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HYPERGLYCEMIA-INDUCED CHANGES IN RESISTIN GENE EXPRESSION IN WHITE ADIPOSE TISSUE IN PIGLETS*

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Abstract

Previous data strongly indicated that resistin, an adipocyte-derived signalling peptide, plays an important role in metabolism and glucose homeostasis. Thus, the aim of the present study was to examine changes in synthesis and concentration of resistin in white adipose tissue in response to hyperglycemia in piglets. In order to develop hyperglycemia, piglets (10-week-old, Polish Landrace fatteners, female) received intraperitoneal (ip) injection of 150 mg streptozotocin (HI, n=6) or 60 mg synthetic glucocorticoid (HII, n=6). An injection of NaCl physiological saline was used as a control (n=6). Plasma resistin level was significantly higher in HII group compared with the control, while no difference was observed in HI. In epicardial adipose tissue (EAT) the resistin mRNA level significantly increased whereas the opposite effect was observed for omental fat tissue (OAT) in both experimental groups. Additionally, the resistin concentration did not change in EAT; however, it was decreased in omental adipose tissue in response to hyperglycemia. The results obtained indicate that activity of resistin strongly depends on glycemic status as well as adipose tissue localization.

Key words: resistin, white adipose tissue, hyperglycemia, piglets

In the recent years, the white adipose tissue (WAT) has been recognized as an important source of a number of bioactive molecules (adipokines) that play a key role in energy homeostasis. Adipokines influence various physiological processes, including food intake (Mars et al., 2005), regulation of lipid and glucose metabolism (Banerjee et al., 2004; Sato et al., 2005), insulin action (Sheng et al., 2008), inflammation (Fu et al., 2006), angiogenesis and cardiac homeostasis (Mu et al., 2006). Among variety of adipokines, resistin is proposed as an important factor in the regulation of glucose homeostasis and insulin sensitivity.

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Resistin, also called ADSF (adipocyte-specific secretory factor), belongs to the cytokine-rich protein family (Steppan et al., 2001). The involvement of resistin in the regulation of glucose homeostasis has been widely investigated although some data currently remain ambiguous. The results depend on the applied animal, which emphasizes the importance of testing and generating an optimal experimental model. According to recent studies, resistin may be involved in glucose metabolism via: suppression of GLUT 4 gene expression (Fu et al., 2006), blockade of insulin signal transduction pathways (Sheng et al., 2008), stimulation of hepatic glucose production (Banerjee et al., 2004) and increasing expression of gluconeogenic enzymes in the liver (Banerjee et al., 2004). It was also reported that resistin down-regulated mRNA and protein expression of insulin receptor in rodent pancreatic beta-cells, but did not affect insulin secretion (Brown et al., 2007).

White adipose tissue is not confined to a specific location but occurs in individual, physically unconnected pads. Furthermore, little is known about coordination of the adipose tissue secretion between depots and secretory pathways of specific adipokines (Kremen et al., 2006). Distinct fat compartments may be associated with diverse metabolic risk. A correlation has been reported between body mass index (BMI) and visceral fat disturbances which appear to be an important indicator for metabolic and/or cardiovascular alterations (Iacobellis et al., 2003). Previous study has documented that visceral adipose tissue (VAT) represents an essential connection between the components of the metabolic syndrome such as glucose intolerance, dyslipidemia, insulin resistance, and cardiovascular disease (Kim et al., 2008). Epicardial fat is a localized form of adipose tissue, present between the outer wall of the myocardium and the visceral layer of pericardium (Iacobellis et al., 2003; Iacobellis and Barbaro, 2008). Epicardial adipose tissue (EAT) as a source of various adipokines acts through paracrine mechanisms on cardiac cells and could play an active role as a risk factor (Karmazyn et al., 2008). The increased morbidity of cardiovascular disease (CVD) with obesity causes adipokines, also resistin, an attractive harbinger of cardiometabolic disorders. In the more recent studies, resistin was associated with an increased risk of coronary artery disease (Korah et al., 2011) and myocardial ischemia (Langheim et al., 2010).

Since the pig genome sequence was obtained, the popularity and value of swine in biomedical research has accelerated. Pigs have many similarities in anatomy and physiology to human, including size, feeding patterns, digestive and cardiovascular anatomy and physiology. For obesity studies, pigs offer several advantages, including fat cell size, and body fat distribution similarity to humans (Schook et al., 2005).

Thus, the aim of the present study was to investigate the effect of hyperglycemia on the resistin synthesis and concentration in white adipose tissue in piglets. To examine if the response to hyperglycemia depends on the type of white adipose tissue we compare epicardial and omental fat tissues.

Material and methods

Animals

Ten-week-old piglets (Polish Landrace fatteners, female, n=18) were purchased from the National Research Institute of Animal Production in Balice, Poland. Animals were housed in individual environmentally controlled pens maintained at 20–23°C with a 12:12-h light-dark cycle. They were fed a commercial feed with a standard grain-based diet fulfilling their daily maintenance requirements and had free access to water. Before the experiment piglets were weighed and separated into 3 equal groups so that the average body weight was as uniform as possible between groups (12.1±1.3 kg). All experimental procedures were performed according to rules accepted by The First Local Ethical Commission for Investigation on Animals (No. 77/2008).

The animals were divided into the control and two experimental (hyperglycemic) groups HI and HII. Piglets received intraperitoneal (ip) injections of 0.9% sterile saline or 3 injections of streptozotocin (HI: STZ, Sigma-Aldrich, USA) or glucocorticoid (HII: Prednisolone, Sigma-Aldrich, USA). STZ-induced hyperglycemia was performed according to the modified protocol described previously (Hara et al., 2008). Briefly, STZ was diluted in cold 0.1 M citric acid buffer, pH 4.5 to the concentration of 0.4% and injected within minutes after reconstitution. STZ was given at the dose of 150 mg/piglets for 3 consecutive days (75; 50; 25 mg). The procedure of glucocorticoid-induced hyperglycemia was based on results obtained by Karlsson et al. (2001). Compactly, prednisolone was reconstituted with 0.9% NaCl and injected for 3 following days at 10, 20 and 30 mg/piglet (total 60 mg/piglet).

Blood and adipose tissue collection

In the next step, the animals were fasted for 24 hours, blood samples from external jugular vein were taken to heparinised tubes and centrifuged (1500 × g, 10 min, 4°C); the plasma was stored at –80°C until further estimations. Directly after a slaughter, epicardial and omental fat tissue samples were collected, immediately frozen in liquid nitrogen and stored at –80°C until further analysis.

EAT was defined and identified as the thinner contiguous adipose tissue beginning ~10 mm to 20 mm away from the coronary vessel extending down on the ventricular myocardium. OAT was sampled adjacent to the spleen in the greater omentum.

Assay

Plasma levels of biochemical factors (glucose, cholesterol, triglycerides) were determined using commercial kits based on enzymatic technique coupled with colorimetric detection (AlphaDiagnostic, Poland). Plasma concentrations of insulin were determined using a commercially available kit (Porcine Insulin RIA, Millipore, USA). The assay sensitivity was 1.611 μIU/ml and intra- and interassay CV were 5% and 10%, respectively. All samples for resistin determination in WAT were homogenized in ice-cold 0.1 M Tris-HCl buffer, pH 7.5. The homogenates were centrifuged at 1000 × g for 20 minutes at 4°C, then the supernatant was collected. Protein concen-

tration was measured using BCA kit (Sigma, USA). Furthermore, resistin (dimeric form) was measured by standard commercial ELISAs according to the manufacturers' recommended protocols (BioVendor, Czech Republic). The intra- and interassay CV for the resistin ELISA were 5.9 and 7.6%, respectively. The assay can measure a concentration as low as 0.012 ng/ml and is sensitive and specific enough to measure resistin protein in various adipose tissues. Briefly, polyclonal coating antibodies for human resistin were bound to enhanced protein – binding 96-well plates. Producer confirmed strong crossreactivity to swine resistin (the porcine amino acid sequence of resistin showed 75.2% identity to the human resistin). Absorbance of the experimental and control samples was measured 20 min after termination of the reaction at 450 nm, using an ELISA plate reader (BioTek, USA).

RNA extraction and cDNA synthesis

Total RNA was extracted from epicardial and abdominal adipose tissues using Trizol Reagent (Sigma-Aldrich, USA). Concentration and purity of the RNA samples were analysed by UV spectroscopy at 260/280 nm, and integrity was confirmed by electrophoresis through 1% agarose gels stained with ethidium bromide. First strand cDNA was transcribed from 1 μg RNA with MultiScribe Reverse Transcriptase (50 U/μl, Life Technologies, USA) using random primers at 25ºC for 10 min followed by 37ºC for 120 min and 85ºC for 5 s (High Capacity cDNA Reverse Trancription Kit, Life Technologies, USA). The cDNA was reconstituted in 50 μl of sterilized water and 500 ng of the cDNA solution was used as a template.

Real-time PCR

Real-time PCR analysis was performed using the StepOnePlus Real-Time PCR System (Life Technologies, USA) with the Universal Master Mix and TaqMan chemistry (Life Technologies, USA). Amplification and detection were carried out in optical-grade 96-well plates with an initial step of 50°C for 2 min, which is the required optimal AmpErase UNG enzyme activity, and then at 95°C for 10 min, to activate the AmpliTaq Gold DNA polymerase and to deactivate the AmpErase UNG enzyme, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The expression of analyzed gene (resistin, TaqMan Gene Expression Assay, ID: Ss03381398_u1; Reference sequence accesion number Nm_213783.1; Probe sequence: CCGGGATGTCGCCAGTTTCCTAATT, forward primer: ATGAAGCCATCAATGAGA, reverse primer: GCCTGAGGGGCAGGTGAC; Amplicon size: 89 bp) was related to the expression of reference gene 18S rRNA (Hs99999901_s1, RefSeq: X03205.1; Life Technologies, USA). The data were calculated according to the ΔΔCt method, using the expression in the white adipose tissue (EAT, OAT) of the control group as the calibrator (relative quantity; RQ=1) and presented as RQ±SD. Samples in which no amplified product could be detected by cycle 40 were considered not to express the gene being assayed.

Data of the experiments were statistically analysed by one way ANOVA followed by the Tukey-Kramer test. All statistical analysis was performed using the commercial package StatView (SAS Institute). Values are expressed as the mean ± SEM and considered significantly different at $P<0.05$.

Results

Plasma biochemical parameters and resistin levels

The obtained plasma biochemical parameters (glucose, total cholesterol, triglycerides) were collected and shown in Table 1. The results confirmed developing of hyperglycemia in experimental groups (HI: 5.52±0.89 mmol/l, HII: 5.01±0.57 mmol/l) in comparison with the control group (2.7±0.18 mmol/l, P<0.01). Plasma insulin level increased in both experimental groups (HI: 117.37±21.13 pmol/l, HII: 190.29±32.25 pmol/l) relative to the control (P<0.05). In hyperglycemic piglets the level of triglycerides was significantly higher (by 195% HI and 650% HII) than in the control group (P<0.01). Total plasma cholesterol concentration was higher in experimental groups (HI: 4.93±0.12 and H II: 7.54±1.05 mmol/l) compared to the control group (2.67±0.13 mmol/l, P<0.05). Furthermore, there was observed an increase in plasma resistin concentration only in HII (40.14±4.5 ng/ml) compared to the control value (11.21±1.86 ng/ml) (P<0.05; Fig. 1).

Table 1. Plasma biochemical parameters (mmol/l) in the control and hyperglycemic piglets

Parameter	Group		
	Control	HI	HII
Glucose (mmol/l)	2.7±0.18 a	5.52±0.89 b	5.01±0.57 b
Triglycerides (mmol/l)	0.20±0.03 a	0.59±0.08 b	0.41±0.07 b
Cholesterol (mmol/l)	2.67±0.13 a	4.93±0.12 b	7.54±1.05 c
Insulin (pmol/l)	73.62±11.12 a	117.37±21.13 b	190.29±34.25 c

a, b, c – P<0.01 – values in rows with different letters differ significantly.

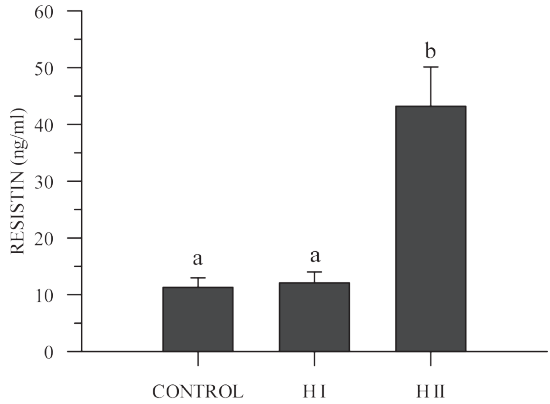

Figure 1. Plasma resistin concentration in the control and hyperglycemic piglets. HI – STZ-induced hyperglycemia; HII – glucocorticoid-induced hyperglycemia. Values marked with different letters differ significantly (P<0.01)

Expression of resistin in epicardial and omental adipose tissues

In epicardial tissue the mRNA expression of resistin was 2.5-fold higher in HI and 2-fold higher in HII compared with the control group (P<0.05; Fig. 2a). Contrary to results obtained for EAT, the resistin mRNA expression was lower in omental adipose tissue in both experimental groups (by 91.9% in HI and 84.7% HII; P<0.01; Fig. 2b).

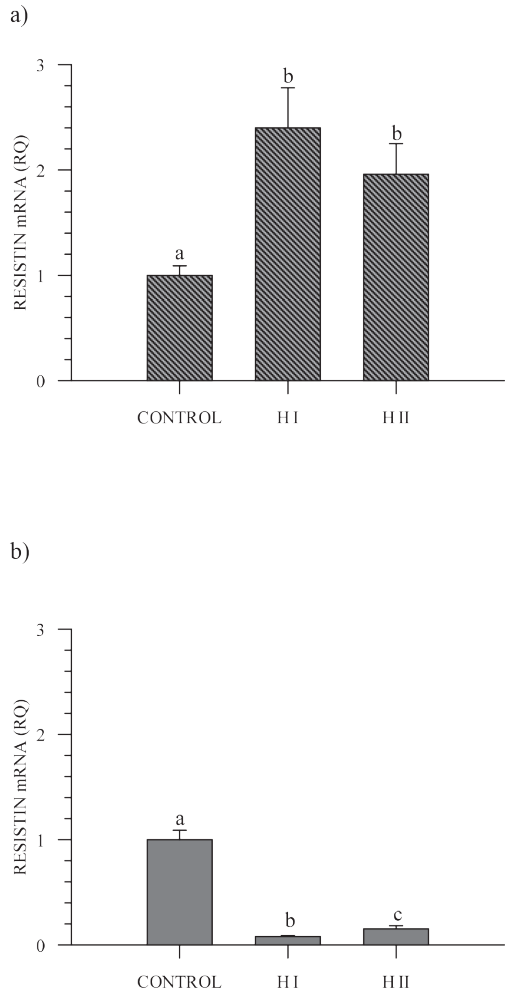

Figure 2. The resistin mRNA expression in epicardial (a) and omental (b) adipose tissues in piglets. a, b – $P<0.05$ – means with different letters differ significantly

Concentration of resistin in epicardial and omental adipose tissues

The concentration of resistin in omental adipose tissue was decreased in hyperglycemic piglets, by 25.71% (HI) and 45.67% (HII) respectively compared to the level observed in the control animals ($P<0.05$; Fig. 3a). Unexpectedly, there was no significant difference in resistin concentration in EAT ($P>0.05$; Figure 3b).

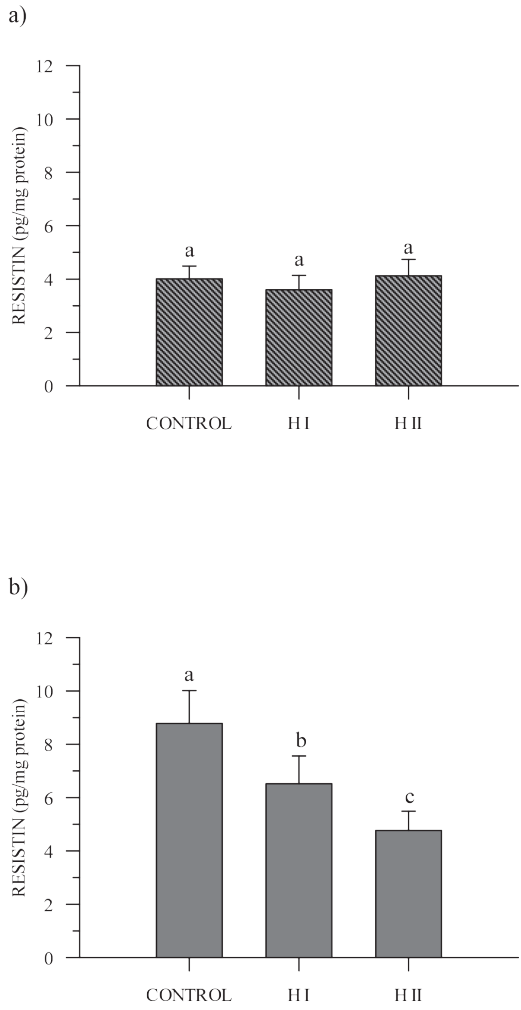

Figure 3. The concentration of resistin in epicardial (a) and omental (b) adipose tissues in piglets. a, b, c – $P<0.05$ – means with different letters differ significantly

Discussion

The current study characterised the changes of resistin mRNA expression and protein concentration in epicardial and omental adipose tissue in response to hyperglycemia induced by two different methods. We found that both procedures of developing hyperglycemia were effective at increasing glucose, triglycerides, cholesterol and insulin concentration in blood. Interestingly, plasma resistin level increased only in piglets with glucocorticoid-induced hyperglycemia. Sasayama et al.

(2014) observed a similar effect of administering another synthetic glucocorticoid, dexamethasone, on serum resistin level in human. Glucocorticoids (GCs) mainly stimulate gluconeogenesis by promoting the expression and activity of key enzymes of gluconeogenesis including: phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase). Certainly, the PEPCK gene contains a glucocorticoid response element (GRE) in its promoter region and is considered a key player in GC-induced hyperglycemia (van Raalte and Diamant, 2014). A previous study showed that mice lacking resistin have reduced glucose production associated with decreased expression of PEPCK and G6Pase (Banerjee et al., 2004). In addition, it was found that mice with chronic hyperresistinemia have increased glucose production along with increased hepatic expression of PEPCK and G6Pase (Rangwala et al., 2004). Therefore, a raised resistin serum level, detected only in HII might be a result of activation by such gluconeogenic factors. Subsequently, Shojima et al. (2002) revealed that resistin expression is greatly affected by a variety of hormones and environmental factors, such as insulin, somatotropin, tumor necrosis factor-α (TNF-α), epinephrine, glucose or dexamethasone. In obese rodents, thiazolidinediones suppress expression of resistin and normalize insulin resistance (Steppan et al., 2001). Thiazolidinediones exert their hypoglycemic effect by binding to peroxisome proliferator-activated receptor γ (PPAR-γ) which is expressed primarily in adipose tissue. This leads to more insulin-sensitive cells and hence reduced insulin resistance (Yki-Järvinen, 2004). These findings suggest that altered expression of resistin may be a key mechanism underlying obesity-induced insulin resistance. Additionally, it is known that thiazolidinediones improve insulin sensitivity by suppressing production of TNF-α in enlarged adipocytes (Miles et al., 1997). Shojima et al. (2002) found that thiazolidinediones markedly suppress expression of resistin in 3T3-L1 adipocytes.

It has been indicated that high levels of glucose significantly enhanced resistin expression, whereas insulin suppressed its expression in adipocytes (Shojima et al., 2002). Kawashima et al. (2003) reported that insulin down-regulated resistin mRNA via PI 3-kinase-, ERK- or p38 MAP-kinase independent pathway in 3T3-L1 adipocytes. Interestingly, insulin-induced decrease of resistin mRNA occurred even in glucose-free medium, suggesting that the effect of insulin is not mediated through increased glucose metabolism. In diabetic individuals, the early state is often characterized by hyperglycemia and hyperinsulinemia (Antuna-Puente et al., 2008). Therefore, it is unclear whether resistin expression is up- or down-regulated at this stage, because hyperglycemia and hyperinsulinemia oppositely regulate resistin expression. Alternatively, it is also possible that insulin resistance (type 2 diabetes) limits the ability of insulin to suppress resistin expression by adipocytes. At more advanced stages of the diabetes type 2, which are marked by hyperglycemia and hypoinsulinemia, resistin would be expected to be up-regulated and thus contribute to hyperglycemia-induced insulin resistance. Additionally, glucocorticoids are known to induce insulin resistance, supposedly by affecting insulin binding, insulin receptor substrate-1 phosphorylation, and glucose transporter translocation (Sakoda et al., 2000). Shojima et al. (2002) observed that dexamethasone increased resistin expression in both 3T3-L1 adipocytes and white adipose tissue. The results suggest

that glucocorticoid-induced up-regulation of resistin expression may also contribute to insulin resistance.

Subsequently, we found the differential expression and concentration of resistin in two distinct visceral adipose tissues, epicardial and omental. In EAT the resistin mRNA level increased whereas its concentration did not change in response to hyperglycemia. In monocytic cells, high glucose concentration induced a significant increase in resistin gene expression by mechanisms involving p38, ERK1/2 and JNK MAPKs and transcription factor NFκB. To date, it remains unknown whether similar signaling occurs in adipocytes (Stan et al., 2011). Another possible explanation for these results may be an oxidative stress which is induced by hyperglycemia. Previous study of Lin et al. (2001) demonstrated that hyperglycemia-induced oxidative stress can affect genes expression in white adipose tissue. Many of these genes are regulated by NFκB, the proinflammatory factor which acts as a transcription factor for various genes, including those to restore appropriate redox homeostasis (Mariappan et al., 2010). Hung et al. (2008) also reported that reactive oxygen species (ROS) increases resistin protein and mRNA expression in vascular smooth muscle cells (VSMC). A functional consequence of resistin gene up-regulation resulted in reduction of glucose uptake. Furthermore, hyperglycemia and hyperlipidemia have been shown to induce proinflammatory cytokines synthesis and release (Fantuzzi, 2005). Moreover, it has been reported that visceral fat is more strongly associated with oxidative stress than subcutaneous fat or general adiposity. Gletsu-Miller et al. (2009) confirmed that fat volumes determine indices of systemic oxidative stress and dimension of inflammatory response. It is also conceivable that resistin may act as pro-inflammatory cytokine (Bakker et al., 2009). It has been indicated that resistin up-regulates inflammatory and adhesion molecules in endothelial cells. Therefore, it is considered as an inflammatory marker of atherosclerosis in humans and may represent a novel link between metabolic signals, inflammation (Bakker et al., 2009) and atherosclerosis (Aldhahi and Hemdy, 2003). In the last decades, much attention has been paid to confirm the role of a constant local EAT inflammation in the whole heterogeneous group of high-risk cardiovascular diseases (CVDs) patients. Mazurek et al. (2003) analysed the pro-inflammatory cytokines profile in epicardial adipose tissue compared to subcutaneous depot. They confirmed that EAT tissue expresses numerous inflammatory mediators including: interleukin-1β, interleukin-6 and interleukin-6 receptor and tumor necrosis factor-α (Mazurek et al., 2003). More recently, Langheim et al. (2010) demonstrated that EAT of patients with acute coronary syndrome (ACS) is characterized by an increased production of resistin. Interestingly, Iacobellis and Leonetti (2005) indicated that there is no fascia separating the myocardium from adipose deposit and both constituents share the same blood supply. In this context, Gao et al. (2007) demonstrated that in mice resistin has a cardioprotective effect. Their study established that resistin can significantly reduce the damage and thus protects the heart against ischemic/reperfusion (I/R) injury. It is suggested that the mechanism is connected with PI3K/Akt/PKCε/KATP-channel-dependent pathway. They postulated that resistin has three main features that would make it an efficient therapeutic agent. Firstly, it is endogenous hormone that should have minimal side effects. Secondly, it has a delayed cardioprotective

effect (still remaining even 24 h after resistin injection). Such a property would be of great benefit for many patients, particularly those undergoing cardiac bypass surgery. Thirdly, it was previously demonstrated that resistin promotes angiogenesis which may improve the recovery of cardiac function after myocardial infarction. Nevertheless, the specific mechanisms causing the increased production of resistin in EAT remain to be elucidated.

Our study established that resistin is expressed in omental adipose tissue in piglets. In contrast to EAT, the simultaneous decrease in resistin expression and concentration in omental adipose tissue was observed during hyperglycemia. Using method of microarrays, Meugnier et al. (2007) demonstrated that 3 h of hyperglycemia caused a marked reduction in the mRNA levels of about 500 genes in skeletal muscle and subcutaneous abdominal adipose tissue. Almost every physiological pathway seems to be affected because hyperglycemia is able to produce a marked reduction in the expression of genes coding proteins involved in almost all the biological processes (Meugnier et al., 2007). The modification of the activity of specific transcription factors by oxidative stress could be a possible mechanism to explain the observed global down-regulation of gene expression during the hyperglycemic clamp. Furthermore, insulin promotes efficient glucose metabolism and various anabolic effects in most tissues. Conceivably, the experimental hyperglycemia could simulate a postprandial glucose excursion in diabetic piglets with limited or non-insulin production and change adipose tissue activities. It was well established that the size of the omental, more than the subcutaneous, fat is strongly related to a higher risk of obesity-related disorders, including insulin resistance, type 2 diabetes, dyslipidemia and CVD. However, the signaling pathways of resistin in omental adipose tissue have to be identified. Dai et al. (2006) previously demonstrated that leukocytes are also an important source of resistin in pig. The changes in resistin gene expression may be explained in part by adipose tissue infiltration by monocytes/macrophages. Likely, adipose tissue macrophages (ATMs) accumulated in both epicardial and omental fat depots might modulate production of resistin during hyperglycemia. The results obtained strongly indicated the opposite response of epicardial and omental adipose tissue to hyperglycemia. Our data confirmed the depot-specific response of abdominal adipose tissue. However, it remains unclear which factor determines distinct fat activities. Because of close anatomic relationship of visceral adipose tissue to the adjacent organ (heart, kidney, blood vessels), numerous local interactions between these tissues are possible (Baker et al., 2006; Chu et al., 2008; Stelmanska et al., 2012). To support this hypothesis, the stronger pericoronary than periaortic fat resistin expression in human, was demonstrated. Moreover, there was a significant correlation of resistin mRNA level with atherosclerosis in both depots (Mazurek et al., 2003; Iacobellis and Leonetti, 2005; Iacobellis et al., 2009).

The obtained results confirmed production of resistin in both abdominal adipose tissues. However, the expression of resistin differs significantly between various fat depots during hyperglycemia in piglets. Our data suggest that resistin is synthesized and acts locally as we observed in abdominal adipose tissue or it is stored and secreted to blood in response to changes in physiological conditions in piglets.

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