Hyperglycemia-induced changes in miRNA expression patterns in epicardial adipose tissue of piglets

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Abstract
MicroRNAs (miRNAs) are a class of molecular posttranscriptional regulators found to participate in numerous biological mechanisms, such as adipogenesis, fat deposition, or glucose metabolism. Additionally, a detailed analysis on the molecular and cellular mechanisms of miRNA-related effects on metabolism leads to developing novel diagnostic markers and therapeutic approaches. To identify miRNA whose activity changed in epicardial adipose tissue in piglets during hyperglycemia, we analyzed the different miRNA expression patterns between control and hyperglycemia groups. The microarray analysis selected three differentially expressed microRNAs as potential biomarkers: hsa-miR-675-5p, ssc-miR-193a-3p, and hsa-miR-144-3p. The validation of miRNA expression with real-time PCR indicated an increased expression levels of ssc-miR-193a-3p and miR-675-5p, whereas the expression level of hsa-miR-144-3p was lower in epicardial adipose tissue in response to hyperglycemia (P < 0.01). The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses suggested that these miRNAs differentially expressed between hyperglycemic and control piglets are involved in insulin, adipocytokine, and phosphatidylinositol 3-kinase–Akt signaling pathways, and development of type 2 diabetes as well. The results suggested that hyperglycemia can significantly affect the expression patterns of miRNA in porcine adipose tissue.

Introduction
Accumulation of visceral adipose tissue has been associated with an increased risk of metabolic syndrome defined by a combination of insulin resistance, hyperglycemia, dyslipidemia, increased expression of inflammatory mediators as well as cardiovascular diseases (CVDs) (Vernon et al. 2001, Blüher 2013, Rosenquist et al. 2013). Pericardial fat is deposited around the heart at two locations: epicardial (EAT) and paracardial adipose tissue separated from each other by the parietal pericardium. EAT is defined as an adipose tissue situated within the pericardium (close anatomic relationship with myocardium) and pericoronary fat situated around the coronary arteries (Iacobellis et al. 2009). It has been established a positive relationship between the amount of EAT and several components of the metabolic syndrome. Iacobellis and Willens (2009) indicated the association with insulin resistance, central adiposity, and clinical parameters of cardiovascular risk, including LDL cholesterol and blood pressure, together with inverse relationship with adiponectin levels. Without doubt,
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E AT is an anatomically unique adipose depot, which demonstrates a transcriptome distinct from other visceral fat pads (perirenal, gonadal, retroperitoneal, omental, and mesenteric) and subcutaneous adipose tissue (SAT) in the same subjects. There are few microarray studies of EAT’s transcriptome in human and other large mammals, especially pig. McAninch and coworkers indicate the genome-wide mRNA profile of EAT versus SAT in patients with coronary artery disease (CAD) and found enrichment in genes involved in endothelial function, coagulation, or immune signaling, and lack of expression of genes associated with protein metabolism and oxidative stress (McAninch et al. 2015). It has been investigated that similar interactions might exist in the adipose tissue depots in a pig model of familial hypercholesterolemia with CAD (Company et al. 2010). Results of transcriptome and gene expression analysis in pigs have been regarded as having a high positive predictive value for subsequent translation to humans.

An identification of reliable biomarkers that can be measured routinely in easily accessible samples, such as plasma or urine, is one of the main challenges in metabolic research. miRNAs are currently being explored for their potential as biomarkers for obesity, diabetes, or CVD because of their stability in the circulation. Additionally, they can be detected in a quantitative manner by methods such as real-time PCR (RT-PCR) and microarrays. The role of miRNAs in diabetes has been associated with several pathogenic features. It was indicated that miR-410, miR-200a, and miR-130a regulate the secretion of insulin in response to stimulatory levels of glucose, and overexpression of miR-410 enhances the levels of glucose-stimulated insulin secretion (Hennessy et al. 2010). Furthermore, miRNA-143 is upregulated during differentiation of human preadipocytes (Esau et al. 2004). miR-30d is upregulated in pancreatic beta cells in response to increasing insulin gene expression (Tang et al. 2009). It was reported that miR-375 is involved in the control of insulin gene expression and secretion (Poy et al. 2004), and overexpression of miR-29 inhibits insulin-stimulated glucose uptake and may cause insulin resistance (He et al. 2007). It has been proposed that peripheral blood mononuclear cells can be used as reporter cells to characterize the miR expression profiling during type 1 (T1D) and type 2 diabetes mellitus (T2D) and gestational diabetes mellitus. Collares and coworkers evaluated shared miRNAs among the major types of diabetes, including hsa-miR-29b, miR-142-3p, and hsa-miR-142-5p (Collares et al. 2013). Many of these shared miRNAs have been associated with metabolic pathways, immunological processes, and tumorigenesis. Recent data confirmed that nine miRNAs were shared among the three types of diabetes, including hsa-miR-126, hsa-miR-144, hsa-miR-27a, hsa-miR-29b, hsa-miR-1307, hsa-miR-142-3p, hsa-miR-142-5p, hsa-miR-199a-5p, and hsa-miR-342-3p (Collares et al. 2013). Thus, the aim of the study was to identify miRNAs whose activities changed in epicardial adipose tissue during hyperglycemia in piglets.

Materials and methods

Animals

The experiments and treatments were conducted in compliance with the European Union regulations concerning protection of the experimental animals. All experimental procedures were performed according to rules accepted by the First Local Ethical Commission for Investigation on Animals (Resolution no. 77/2008). Eight-week-old piglets (Sus scrofa domesticus, Pulawska, n=12) were purchased from the National Research Institute of Animal Production in Krakow-Balice, Poland. The Pulawska breed is kept locally and represents the group that constitutes the genetic reserve. Animals were housed in individual environmentally controlled cages maintained at 20–23°C with a 12h light:12h darkness cycle. They were fed a commercial feed with a standard grain-based diet fulfilling their daily maintenance requirements and had free access to water. The animals were divided into control and experimental (hyperglycemic, H) groups. Piglets received intraperitoneal injections of 0.9% sterile saline (control) or three injections of streptozotocin (experimental; STZ, Sigma-Aldrich). STZ-induced hyperglycemia was performed as described previously by Oclor and coworkers (2015). Concisely, STZ was diluted in cold 0.1 M citric acid buffer, pH 4.5 to the concentration of 0.4% (w/v), and injected within minutes after reconstitution. STZ was given at a dose of 150 mg/piglet for three consecutive days (75, 50, and 25 mg). Blood samples from the external jugular vein were collected in heparinized tubes and centrifuged (1500 × g, 10 min, 4°C); the plasma was stored at –80°C until further estimations. The animals were slaughtered 24 h after the last administration of STZ, and their epicardial adipose tissue was isolated, immediately frozen in liquid nitrogen, and stored at –80°C until further RNA analysis. EAT was defined and identified as the thinner contiguous adipose tissue beginning ~10–20 mm away from the coronary vessel extending down on the ventricular myocardium.
Sample preparation

Total RNA was extracted from EAT using miRCURY Isolation Kit—Cell & Plants (Exiqon, Vedbaek, Denmark) in accordance with the manufacturer’s instructions. For optimal isolation of RNA from tissues with high lipid content, the modified protocol using lysis additive was applied. RNA quality was assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific) and a 2100 Bioanalyzer (Agilent). Samples with RNA integrity number (RIN) values higher than seven were used to perform array.

LNA-based miRNA microarray

The microarrays were miRCURY v10.0 locked nucleic acid (LNA) miRNA array from Exiqon. The Exiqon probe set consists of 1700 custom-made capture probes that are enhanced using LNA technology, which is claimed to normalize the \( T_m \) of the capture probes, as insertion of one LNA molecule into the capture probes increases the \( T_m \) by 2–8°C. Total RNA (2 μg) was labeled with Hy3 dye according to the manufacturer’s protocol using the labeling kit from Exiqon. For the labeling reaction, RNA was incubated with the Hy3 dye, labeling enzyme, and spike-in miRNAs, in a total volume of 12.5 μL, for 1 h at 16°C. The enzyme was then heat inactivated at 65°C for 15 min. The samples were incubated at 95°C for 2 min and protected from light. A total of 32.5 μL of hybridization buffer was added to make up the volume required by the hybridization station. The samples were briefly spun down and filtered through a 0.45-micron durapore filter (Millipore). Samples were then loaded onto the MAUI (BioMicro Systems, Inc., Salt Lake City, UT, USA) hybridization station. Low- and high-stringency washes were carried out to minimize nonspecific hybridization, and the microarrays were then dried. Images were acquired using a GenePix 4200A microarray scanner (Axon Instruments–MDS, Burlingame, CA, USA).

Microarray data processing

Data were analyzed using GenePix Pro 6 software (Axon, Foster City, CA, USA). Following quantile normalization of the entire chip, the distribution of intensities was plotted for all of the human-annotated miRNA probes, and this was compared with background signal intensities, with a cutoff of 400 units being taken as an expressed miRNA (total of 280 porcine miRNAs). The corrected signal intensities were transformed into a ratio (sample/common reference). The ratios were log transformed and then normalized using a global loess normalization procedure. Differential expression was determined using the significance analysis of microarray (SAM) approach, and miRNAs with a false discovery rate (FDR) of 10% or better and modulated by >30% were selected for further validation studies.

Direct miRNA quantification

The validation of miRNA expression data for three miRNAs (miR-675-5p, miR-193a-3p, miR-144-3p) by quantitative RT-PCR was performed using total RNA from the 24 porcine samples studied and microRNA-specific primers (TaqMan MicroRNA Assay, Life Technologies). Briefly, reverse transcription (TaqMan MicroRNA Reverse Transcription Kit, Life Technologies) was carried out in a total reaction volume of 15 μL containing 5 μL total RNA (concentration 10 ng/μL), 3 μL of reverse transcription primer, 1.50 μL of 10× reverse transcription buffer, 1.00 μL MuLV Reverse Transcriptase (50 U/μL), 0.15 μL of 100 mmol/L dNTPs (with dTTP), 0.19 μL of RNase inhibitor 20 U/μL, and 4.16 μL of nuclease-free water (all reagents supplied by Life Technologies). Reactions were incubated according to the manufacturer’s recommendations. Quantitative RT-PCRs were performed in triplicate; the 10 μL PCR reaction contained 1.33 μL reverse transcription product, 10 μL TaqMan 2x Universal PCR Master Mix without AmpErase UNG, 1 μL microRNA primer (Life Technologies), and 7.67 μL of nuclease-free water (Table 1). The reactions were incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 35 s. The highly conserved and universally expressed 18S rRNA was used as normalizing endogenous controls in the quantitative RT-PCR. Fold changes (FCs) in expression were calculated using the \( 2^{-\Delta\Delta Ct} \) method.

In silico functional profiling of target genes

The binding of miRNA to target mRNA occurs between the ‘seed’ region of miRNA (nucleotides from 2 to 7 at the 5’ end of mature miRNA) and the 3’ untranslated region of the mRNA. Potential miRNA target genes were identified and retrieved using the algorithms implemented by TargetScan 6.2 (Grimson et al. 2007). miRNA pathway analysis was performed using DIANA-mirPath. This allowed the identification of molecular pathways potentially altered by the expression of single or multiple miRNAs (Papadopoulos et al. 2009). An enrichment analysis of miRNA target genes comparing each set of miRNAs to all available pathways.
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The obtained plasma biochemical parameters (glucose, total cholesterol, triglycerides) were collected and shown in Table 2. The results confirmed the development of hyperglycemia in experimental group (H: 5.25 ± 0.79 mmol/L) relative to the control group (1.57 ± 0.24 mmol/L, P < 0.01). Plasma insulin level increased in experimental group (H: 120.15 ± 18.02 pmol/L) in comparison with the control (62.37 ± 9.98 pmol/L, P < 0.01). Total plasma cholesterol concentrations were higher in experimental groups (H: 6.07 ± 0.91 mmol/L) compared to the control groups (4.67 ± 0.6 mmol/L, P < 0.05). In hyperglycemic piglets, the level of triglycerides was significantly higher (0.56 ± 0.09 mmol/L) than in the control group (0.23 ± 0.04 mmol/L, P < 0.05).

Expression of miRNAs in EAT during hyperglycemia

miRNA expression was characterized using the Exiqon microarray platform in EAT samples (n = 4) that passed the quality control tests. Approximately 50 miRNAs in EAT has been detected (Fig. 1). The three miRNAs (hsa-miR-675-5p, ssc-miR-193a-3p, hsa-miR-144-3p; difference in log FC greater than 1 between the control and experimental groups) were selected for validation (n = 18). Relative levels of expression for selected miRNAs were validated by RT-PCR. The FC detected in the RT-PCR experiment was greater than that in the microarray experiment for hsa-miR-675-5p (FC: 10.18 vs 2.18) and ssc-miR-193a-3p (FC: 14.82 vs 2.19). miR-144-3p was downregulated in EAT of hyperglycemic piglets (FC: 0.34 vs 0.48) (Fig. 2).

In silico functional profiling

TargetScan predicted a total of 1099 target genes for three coexpressed miRNAs in EAT in piglets (data not shown).
The predicted target genes were further classified to identify pathways that were actively regulated by miRNAs according to DAVID KEGG analysis (Table 3). It is worth noting that the targets of the majority of upregulated miRNAs in the hyperglycemic piglets belonged to the insulin and adipocytokine signaling pathways.

Discussion

There is an increasing evidence that miRNAs play a role in regulating glucose and lipid metabolism through the control of pancreatic islet cell function, adipocyte insulin resistance, hepatocyte insulin signaling, and glucose homeostasis, and hence may be involved in the pathogenesis of disorders such as T2D (Fernandez-Velverde et al. 2011). miRNAs in adipose tissue are strongly deregulated in response to hyperglycemia-induced molecular changes and environmental signals. For instance, the expression of miR-29 family is upregulated in adipocytes in response to high glucose (Herrera et al. 2010). Likewise, miR-320 increases insulin sensitivity of insulin-resistant adipocytes (Ling et al. 2009), and miR-27b impairs human adipocytes differentiation (Karbiener et al. 2009). In this study, we used microarrays to obtain the expression profiles of miRNAs in EAT from hyperglycemic piglets and compared them with expression profiles from control. As mentioned previously, EAT has anatomical proximity to the heart. Therefore, it is reasonable to expect EAT to be closely associated with derangements in the cardiac morphology and function during hyperglycemia. Additionally, the recent data indicated that microRNAs are transmitted...
from one tissue to another (Dinger et al. 2008). Our results demonstrated that miRNAs such as miR-193a-3p and miR-675-5p were upregulated in EAT of hyperglycemic piglets, whereas miR-144-3p was downregulated ($P < 0.01$). The in silico prediction of miRNA target genes and functional analysis can provide clues as to what biological processes may be disrupted by altered miRNA expression. Based on published reports and bioinformatics-based data, we identified the potential glucose metabolism-related mRNA targets for these miRNAs. miR-675-5p or miR-193a-3p may influence particular elements of the insulin pathway. The actions of endogenous PTEN include reducing insulin-mediated stimulation of glucose transport in 3T3-L1 adipocytes and likely extend to other physiological processes regulated by Akt protein kinases in adipocytes (Magnuson et al. 2012). Under normal conditions, activation of PI3K leads to activation of Akt, which phosphorylates and inhibits many downstream substrates, including BAD, FOXO transcription factors, GSK3, and TSC2). Through these and other targets, Akt activity stimulates glucose uptake, cell growth, and proliferation, and inhibits apoptosis. Akt-directed phosphorylation of TSC2 relieves its inhibition of target of rapamycin (TOR).

In addition to promoting glucose uptake, insulin inhibits production and releases glucose by blocking hepatic gluconeogenesis. Gluconeogenesis is controlled through the transcriptional modulation of PCK and glucose-6-phosphatase (G6P), the rate-limiting enzymes in the process, which have been shown to be regulated by glucagon and insulin (Ranmannan et al. 2011, Oh et al. 2013). In our study, miR-675-5p was significantly upregulated in EAT during hyperglycemia. The in silico prediction of miR-675-5p indicated that PCK-2 is targeted by this miR. Glyceroneogenesis is an abbreviated version of gluconeogenesis in which glycerol-3-phosphate is produced from substrates such as pyruvate, lactate, or alanine. Glyceroneogenesis, like gluconeogenesis, is regulated by the activity of PCK. PCK is involved in adipose tissue triglyceride storage and may play a crucial role in lipid storage regulation. It is implicated in glyceroneogenesis, impacting on both the storage and the regulated release of fatty acids via a triglyceride–fatty acid cycle in adipose

### Table 3 KEGG pathway enrichment analysis of miRNA target genes.

<table>
<thead>
<tr>
<th>miRNAs</th>
<th>Significant pathways</th>
<th>$P$-value</th>
<th>Target genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>mir-144-3p</td>
<td>Insulin signaling pathway (04910)</td>
<td>0.006</td>
<td>GSK3B, SOS2, CALM1, SOCS2, PIK3CB, PPP1CC, PCK2, KRAS, TSC2, EIF4E, MAPK8, PRKX, PRKAA1, AKT3, MTOR</td>
</tr>
<tr>
<td>miR-675-5p</td>
<td>Adipocytokine signaling pathway (04920)</td>
<td>0.001</td>
<td>PCK2, CAMKK2, JAK2, PPARA, MAPK8, PRKAA1, AKT3, MTOR, RXRB</td>
</tr>
<tr>
<td>miR-193a-3p</td>
<td>PI3K–Akt signaling pathway (04151)</td>
<td>0.001</td>
<td>PRLR, GSK3B, RBL2, PPP2R5E, MYB, SOS2, ITGB8, ITGA8, PIK3CB, YWHAH, GN61, IL7, EGFR, GNB1, PPP2R2D, KRAS, CDK6, PT2, TSC2, CCRD1, JAK2, EIF4E, PPP2R2A, YWHAZ, KITLG, PRKAA1, LAMC1, ITGA7, AKT3, COL11A1, FN1, PK2, MTO, PTEN, SGK3, FGFR7, TEK, SOCS2, PIK3CB, PRKCE, MAPK8, MTO, CACNA1D</td>
</tr>
<tr>
<td>miR-144</td>
<td>Type 2 diabetes mellitus (04930)</td>
<td>0.012</td>
<td>trendy</td>
</tr>
</tbody>
</table>

$P$-values computed for each pathway were adjusted using the method of Benjamini and Hochberg to control the FDR, and adjusted $P < 0.01$ was considered significant.
tissue (Beale et al. 2007, Karolina et al. 2011). Moreover, the rate of lipid release is a balance between lipolysis and esterification. Notwithstanding, dysfunctional adipogenic Pck1 expression leads to obesity (due to an increase in triglyceride storage) and insulin resistance (due to a decrease in triglyceride storage) in association with increased and decreased Pck1 activity, respectively (Beale et al. 2007). Beale and coworkers discussed a novel hypothesis suggesting that dysfunctional regulation of Pck1 in adipose would be a causal factor in T2D and obesity (Beale et al. 2007). Furthermore, mutations that increase Pepck1/Pck1 activity would increase blood glucose, in association with compromised insulin-mediated control of hepatic gluconeogenesis. By contrast, mutations associated with impaired hepatic Pck1 expression have been reported to induce hypoglycemia.

Interestingly, Karolina and coworkers reported that miR-144 shows the highest upregulation in T2D in the pancreas, liver, skeletal muscle, adipose and blood (Karolina et al. 2011). miR-144 also exhibited an approximately linear relationship with increasing glycemic status in T2D patients. In addition, it was indicated that miR-144 negatively modulates IRS1. miR-144 targets IRS1, a gene highly involved in insulin signaling pathway, and upregulation of this miRNA exhibits a linear relationship with the glycemic status in T2D patients (Iwaya et al. 2012). However, we observed a similar downexpression of miR-144 in our samples. Clearly, the role of miR-144 in adipose tissue during hyperglycemia needs to be robustly investigated. It was indicated that miR-144 is involved in the regulation of genes of mammalian TOR (mTOR) pathway (Iwaya et al. 2012).

Many paths can lead to systemic insulin resistance, and there are considerable intertissue communications to coordinate the whole-body metabolism in diverse situations such as eating or fasting. The miRNA profile of insulin resistance tissues changes years before diagnosis of T2D. Thus, miRNAs might be not only markers of early-onset disease but also responsible for its progression and thus a good target for early intervention.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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