Adipokine gene transcription level in adipose tissue of runt piglets

T.G. Ramsay *, M.J. Stoll, T.J. Caperna

Animal Biosciences and Biotechnology Laboratory, Animal and Natural Resources Institute, USDA/ARS, Beltsville Agricultural Research Center, USDA, Beltsville, MD 20705, USA

1. Introduction

Mortality and morbidity of live-born piglets continues to be a major economic burden for swine production. Despite changes in management practices and new genetics, estimates of pre-weaning mortality in the U.S. have remained constant at 9–12%, over the last 20 years (APHIS, 2005). The greatest incidence of death occurs within the first 3–4 days following birth, especially among neonates of low birth weight ("runt") or decreased vitality (Randall, 1972; Stanton and Carroll, 1974; Lay et al., 1999; Tuchscherer et al., 2000; O’Reilly et al., 2006).

The runt pig may be more susceptible to metabolic or physiological stressors than the normal sized littermate (Randall, 1972). As this neonatal period is the time period of maximal adipose tissue proliferation and differentiation in the postnatal pig (Mersmann et al., 1975), adipose tissue may be quite susceptible to stressors at this critical time of tissue development. Stressors such as adaptation to environmental temperature, adaptation to postnatal nutrient metabolism, psychological stress inherent to competition for nutrients, etc are all challenges for the runt pig. Thus the runt pig may serve as a model for examining stress in the pre-weaning piglet.

Identification of specific proteins which either respond or are resistant to stress may serve as functional markers for pre-weaning survival. Recent studies have shown that adipose tissue is a source of numerous growth factors and cytokines, referred to as adipokines, which include tumor necrosis factor (TNFα), IL1β, IL6, resistin, leptin, adiponectin (ADIPOQ), and visfatin (Trayhurn and Wood, 2005; Fain, 2006; Tilg and Moschen, 2006). Hausman et al. (2006) used microarray and proteomic analyses to demonstrate that neonatal pigs adipose tissue expresses a large variety of cytokines.

Stress may impact adipokine expression and subsequent peripheral responses that affect animal viability. The present study used the runt pig as a model for neonatal stress to test the hypothesis that stress during the pre-weaning period can alter adipokine gene transcription levels. Therefore, the present study was designed to determine whether the adipose tissues from runt pigs differ in the mRNA abundance of adipokines from their normal sized littermates.

2. Materials and methods

2.1. Animals and sampling

Nineteen second parity sows (Sus scrofa) of similar genetics (Yorkshire x Landrace crosses) and body weight were fed a standard swine diet during gestation. Sows were individually housed and were monitored to ensure collection of piglets within the first 24 h of birth (day 1). These nineteen sows produced 21 pairs of runts and control littermates. Piglets (Landrace x York) were weighed on day 1 (d1) and then were randomly assigned across litters for sampling at d1 (n = 5/group; 3 female, 2 male), d7 (n = 7/group; 4 female, 3 male) or d21 (n = 9/group; 8 female, 1 male) of age. Runt pigs were defined as born at less than 1 kg bwt (Hegarty and Allen, 1978), while sex-matched littermates were identified as those closest to the mean litter weight.
Runt pigs (n = 21) used in this study were born at a mean body weight of 0.84 ± 0.03 kg while control littersmates (n = 21) weighed 1.49 ± 0.05 kg. All sampling was performed between 0900 and 1100 h and animals were only sampled in the fed state. Tissue sampling was performed following euthanasia induced with pentobarbital sodium (200 mg/kg). Animal handling and euthanasia procedures were approved by the USDA-ARS Beltsville Area animal care and use committee.

Dorsal subcutaneous (SQ) adipose tissue samples were collected between the second and fourth thoracic vertebrae while perirenal (PR) adipose tissue was collected from around both kidneys. No PR was apparent in the d1 pigs and could not be collected, thus PR sampling was begun at d7. Tissues were diced and frozen in liquid nitrogen immediately upon removal from the carcass and stored at −80 °C. Duplicate tissue samples (100 mg) in Qsarol extraction solution were disrupted using a tissue homogenizer and total RNA was isolated using Qiagen RNeasy spin columns according to the manufacturer’s protocol (Qiagen, Valencia, CA). Integrity of RNA was assessed via agarose gel electrophoresis and RNA concentration was determined spectrophotometrically using A260 and A280 measurements. Following verification of RNA integrity, total RNA from the duplicate tissue samples was pooled for each pig prior to reverse transcription and real time PCR analysis.

2.2. Real time PCR analysis of mRNA abundance

The adipokine genes selected for analysis included leptin (LEP), adiponectin (ADIPOQ), TNFα, IL1β, IL6, IL8, IL10, IL15, haptoglobin (HP), vascular endothelial growth factor (VEGFA), macrophage migration inhibitory factor (MIF) and monocyte chemotactic protein, also known as chemokine (C–C motif) ligand 2 (CCCL2). These genes were selected as they all have been demonstrated to be expressed by adipose tissue from mature animals or humans as described in the Introduction and their ontogeny of development in neonatal adipose tissue has been characterized (Ramsay and Caperna, 2009). Leptin, adiponectin and fatty acid synthase (FASN) were selected because they are markers for differentiation (McNeel et al., 2000). The interleukins 1β, 6, 8 and 15 were included because they have been identified by Hausman et al. (2006) to be expressed by neonatal pig adipose tissue using proteomics. Interleukin 10 transcription level was examined as it has been demonstrated to be expressed by porcine adipose tissue (Brix-Christensen et al., 2005), but more importantly it is considered to be an anti-inflammatory cytokine. Tumor necrosis factor α was examined as it has been the most characterized of all adipokines and has key regulatory roles in the expression of a variety of proteins. Several of the proteins that are regulated by TNFα include other adipokines. These TNFα regulated adipokines include MIF, VEGFA, HP and CCL2, which were included in this study. Also, Hausman et al. (2006) have previously demonstrated the secretion of VEGFA and TNFα by neonatal adipose tissue in vitro. Cyclophilin A (PPIA) is a housekeeping gene (Choi et al., 2002) that was used as a relative standard for comparisons. Preliminary experiments confirmed the transcription of all of these genes by isolated adipocytes and also isolated stromal-vascular cells prepared from the adipose tissue of 21 day old pigs (data not presented).

The primers used for generating the adipokine amplicons are reported in Table 1 and all primer sets were designed to span an intron. The amplicons were excised from an agarose gel, re-amplified, and run through a GenElute PCR clean-up kit (Sigma-Aldrich, St. Louis, MO). The amplicons were subsequently sequenced to confirm identity using automated fluorescent DNA sequencing (ABI 310, Perkin Elmer Applied Biosystems, Foster City, CA).

<table>
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MCP = monocyte chemotactic protein; VEGFA = vascular endothelial growth factor; MIF = macrophage migration inhibitory factor.

Carlsbad, CA) according to the manufacturer’s recommendations, as previously described (Ramsay and Caperna, 2009).

Real time PCR was done in duplicate using the IQ Sybr Green Supermix kit (Bio-Rad) for each duplicate of tissue. A 24 µL reaction mix was made containing 12.5 µL sybr green supermix, 1.0 µL forward primer (10 µM), 1.0 µL reverse primer (10 µM) and 9.5 µL sterile water. This reaction mix was added to each well, followed by 1.0 µL RT product (25 µL total volume).

Parameters for all reactions except cyclophilin were as follows: 1 cycle 95 °C for 15 min (PCR activation), followed by 30 cycles, 94 °C for 15 s, 58 °C for 30 s, 72 °C for 30 s, with a final extension at 72 °C for 8 min. Parameters for cyclophilin were as follows: 1 cycle 95 °C for 15 min (PCR activation), followed by 30 cycles, 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, with a final extension at 72 °C for 7 min. Melting curve analysis was performed on all real time PCR reactions to confirm specificity and identity of the real time PCR products. A non-template control was run for every assay. Specificity of real time PCR products was further confirmed by agarose gel electrophoresis. The two-step real time PCR reactions were optimized for linearity (exponential amplification) from >20 to <30 cycles under the conditions described above.

2.3. Quantification of mRNA abundance

At the end of the PCR, baseline and threshold crossing values (Ct) for all analyzed genes were calculated using the Bio-Rad software and the Ct values were exported to Microsoft Excel for analysis. The relative transcription level of the genes of interest, standardized against the amount of cyclophilin A mRNA, was calculated using the ΔΔCT method (Winer et al., 1999; Livak and Schmittgen, 2001). Values are presented as the mean ± SEM of duplicate determinations from tissues from all animals in each group.
2.4. Statistical analysis

Data were analyzed by one-way analysis of variance using SigmaStat software (SPSS Science, Chicago, IL) to test for age effects. Mean separation was analyzed using Student–Newman–Keuls test. Paired t-test was used to compare gene transcription level between each pair of runts and controls within an age. Means were defined as significantly different at $P<0.05$.

3. Results

Animals selected for sampling at day 1 of age differed in body weight at time of final sampling. Data in Fig. 1 demonstrate that runt animals were smaller than littermate control pigs at each of the three ages ($P<0.05$). Animals that were born at weights below one kg remained smaller at 21 days of age when the experiment terminated.

Leptin was lower in the SQ of the runt pig at d1 and d7 ($P<0.05$, Fig. 2A), but was similar to control mRNA abundance at day 21 ($P>0.05$). Leptin mRNA abundance increased with age in SQ ($P<0.001$). Perirenal adipose tissue could not be detected or sampled at d1 of age; therefore the data only include d7 and d21. Leptin mRNA abundance was elevated 88% at d7 in PR from runt pigs relative to controls ($P<0.01$), but no difference was apparent at day 21 ($P>0.05$). Leptin mRNA abundance increased with age in control animals ($P<0.05$). Adiponectin was depressed in the SQ of runt pigs at d1 of age relative to control pigs ($P<0.01$; Fig. 2B) but no differences were detected between SQ and PR from runts and controls at d7 or d21 ($P>0.05$). Adiponectin mRNA abundance increased with age in both SQ ($P<0.001$) and PR ($P<0.001$). The enzyme fatty acid synthase serves as a differentiation marker, along with the adipokines leptin and adiponectin. Fatty acid synthase transcript level was lower in SQ of runt pigs at d1 than in control pigs ($P<0.001$, Fig. 2C). However, no differences between runts and controls were detected in FASN mRNA abundance in the SQ at d7 or d21 ($P>0.05$). There was a significant increase in FASN mRNA abundance in SQ at d7 of age, which subsided by d21 ($P>0.001$). The relative transcript level of FASN was not detectably different in the PR from runt pigs than from control pigs ($P>0.05$). Fatty acid synthase mRNA abundance decreased with age ($P<0.001$).

The interleukins that have been previously demonstrated to be expressed or secreted by mammalian adipose tissue were examined in Fig. 3. The mRNA abundance of IL1β in the SQ in runt pigs on d1 was greater by 61% relative to the level in the SQ of control pigs ($P<0.05$; Fig. 3A). This difference was no longer apparent in the SQ at day 7 or day 21 ($P>0.05$). There was no effect of age at sampling on SQ IL1β mRNA abundance ($P>0.05$). Neither was IL1α mRNA increased in PR by runting or age ($P>0.05$). Similar to IL1β, IL6 was elevated in the SQ of the d1 runt pig relative to the control pig ($P<0.05$; Fig. 3B). However, IL6 mRNA abundance was similar between SQ of runt and control pigs at both d7 and d21 ($P>0.05$). Age had no effect on IL6 transcript level in the SQ ($P>0.05$). Perirenal IL6 mRNA abundance at d7 was also greater in runts relative to controls ($P<0.05$), but was similar between the two groups at d21 ($P>0.05$). There was an increase in IL6 mRNA abundance with age in the PR ($P<0.001$). Interleukin 10 was unaffected by runting in the SQ at any age ($P>0.05$; Fig. 3C); however, IL10 mRNA abundance in SQ was increased at d21 of age ($P<0.05$). The IL10 transcript level in PR was greater by 87% in the runt pig at d7 relative to the littermate control ($P<0.05$). Interleukin 10 mRNA abundance in PR increased with age ($P<0.05$). No differences in IL8 or IL15 mRNA abundance were detected between runt and control pigs at any age examined ($P>0.05$; data not presented), although IL15 mRNA abundance increased between d7 and d21 of age (data not presented; $P<0.001$).

Tumor necrosis factor α gene transcription level did not differ between runt and control pigs within the adipose tissues examined ($P>0.05$, Fig. 4A); although TNFα mRNA abundance increased in both tissues between d7 and d21 ($P<0.001$). Haptoglobin mRNA abundance was not affected in the SQ by runting ($P>0.05$, Fig. 4B); although mRNA abundance in the SQ was affected by age ($P<0.05$). Haptoglobin gene transcription level was greater in the PR of runt pigs at d7 of age ($P<0.05$). Haptoglobin mRNA abundance was similar in the PR between animal groups at d21 ($P>0.05$). The MIF mRNA abundance was 80% greater ($P<0.05$) at d1 in the SQ of the runt pig relative to the control littermate (Fig. 4C). However, no differences in MIF transcription level were detected in the SQ between runts and controls at d7 or d21 ($P>0.05$). The MIF transcription level in PR from runt pigs at d7 was 196% ($P<0.05$) of the level in control PR. Furthermore, MIF mRNA abundance was elevated by 30 fold in PR of runt pigs at d21 compared to controls ($P<0.001$). No differences in CCL2 or VEGFA mRNA abundance were detected between runt and control pigs at any age examined ($P>0.05$; data not presented). The mRNA abundance of CCL2 increased with age in the SQ ($P<0.001$, data not presented). Transcription level of VEGFA increased between d7 and 21 in the PR ($P<0.001$, data not presented).

4. Discussion

Runting originates in the embryonic/fetal stages of life, but the greatest stress upon the runt, as with all pigs, is immediately after birth when dealing with environmental, physiological and psychological changes (Curtis, 1974; Stanton and Carroll, 1974). The runt is perhaps more susceptible to these stressors due to its relative immaturity in comparison to its normal sized littermates (Morise et al., 2009; Quiniou et al., 2002; Stanton and Carroll, 1974); a component of this immaturity is the relatively limited development of adipose tissues (Laws et al., 2008; Morise et al., 2009), as suggested by Rehfeldt and Kuhn (2006) wherein % carcass lipid was reduced in low birth weight newborn pigs versus median birth weight pigs.

The present data demonstrate that the mRNA abundance of a number of adipokines is affected by the susceptibility of the runt to stress during this neonatal transitional period. Leptin and adiponectin were reduced while IL1β, IL6 and MIF mRNA abundance were greater in the SQ of the runt pig on d1, relative to the control littermate. These effects were temporary as gene transcript levels were normalized by d7, except for leptin; following the period of maximal mortality/highest stress (Curtis, 1974; Stanton and Carroll, 1974; Morise et al., 2009). However, the internal PR did not necessary reflect the changes in SQ as leptin, IL6, IL10, haptoglobin and MIF were elevated in the runt PR at d7; despite either no differences in SQ mRNA abundance between runt and control, or greater SQ mRNA abundance in runt than control at that time.
Fig. 2. Relative leptin (A), adiponectin (B) and fatty acid synthase (C) expression in subcutaneous (SQ) and perirenal (PR) adipose tissues from neonatal pigs. Adipose tissue was collected from pigs at d1 (n=5), d7 (n=7) or d21 (n=9) of age. Reverse transcription and real time PCR was then performed as described in the Materials and methods. Data are expressed relative to cyclophilin A expression in each tissue sample. *Columns within an age are different (P<0.05).
Fig. 3. Relative interleukin 1β (IL-1β, A), IL-6 (B) and IL-10 (C) expression in subcutaneous (SQ) and perirenal (PR) adipose tissues from neonatal pigs. Adipose tissue was collected from pigs at d1 (n = 5), d7 (n = 7) or d21 (n = 9) of age. Reverse transcription and real time PCR was then performed as described in the Materials and methods. Data are expressed relative to cyclophilin A expression in each tissue sample. *Columns within an age are different (P<0.05).
Fig. 4. Relative tumor necrosis factor α (A), haptoglobin (B) and macrophage migration inhibitory factor (MIF, C) expression in subcutaneous (SQ) and perirenal (PR) adipose tissues from neonatal pigs. Adipose tissue was collected from pigs at d1 \((n=5)\), d7 \((n=7)\) or d21 \((n=9)\) of age. Reverse transcription and real time PCR was then performed as described in the Materials and methods. Data are expressed relative to cyclophilin A expression in each tissue sample. *Columns within an age are different \((P<0.05)\).
point. The inability to examine PR adipokine mRNA abundance at d1 limits the interpretation; however, these differences in the time frame of changes in transcript level between tissues imply that the internal PR and external SQ sites of adipose tissue deposition differ in the kinetics of their response to the stresses impacting the runt. Numerous studies have documented the intrinsic differences in the development, physiology and metabolism between internal and external sites of adipose tissue deposition of swine (Anderson et al., 1972; Anderson and Kauffman, 1973; Budd et al., 1994; Gardan et al., 2006; Ramsay et al., 2008). The developmental changes induced with runting may cause internal and external sites of adipose tissue deposition to respond differently to physiological and metabolic stressors.

Leptin, adiponectin and FASN are considered markers for porcine adipocyte differentiation (Chen et al., 1997; McNeel et al., 2000; Samulini et al., 2008). The lower mRNA abundance of these genes in the SQ of the neonatal runt pig (d1) relative to the normal sized, littermate control may suggest a reduced adiposity, as reported for the runt pig at birth (Randall, 1972). Reduced serum leptin (Jaquet et al., 1998) and adiponectin (Cianfarani et al., 2004; Kamo et al., 2004) concentrations have previously been reported in human infants whom are small for gestational age. However serum leptin is not altered in runt piglets (Morise et al., 2009). The reduced level of SQ FASN implies a reduced capacity for lipid synthesis and thus adiposity in the newborn runt (not measured in the present study). This limited lipid storage is rapidly compensated, as runt pigs can have as much or more total body fat than larger littersmates by weaning (Laws et al., 2008). Further, the similar transcript levels between the runts and controls for all genes that were investigated in the SQ at d7 and d21, except for leptin, indicate that adipocyte differentiation and basic physiologic responses of adipocytes in pigs that survive beyond the first few days are equivalent between runts and littersmates. Unlike the SQ, PR leptin mRNA abundance was elevated in the runt pig at d7. This was a surprising finding as runt pigs have been reported to have less PR (Morise et al., 2009) and in the present study no PR was apparent in any pigs at d1.

The interleukins are a large and diverse family of proteins with a variety of regulatory functions. Among the studied interleukins, only IL1β and IL6 mRNA abundance were affected by runting in the SQ of the runt. Interleukin 1β has been demonstrated to inhibit insulin signaling (Lagathu et al., 2006), inhibit leptin expression and secretion (Bruun et al., 2002) as well as promote IL6 expression by adipocytes (Flower et al., 2003). The increase in IL1β mRNA abundance in the SQ of the runt pig in the present study may have contributed to the observed decrease in leptin transcript level and the elevated IL6 transcript level in the SQ of the runt pig.

Interleukin 6 demonstrated the greatest difference in gene transcript levels between runts and controls within the SQ at d1. Recent findings have implicated IL6 as functioning in the inhibition of insulin signaling (Rotter et al., 2003; Senn et al., 2003), stimulation of lipolysis (Truejillo et al., 2004; Petersen et al. 2005) and inhibition of lipoprotein lipase activity (Greenberg et al., 1992). Those reported metabolic changes would have deleterious effects on the neonatal runt pig as an inhibition of lipid accretion, uptake and storage would reduce the ability of the neonate to respond to thermal or metabolic stress, which could contribute to the reported higher mortality of the runt pig (Randall, 1972). If peripheral IL6 concentrations were affected by an increase in IL6 expression in the neonatal adipose tissue as suggested in the present study, liver glycogen storage (Tsigos et al., 1997) and thus glucose homeostasis would also be impaired during the critical first days postnatal. Since the neonatal pig has only 1–2% body fat it may be unlikely that IL6 produced within the adipose tissue could affect peripheral immune or metabolic function. However, IL6 of adipose origin may have a significant impact within the localized environment of the adipose tissue to affect its development during the first critical days of life.

Interleukin 10 is an anti-inflammatory adipokine that has been reported to down-regulate TNFα, IL6 and IL8 proteins in neonatal whole
relative to a normal birth weight pig from the same litter. Specifically, IL6 mRNA abundance in the SQ of the runt is much higher during the first 24 h post parturition, while MIF mRNA abundance in the PR of the runt is much higher than in PR of normal sized littermates at least until d21 post parturition. Confirmation of these changes in gene transcript level by measuring the secretion of IL6 and MIF would provide further support for pursuing the association of these adipokines with neonatal survivability/stress response.

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References


