RAPID COMMUNICATION

Food restriction regulates adipose-specific cytokines in pituitary gland but not in hypothalamus

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Abstract

White adipose tissue is now recognized as the source of a growing list of novel adipocyte-specific factors, or adipokines. These factors regulate energy homeostasis, including the response to food deprivation. We hypothesized that the brain and pituitary gland would also express adipokines and their regulatory factors and subsequently demonstrated that the rodent brain-pituitary system expresses mRNA and protein for leptin and resistin. We now report that the adipokines FIAF and adiponutrin, as well as the nuclear hormone receptor PPAR γ , are expressed in pituitary, brain and adipose tissue. In pituitary gland, 24 h of food restriction reduced PPAR γ expression by 54% whereas both adiponutrin and FIAF were increased 1.7 and 2.3

fold, respectively. These changes in expression were similar to those observed in fat, except for adiponutrin, which by contrast is dramatically reduced 95% by fasting. Furthermore, whereas PPAR γ 2 is the main isoform affected by fasting in adipose tissue, our data suggest that only PPAR γ 1 is present and downregulated by fasting in pituitary tissue. In contrast to the sensitivity of pituitary tissue to the effects of fasting, no significant change in expression was observed in basal hypothalamus for any of the genes studied. Overall, our data suggest that pituitary-derived adipokines may play an unexpected role in the neuroendocrine regulation of energy homeostasis.

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Introduction

Classically viewed as a passive energy storage depot, white adipose tissue (WAT) is now recognized as a source of numerous secreted factors which play an important role in mammalian physiology, and in particular, energy homeostasis (Ahima & Flier 2000, Fruhbeck *et al.* 2001, Trayhurn & Beattie 2001). In the last decade, a growing list of novel, adipocyte-secreted factors, or adipokines, were discovered. This list includes, but is not limited to, leptin (Himms-Hagen 1999), resistin (Banerjee & Lazar 2003), adiponectin/acrp30 (Berg *et al.* 2002), FIAF (fasting-induced adipose factor) (Kersten *et al.* 2000), and adiponutrin (Baulande *et al.* 2001, Polson & Thompson 2003). These adipokines regulate glucose and fatty acid homeostasis, appetite control, energy expenditure, and the response to starvation. Hence, the perception of WAT function has changed significantly to accommodate its

emerging role as an endocrine organ (Trayhurn & Beattie 2001).

A common characteristic of adipokines is their purported adipose-specific expression and release. Leptin, although inarguably produced chiefly by adipocytes, is expressed by many peripheral tissues including the human placenta, stomach, and skeletal muscle (Ahima & Flier 2000). In addition we identified leptin mRNA and protein expression in the rodent brain and pituitary gland (Morash *et al.* 1999, Wilkinson *et al.* 2000, Ur *et al.* 2002). Another supposedly adipose-specific factor, resistin, is also found in the mouse brain and pituitary gland (Morash *et al.* 2002). Furthermore, both resistin (Song *et al.* 2002) and leptin (De Vos *et al.* 1996) are target genes for the transcription factor peroxisome proliferator-activated receptor gamma (PPAR γ), the presence of which has been recently demonstrated in anterior pituitary cells (Heaney *et al.* 2003). Similar to the adipokines, PPAR γ is

Table 1 Primer sequences and PCR conditions

	Sequence 5'-3'	Product Size (bp)	Anneal (°C)	Cycles Pit/Fat	Reference
Primer					
Adiponutrin					
Sense	GTTTGCAGGCTGCGGCTTCC	551	60	29/25	(Polson and Thompson 2003)
Antisense	GGCAGATGTCATGCTCACCG				
FIAF					
Sense	GGAGCGGCACAGTGGACTTT	548	58	29/25	(Yoon <i>et al.</i> 2000)*
Antisense	TACCCTTTTACGCTCCTGCC				
PPAR γ (1 & 2)					
Sense	TGATATCGACCAGCTGAACC	704	57	32/26	(Yang <i>et al.</i> 1999)
Antisense	TGGCGAACAGCTGAGAGGAC				
PPAR γ 2					
Sense	GCTGATGCACTGCCTATGAG	538	57	40/29	—
Antisense	TCTGAAACCGACAGTACTGAC				

Pit – pituitary gland. *Primer sequence modified from original reference.

predominantly expressed in adipose tissue where it is implicated in adipocyte differentiation, lipid storage and glucose homeostasis (Kliwer *et al.* 1994).

Thus, given the presence of resistin, leptin and PPAR γ in pituitary tissue, we hypothesized that other adipokines should also be expressed in the pituitary where they may play a role in the neuroendocrine regulation of energy balance. We show here that this hypothesis is correct and, moreover, that pituitary adipokine gene expression is specifically sensitive to food availability, whereas hypothalamic expression is unaffected.

Materials and Methods

Animals

Pubertal CD1 mice (male; 22–24 g; age 30–32 days) were obtained from Charles River Breeding Farms (Quebec, Canada), maintained under a photoperiod of 14 h light: 10 h darkness (lights on: 0700 h) and given free access to Purina Rat Chow and drinking water. For fasting studies, food was removed in the late morning for a 24 h period. The experimental protocol was reviewed and approved by the Dalhousie University Committee on Laboratory Animals. Mice were killed by decapitation and samples of frontal cerebral cortex, basal hypothalamus, and visceral (epididymal) fat were dissected and frozen in liquid nitrogen. The pituitary gland was removed intact (anterior plus posterior).

RNA isolation and semi-quantitative RT-PCR analysis

Total RNA was isolated from brain and pituitary using the RNeasy mini kit (Qiagen) and from adipose tissue using Trizol reagent (Gibco; Burlington, ON, USA). The RNA was DNase treated using the RNase-free DNase kit (Qiagen). RNA (1 μ g for brain and pituitary; 0.35 μ g for

adipose tissue) was denatured at 65 °C for 5 min and reverse transcribed in a final volume of 30 μ l using Omniscript reverse transcriptase (Qiagen) at 37 °C for one hour. Semi-quantitative PCR amplification was performed using intron-flanking primers and HotStarTaq DNA polymerase (Qiagen), as previously described (Morash *et al.* 2002). Reactions were normalised by evaluating the house-keeping transcript 18S using commercial primers (Classic 18S primers; Ambion). The sequences of the primers and PCR reaction conditions are outlined in Table 1. For PPAR γ , two sets of primers were utilised which detect either general PPAR γ expression (isoforms 1 and 2) or PPAR γ 2 specifically. For RT-PCR analysis of pituitary tissue, each 'n' value represents tissue pooled from 2 mice.

Cloning and sequencing of PCR products

Total RNA from pituitary and gonadal fat was reverse transcribed and PCR-amplified as described above. Portions of the resulting PCR reaction were cloned into pGEM-T Easy (Promega; Madison, WI, USA) and transformed into *E.coli*. Individual transformants were sent for sequence analysis to Cortec DNA Service Laboratories Inc. (Kingston, ON). The PCR-generated amplicons were found to be 100% identical to the corresponding regions of the genes of interest.

Northern blot analysis

Tissues (hypothalamus, cortex, pituitary and visceral fat) were collected from 40 male CD1 mice at postnatal day (PD) 28 for isolation of total RNA using Qiagen RNeasy midi kits (brain/pituitary) or Trizol reagent (Gibco, BRL). Poly A+ RNA was isolated from the total RNA using Oligotex mRNA mini kit (Qiagen). Approximately 5–7 μ g of poly A+ RNA was analyzed by Northern

analysis using the NorthernMax kit (Ambion). RNA was separated by agarose gel electrophoresis and subsequently transferred to BrightStar-Plus nylon membrane (Ambion). Probes for adiponutrin and FIAF were generated by PCR amplification using the primers outlined in Table 1. Amplicons were isolated by agarose gel electrophoresis, purified (QIAquick gel extraction kit; Qiagen) and quantitated. Probes were radiolabeled with αP^{32} dCTP using Rediprime II random prime labelling system (Amersham). The labeled probes were separated from un-incorporated radionucleotides using Probe Quant G-50 micro columns (Amersham). The nylon blot was prehybridized in 13 ml of hybridization solution at 42 °C for 1 h. Probes were denatured by boiling for 5 min, quick chilled for 5 min and added to the prehybridized nylon blot (approximately 1×10^6 c.p.m./ml hybridization buffer). The blot was hybridized at 42 °C overnight and subsequently washed twice for 5 min in $2 \times$ SSC/0.1%SDS at room temperature followed by two 15 minute washes (42 °C) in $0.1 \times$ SSC/0.1%SDS. Subsequently, the blot was exposed for 6–18 h to X-ray film (Eastman Kodak).

Statistical analysis

Data were analyzed by Student's *t*-test and are reported as mean $\pm$ S.E.M. Significance was set at $P < 0.05$.

Results

Adipokine and PPAR γ mRNA expression in pituitary and brain tissue

Adiponutrin, FIAF and PPAR γ mRNA expression was assessed by RT-PCR analysis and products of the expected sizes were reproducibly detected in fat, hypothalamus, cerebral cortex and whole pituitary gland (Fig. 1A). As expected, abundant expression was observed in adipose tissue for all genes studied. Moreover, FIAF, adiponutrin, and PPAR γ mRNA were all readily detectable in brain and pituitary tissues, albeit at lower levels than adipose tissue. Of note is the observation that only general PPAR γ (non-isoform specific) expression was observed in brain and pituitary, with specific PPAR γ 2 expression not detected after 40 PCR cycles. In adipose tissue, specific PPAR γ 2 expression was less abundant than general PPAR γ . PCR product identities were confirmed by sequencing and comparison with the GenBank database.

Using probes generated in the RT-PCR experiments, adiponutrin, and FIAF expression was also assessed by Northern analysis in pituitary, brain and adipose tissue (Fig. 1B). As expected, high levels of expression were observed in visceral fat. Again, adiponutrin and FIAF mRNA were readily detected in brain and pituitary gland. Northern analysis for PPAR γ mRNA was not performed

A. RT-PCR

B. Northern Analysis

Figure 1 (A) RT-PCR analysis of adipokine gene expression in male (PD28) CD1 mouse pituitary (P), hypothalamus (H), cerebral cortex (C) and visceral fat (F) tissue. PCR parameters and amplicon sizes are outlined in Table 1. No PCR products were detected in the negative (-) controls, which were performed by omission of reverse transcriptase. **Figure 1** (B) Northern analysis of adipokine gene expression in cerebral cortex (C), hypothalamus (H), pituitary (P) and visceral fat (F) tissue from male (PD28) mice. Each lane contains approximately 5–7 μ g of poly A+ mRNA. The expected size of the native mRNA transcript is indicated.

because of the existing strong evidence of expression in pituitary and brain (Rohn *et al.* 2001, Heaney *et al.* 2003).

Effects of fasting on adipokine and PPAR γ mRNA in pituitary and adipose tissue

In two separate experiments, we used semi-quantitative RT-PCR to determine the effect of a 24-h fast on PPAR γ , FIAF, and adiponutrin mRNA expression (Fig. 2). In adipose tissue, fasting resulted in a 2.5-fold increase in FIAF expression ($P < 0.025$), whereas adiponutrin was dramatically reduced by 95% ($P < 0.005$). Fasting also caused significant reductions in PPAR γ 2 expression in fat (61%, $P < 0.01$) but had no effect on PPAR γ (1 and 2). In pituitary tissue, fasting also resulted in marked alterations in expression, however some differences were observed when compared with fat. In parallel with adipose tissue,

Figure 2 Effect of 24 h of fasting on adipose (A) and pituitary (B) adipokine mRNA expression. Semi-quantitative RT-PCR was used to analyse changes in gene expression. Data are represented as percentage change (mean $\pm$ S.E.M.) based on fed control groups (control=clear bars, fasted=solid bars). Cumulative data represent the pooled result of two separate fasting experiments ($n=7$ for adipose tissue, except PPAR γ 1&2 where $n=3$; $n=8$ for pituitary tissue). For pituitary tissue, each 'n' represents two pooled pituitary glands. * $P<0.025$, ** $P<0.01$, *** $P<0.005$ (by t -test).

FIAF expression was increased 2.4-fold in pituitary tissue ($P<0.005$), but unlike adipose tissue, pituitary adiponutrin expression was significantly increased (1.7 fold; $P<0.005$). Pituitary PPAR γ (1 and 2) expression was reduced by fasting by 54% ($P<0.005$). Since PPAR γ 2 expression was undetectable in pituitary, the change in expression observed putatively reflects that of PPAR γ 1. In contrast to the effect of fasting on gene expression in pituitary and fat tissue, no effect was observed in basal hypothalamus ($n=5$) for any of the genes studied (adipo-

nutrin: $P=0.47$; FIAF: $P=0.16$; PPAR γ (1 and 2): $P=0.29$; data not shown).

Discussion

In previous studies we tested the hypothesis that the brain and pituitary gland should express the adipokines leptin and resistin. This hypothesis proved to be correct (Morash *et al.* 1999, 2002), and in the case of leptin was independently confirmed (Jin *et al.* 2000, Beretta *et al.* 2002, Ehrhardt *et al.* 2002). Thus, brain-derived leptin could be the endogenous ligand for some populations of central leptin receptors. Receptors for resistin have not yet been reported, but resistin is regulated via PPAR γ and C/EBP α (Song *et al.* 2002), both of which are found in brain and pituitary (Cardinaux *et al.* 2000, Rohn *et al.* 2001, Day *et al.* 2003, Heaney *et al.* 2003). Our detection of resistin mRNA and protein in brain and pituitary therefore suggests a role for resistin in the neuroendocrine system. We have extended this logic to demonstrate, for the first time, the expression of the novel adipokines adiponutrin and FIAF in mouse brain and pituitary. Furthermore, we have shown that these genes may be differentially regulated in fat, pituitary and brain tissue.

Adiponutrin is a transmembrane protein which, although not secreted, shows similar characteristics to other adipokines (Baulande *et al.* 2001) and is reportedly expressed solely in white and brown adipose tissue. Adiponutrin mRNA levels are elevated in fat from obese *fa/fa* rats whereas fasting dramatically reduces mRNA levels (Baulande *et al.* 2001, Polson & Thompson 2003). We confirmed the fasting-induced decline in adipose adiponutrin mRNA but also demonstrated an unexpected increase of gene expression in pituitary tissue. The reason for the fasting-induced increase in pituitary expression is unclear, however it indicates that pituitary adiponutrin expression may be regulated by mechanisms distinct from those operating in adipocytes.

FIAF (fasting-induced adipose factor) is a member of the angiopoietin family of secreted proteins (Kersten *et al.* 2000, Yoon *et al.* 2000) and is predominantly localized to adipose tissue. FIAF is reported to be a PPAR γ target gene in adipose tissue but, by contrast, a PPAR α target gene in the liver (Kersten *et al.* 2000, Yoon *et al.* 2000). As its name implies, FIAF is increased in response to fasting in adipose tissue (Kersten *et al.* 2000, Yoon *et al.* 2000). We confirmed this observation and showed that pituitary FIAF mRNA is similarly sensitive to food restriction. In fat, however, leptin-induced appetite suppression does not result in increased FIAF mRNA (Yoon *et al.* 2000). This raises the possibility that upregulation of FIAF expression is inhibited by leptin.

PPAR γ is a ligand-activated transcription factor belonging to the nuclear hormone receptor superfamily

(Zhu *et al.* 1995). Alternative promoter usage and differential splicing of the PPAR γ gene results in two isoforms, $\gamma 1$ and $\gamma 2$, which differ by 30 additional amino acids at the N-terminal of $\gamma 2$ (Zhu *et al.* 1995). PPAR γ expression is largely restricted to adipose tissue and large intestine (Kliwer *et al.* 1994, Escher *et al.* 2001) with variations in the relative abundance and distribution of the $\gamma 1$ and $\gamma 2$ isoforms (Escher *et al.* 2001). Recently, PPAR γ immunoreactivity was reported in normal corticotrophic pituitary cells with abundant expression observed in pituitary tumours (Heaney *et al.* 2003). In the present study we demonstrate for the first time mRNA expression of PPAR γ in normal pituitary tissue, our data suggesting that this is limited to the PPAR $\gamma 1$ isoform. The effects of high affinity PPAR γ ligands such as the insulin-sensitizing thiazolidinediones (TZDs) implicate PPAR γ in insulin pathways (Wang & Tafuri 2003). Our data, that pituitary gland PPAR γ mRNA is sensitive to food restriction, suggest an additional possible target for TZDs and insulin. Indeed, insulin receptors and proteins thought to be involved in key steps of post-receptor signal transduction (e.g. IRS-1) are widely present in pituitary and brain (Unger & Betz 1998), indicating a possible link between a peripheral metabolic signal (insulin) and neuroendocrine pathways involving adipokines.

The lack of effect of food restriction on basal hypothalamic expression for any of the genes studied was unexpected. The hypothalamic arcuate nucleus is accepted to be a key target for hormones such as leptin and insulin in the control of energy homeostasis (Niswender & Schwartz 2003). It is possible that our dissection of the basal hypothalamus might conceal changes in mRNA expression within smaller, discrete nuclei. The application of *in situ* hybridization analysis to this problem is required. Nonetheless, the apparently specific effect of fasting on pituitary gene expression is intriguing. There is a remarkable convergence of adipokine gene expression with leptin and insulin signaling pathways in the anterior pituitary (Unger & Betz 1998, Horsch & Kahn 1999, Gautron *et al.* 2002). It is feasible that pituitary adipokine expression serves as a link between peripheral metabolic signals and the regulation of pituitary hormone secretion. In keeping with this supposition, preliminary data from our laboratory reveal that ACTH-secreting AtT20 cells abundantly express adiponutrin and PPAR γ mRNA (Wiesner *et al.* 2003).

In summary, we report that multiple adipose factors are expressed in pituitary tissue and brain, and that their mRNA levels in pituitary are sensitive to fasting. The presence of PPAR γ and other novel adipose-related transcription factors in pituitary gland and brain indicate the capacity for regulation of adipokine expression in these tissues. The exact role of these adipokines in brain-pituitary function remains to be elucidated, though our data suggest they may be implicated in the neuroendocrine response to starvation.

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