bonferroni correction to compare relative expression between adipose tissues and skeletal muscle of healthy cats. The level of significance was set at P<0.05.

**Results**

*Partial mRNA sequences of glucose metabolism-related genes in cats*
By applying a PCR-based approach we partially determined the feline-specific mRNA sequences and deduced the corresponding protein sequences of GLUT1, GLUT4, PPARγ1, PPARγ2, PAI-1, MCP-1 and insulin receptor. In addition, we extended the known partial mRNA sequence of feline adiponectin (Fig. 1 and 2). The GenBank accession numbers and mRNA and protein similarities to canine and human sequences are given in Table 3.

*mRNA expression in insulin sensitive tissues in cats*

Expression of adiponectin, GLUT1, GLUT4, PPARγ1, PPARγ2, PAI-1 and insulin receptor was successfully quantified in adipose tissue and muscle of healthy cats. With feline-specific primers, MCP-1 mRNA was detected in subcutaneous and visceral fat, but in most samples of skeletal muscle melting curves showed 1-2 additional peaks or a single broad peak with a lower melting temperature. Therefore, the primers designed for MCP-1 were not considered to be suitable for transcript quantification in feline skeletal muscle.

Visceral adipose tissue had significantly higher levels of mRNA expression of adiponectin, GLUT1, GLUT4 and PPARγ2 than subcutaneous adipose tissue. All targets had lower levels of mRNA expression in skeletal muscle than in subcutaneous or visceral fat (Table 4).

**Discussion**

To explore glucose metabolism in obese and diseased cats, it is important to develop molecular tools to investigate transcriptional changes that occur in insulin sensitive tissues. For this purpose, based on current knowledge of the pathogenesis of obesity and type 2 diabetes mellitus, we determined gene sequences and designed primers for quantification of
In this study, we determined seven new feline-specific partial mRNA sequences of insulin signalling-related genes and elongated the known feline adiponectin sequence. Feline cDNA was amplified by PCR using canine specific primers determined from conserved regions in canine and human sequence alignments. In approximately 85% of cases, we obtained single amplicons that could be directly sequenced without the need to clone into a vector. Even though complete feline cDNA sequences were not obtained, high similarities (86-95% identity) were evident among feline, canine and human gene sequences (Table 3). Similar or even higher degrees of sequence identity (81-98%) were observed for the deduced corresponding protein sequences. As expected from the closer phylogenetic relatedness of cats and dogs, feline sequences exhibited a higher degree of similarity to canine than to human sequences.

Feline-specific PCR primers nested within the newly determined mRNA sequences were tested using cDNA derived from adipose tissue and skeletal muscle of healthy cats. Primer pairs for adiponectin, GLUT1, GLUT4, PPARγ1, PPARγ2, PAI-1 and insulin receptor were appropriate to quantify cDNA transcripts in each of the examined insulin sensitive tissues. Primers for MCP-1 were suited for mRNA quantification in subcutaneous and visceral fat but not in skeletal muscle.

The reverse transcriptase-PCR procedures were established in order to advance knowledge on disorders of glucose metabolism in cats. The prevalence of diabetes mellitus in cats is 1/50-1/400 and recent evidence suggests that the incidence is increasing (Panciera et
al., 1990; Baral et al., 2003). Predisposing factors, such as obesity due to overfeeding and physical inactivity, have increased dramatically in the feline population (Rand et al., 2004). In the cat and several other species, obesity promotes insulin resistance and increases the workload of pancreatic islet β-cells, ultimately leading to β-cell exhaustion and development of overt diabetes mellitus (Appleton et al., 2001; Wellen and Hotamisligil, 2005).

Conclusion

In this study, feline-specific mRNA sequences of several insulin signalling-related genes were determined. Primers were designed for quantification of levels of expression of these genes in tissues by real-time PCR. These tools should prove useful for future studies on gene dysregulation and metabolic abnormalities in disorders causing impaired insulin sensitivity, such as diabetes mellitus, in cats.

Acknowledgements

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References


## Tables

### Table 1

Primers used for amplification of feline cDNAs

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense (5′-3′)</th>
<th>Antisense (5′-3′)</th>
<th>Approximate product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adiponectin</td>
<td>GGCCTGGAGTCTCCTGCTCCC</td>
<td>GTACTGGTCATAGGTGAAGAG</td>
<td>500</td>
</tr>
<tr>
<td>GLUT1</td>
<td>GGGCTTCTCGAAACTGGGCAA</td>
<td>GGCTCGGGCTGACATCTGTCA</td>
<td>1,250</td>
</tr>
<tr>
<td>GLUT4</td>
<td>AAGATGCCGTCGGGCTTCCA</td>
<td>CTCACCCTCTGCTCTAGAAGAG</td>
<td>1,450</td>
</tr>
<tr>
<td>PPARγ1</td>
<td>TCTCTCCTAATGGAAGACC</td>
<td>GCATTATGAGACATCCCACC</td>
<td>450</td>
</tr>
<tr>
<td>PPARγ2</td>
<td>GCGATTCTTTCACGTGAC</td>
<td>GCATTATGAGACATCCCACC</td>
<td>550</td>
</tr>
<tr>
<td>PAI-1</td>
<td>TCGTCCAGGGATCTGAGGC</td>
<td>GGGCTGTCATGATGATCTGBC</td>
<td>800</td>
</tr>
<tr>
<td>MCP-1</td>
<td>TGCCGTGCTAGCTAGCCG</td>
<td>CATGGAATCTGGACCCACTT</td>
<td>250</td>
</tr>
<tr>
<td>Insulin receptor</td>
<td>TCTCCAGAAACCAGGTAAG</td>
<td>CATGGATATCCGGAACAAACC</td>
<td>1,800</td>
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</tbody>
</table>
Table 2

Feline-specific primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense (5′-3′)</th>
<th>Antisense (5′-3′)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adiponectin</td>
<td>CGGGTGAAAAGGGTGAGAAA</td>
<td>TGAACGCTGAGCGGTATACG</td>
<td>162</td>
</tr>
<tr>
<td>GLUT1</td>
<td>AGGAGAACCGGGCCAAGA</td>
<td>AGCGGAACAGCTCCAGGAT</td>
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<tr>
<td>GLUT4</td>
<td>TGGACGTGCAACTTCATCATC</td>
<td>CAGAAGGACCAGCAGACAGAA</td>
<td>90</td>
</tr>
<tr>
<td>PPARγ1</td>
<td>GCCGTGTCTGTGGAGATAAAGC</td>
<td>TCTGATTGTCCCGGGAAGAA</td>
<td>89</td>
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<tr>
<td>PPARγ2</td>
<td>GTCTGCAAGCACTTCACAAGAAA</td>
<td>ATCCACGGAGCTGATTCAAA</td>
<td>85</td>
</tr>
<tr>
<td>PAI-1</td>
<td>CAGAGCCAGGTCATCGTAA</td>
<td>AGTAGAGGCGCATTACCCAGCAT</td>
<td>122</td>
</tr>
<tr>
<td>MCP-1</td>
<td>TGCAGAGGCTGTGAGCTTAA</td>
<td>TTGCGTCAGCGAGATCTT</td>
<td>102</td>
</tr>
<tr>
<td>Insulin receptor</td>
<td>CTGCCCTGCCCAGTCTCATC</td>
<td>GCAGACGGTGGCGAACAATTT</td>
<td>85</td>
</tr>
</tbody>
</table>
Table 3

GenBank accession number and mRNA and protein homology of genes investigated in the cat

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank accession number</th>
<th>Canine identity</th>
<th>Human identity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mRNA</td>
<td>Protein</td>
</tr>
<tr>
<td>Adiponectin</td>
<td>DQ640898</td>
<td>93%</td>
<td>95%</td>
</tr>
<tr>
<td>GLUT1</td>
<td>DQ640900</td>
<td>95%</td>
<td>98%</td>
</tr>
<tr>
<td>GLUT4</td>
<td>DQ640899</td>
<td>90%</td>
<td>96%</td>
</tr>
<tr>
<td>PPARγ1</td>
<td>DQ640901</td>
<td>95%</td>
<td>97%</td>
</tr>
<tr>
<td>PPARγ2</td>
<td>DQ640902</td>
<td>95%</td>
<td>97%</td>
</tr>
<tr>
<td>PAI-1</td>
<td>DQ835567</td>
<td>91%</td>
<td>94%</td>
</tr>
<tr>
<td>MCP-1</td>
<td>DQ835566</td>
<td>93%</td>
<td>88%</td>
</tr>
<tr>
<td>Insulin receptor</td>
<td>DQ835565</td>
<td>94%</td>
<td>97%</td>
</tr>
</tbody>
</table>
Table 4

mRNA quantities in insulin sensitive tissues of healthy cats

<table>
<thead>
<tr>
<th>Gene</th>
<th>Subcutaneous fat mRNA</th>
<th>Visceral fat mRNA</th>
<th>Skeletal muscle mRNA</th>
</tr>
</thead>
</table>
| Adiponectin   | $0.7 \times 10^6 \pm 0.2 \times 10^6$ | $1.3 \times 10^6 \pm 0.1 \times 10^6$ | $3.8 \pm 0.5$ |*
| GLUT1         | $1.1 \times 10^2 \pm 0.3 \times 10^2$ | $3.2 \times 10^2 \pm 0.4 \times 10^2$ | $2.9 \pm 0.6$ |*
| GLUT4         | $31.0 \pm 7.6$ | $48.6 \pm 4.1$ | $1.1 \pm 0.1$ |*
| PPARγ1        | $9.2 \times 10^3 \pm 2.7 \times 10^3$ | $9.5 \times 10^3 \pm 1.8 \times 10^3$ | $8.6 \pm 2.3$ |*
| PPARγ2        | $0.7 \times 10^4 \pm 0.2 \times 10^4$ | $1.5 \times 10^4 \pm 0.2 \times 10^4$ | $11.0 \pm 3.1$ |*
| PAI-1         | $2.1 \times 10^2 \pm 0.7 \times 10^2$ | $2.4 \times 10^2 \pm 0.7 \times 10^2$ | $0.7 \pm 0.1$ |*
| MCP-1         | $1.1 \pm 0.3$ | $1.0 \pm 0.3$ | NA |**
| Insulin receptor | $1.6 \times 10^2 \pm 0.5 \times 10^2$ | $3.1 \times 10^2 \pm 0.6 \times 10^2$ | $3.1 \pm 0.7$ |*

Values are expressed as mean ± SEM. Transcript quantities are normalised to GAPDH and expressed relative to an internal calibrator.

* mRNA expression lower in skeletal muscle than in subcutaneous and visceral fat ($0.05<P<0.001$)

** Not available
Figure legends

Fig. 1. Partial feline-specific mRNA sequences. The mRNA product size is indicated. For each feline mRNA product the expected exon position within the corresponding gene is indicated according to complete canine and human sequences. Exons are alternatively depicted in bold and normal type.

Fig. 2. Deduced feline-specific protein sequences. Differing amino acids are specified for canine and human sequences. Identical amino acids are indicated by a horizontal line.