

Expression of leptin receptor and suppressor of cytokine signaling-3 genes in adenohipophysis of normal-fed and fasted cows

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SUMMARY

Expression of leptin receptor (LR) and suppressor of cytokine signaling (SOCS)-3 genes was investigated in normal-fed and fasted cows. Fasting did not affect LR mRNA, but increased SOCS-3 mRNA in the adenohipophysis, suggesting that heightened responsiveness of fasted cows to leptin is not dependent upon alterations in LR or SOCS-3 mRNA in the adenohipophysis. *Reproductive Biology* 2005(5):237-245.

Key words: adenohipophysis, leptin receptor, SOCS-3, bovine

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INTRODUCTION

Leptin prevents fasting-induced reductions in the frequency of LH pulses in the rat [18], castrated ram [19], and peripubertal heifer [16]. In mature cows, 2 to 3 days of fasting does not affect the frequency of pulses of LH, yet leptin increases mean concentrations of LH and size of individual pulses of LH [2, 23] without effects on the frequency of pulses of LH. Although leptin stimulates GnRH release in fasted cows [24], evidence suggests that leptin can also act directly at the adenohipophysis to increase basal and GnRH-stimulated release of LH from adenohipophyseal (AP) explants of fasted cows [3]. However, leptin fails to stimulate the hypothalamic-adenohipophyseal axis of well-nourished sheep [14, 19] and cattle [2, 23], suggesting that physiological resistance to leptin may occur in animals that are in neutral or positive energy balance. Resistance to leptin is observed in humans [10] and rodents [13] and is speculated to involve decreased numbers of leptin receptor (LR) and a diminished ability to stimulate downstream cellular signals [20]. Moreover, excessive activity of suppressor of cytokine signaling (SOCS)-3 has been implicated in the development of leptin resistance syndromes [8]. The LR is present in the hypothalamus and adenohipophysis, and the expression of LR is increased in the hypothalamus of nutritionally-restricted sheep [1, 11]. Herein, we tested the hypothesis that 72 h of fasting would increase LR mRNA and decrease SOCS-3 mRNA in the adenohipophysis of cows, alterations that could account for the increased responsiveness of the adenohipophysis to leptin.

MATERIALS AND METHODS

Ten ovariectomized cows, each bearing an estradiol implant designed to maintain basal physiological concentrations of estradiol [12], were used for this study. Cows in moderately thin body condition (BC = 4; scale of 1 to 9) were fed to maintain body weight and condition (Full-fed; n=5) or were fasted for 72 h with free access to water (Fasted; n=5). At the end of

the 72-h period, cows were euthanized and the adenohipophyses removed and dissected from the neurohipophyses. Adenohipophyseal tissue was snap frozen in liquid nitrogen for isolation of RNA. All animal-related procedures were approved by the Institutional Agricultural Animal Care and Use Committee of the Texas A&M University System.

Real-time polymerase chain reaction (PCR)

Total cellular RNA was isolated from 0.1 g of AP tissue as described previously [21] and subsequently treated with RNase-free DNase (Promega Co., Madison, WI, USA). Total RNA (5 µg) was reverse transcribed to cDNA using SuperScript II reverse transcriptase (Gibco BRL, Gaithersburg, MD, USA) and oligo dT₁₄ and random primers. Real-time PCR was performed using SYBR Green PCR Master Mix and the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Primers (Table 1) were designed using the Primer Express software (Applied Biosystems). Reactions were performed in triplicate for each cDNA sample. Amplification was performed in 40 cycles of 95° C for 15 sec followed by 60° C for 1 min. A final cycle with an increase in temperature to 95° C was used to estimate melting temperature of PCR products. No-template control was used to verify the absence of primer-dimer amplification. RNA samples used to generate cDNAs were used to confirm absence of genomic DNA carryover.

Data for LR and SOCS-3 gene expression in adenohipophysis were analyzed using the Q-Gen software application [17]. The efficiency of PCR amplification for targets (LR and SOCS-3) and reference (cyclophilin) genes were estimated based on amplification of serial dilutions of a reference cDNA sample. Threshold cycle (CT) values of the reference cDNA were regressed against the logarithmic transformation of proportions of cDNA in each reaction. The slope of the regressed equation was used to estimate the efficiency of amplification for target and reference genes for each PCR run, such as efficiency is equal to $10^{(-1/\text{slope})}$. Mean gene expression for LR and SOCS-3 for each adenohipophyseal sample was normalized to the expression of a reference gene (cyclophilin) and calculated as described

Table 1. Sequence of primers used for real-time PCR amplification of LR, SOCS-3, and cyclophilin.

Gene	Oligonucleotide sequence (5' – 3')	Genbank accession	
	Forward primer	Reverse primer	
LR	CAATGCAGCAGTGCTCAATC	GGGCTGTCTCCCTGCTCTCAT	U62385
SOCS-3	CCAGCCTGGCCTCAA	CTTGCGCACTGGGTTCAC	NM_174466
Cyclophilin	CCAACGGCTCCCAGTTCTT	ACTAGGTGCTTCCCACATCCAAA	D14074

previously [17]. The *t*-test procedure was used to compare data from Full-fed and Fasted groups.

RESULTS

Regression of CT values determined for amplification of a reference cDNA against the amount of cDNA in the reaction indicated a linear relationship ($p < 0.0001$) for both LR ($R^2 = 0.9792$) and cyclophilin ($R^2 = 0.996$). Estimated efficiency of amplification for LR and cyclophilin was 2.46 and 2.43, respectively. Similarly, there was a linear relationship ($p < 0.0001$) for both SOCS-3 ($R^2 = 0.9786$) and cyclophilin ($R^2 = 0.992$) and estimated efficiency of amplification for SOCS-3 and cyclophilin in this assay was 3.35 and 2.22, respectively. Comparison of normalized expression between Full-fed and Fasted groups indicated that dietary treatment did not ($p > 0.1$) affect the expression of LR in adenohipophysis (fig. 1). In contrast, fasting increased ($p < 0.05$) the expression of SOCS-3 in the adenohipophysis (fig. 2).

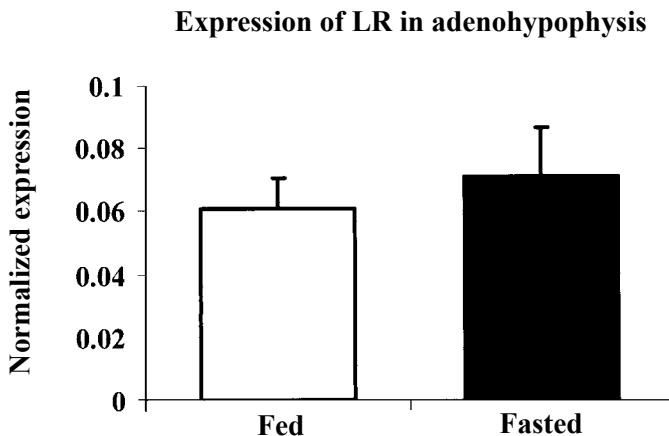


Figure 1. Normalized mean expression of leptin receptor (LR) in adenohipophyses of normal-fed ($n=5$) and fasted ($n=5$) cows. Three days of fasting did not affect ($p > 0.1$) expression of the LR gene.

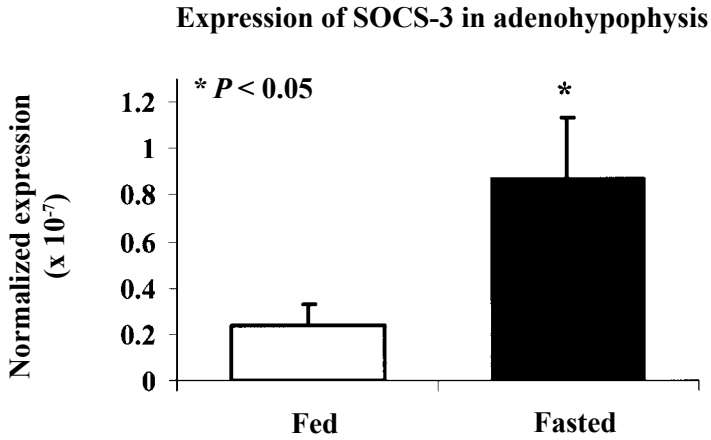


Figure 2. Normalized mean expression of suppressor of cytokine signaling (SOCS)-3 in adenohipophyses of normal-fed (n=5) and fasted (n=5) cows. Three days of fasting increased ($p < 0.05$) the expression of SOCS-3 in fasted cows.

DISCUSSION

Acute or chronic restrictions of food intake increase expression of LR in the hypothalamus in rodents [7] and sheep [1, 11]. In addition, reports in the mouse suggested that 48 h of fasting can increase expression of LR in the adenohipophysis [9]. In contrast, 3 days of fasting did not affect expression of LR in the adenohipophysis of cows in the current study, suggesting that increased expression of LR in the adenohipophysis does not account for the ability of leptin to stimulate basal release of LH from perfused AP explants from fasted cows [3], nor to increase mean concentrations of circulating LH [2, 23] in fasted cows. Although levels of LR mRNA may not represent those of protein, changes in LR protein are not expected to occur in the absence of changes in LR mRNA. However, LH-synthesizing cells form only approximately 12% of the endocrine cell types of the bovine adenohipophysis [5] and less than 30% of gonadotropes of the pars distalis of the ovine adenohipophysis are immunoreactive for LR [15]. Therefore, due to the heterogeneity of cells that constitute the adenohipophysis, overall LR gene expression may obscure changes within individual AP cell types. Our attempt to

determine the proportion of LH β -containing gonadotropes that express LR was unsuccessful because of very low levels of detectable LR mRNA in the adenohipophysis of cows using *in situ* hybridization histochemistry (unpublished) and unavailability of antibodies that unambiguously detect LR protein in bovine tissue.

Binding of leptin to LR stimulates downstream signaling events that lead to increased expression of the SOCS-3 gene [25], which in turn, suppresses LR signaling. Excessive activity of SOCS-3 is involved in leptin resistance [10]. In rats fasted for 48 h, SOCS-3 mRNA decreases in the arcuate and dorsomedial nuclei of the hypothalamus [4], suggesting that increased responsiveness to leptin may occur as result of a decrease in the suppression of LR signaling by SOCS-3. However, in contrast to our hypothesis, SOCS-3 mRNA was increased in the adenohipophysis of fasted cows. Therefore, it is possible that leptin acts at the gonadotrope level through alternate cellular mechanisms that are independent from the JAK/STAT pathway, as demonstrated for the regulation of growth and fertility in mice [6]. Although SOCS-3 expression is induced by other members of the cytokine family in addition to leptin [22], it is not clear whether they are influenced by nutrient restriction. In addition, differential regulation of SOCS-3 among AP cell types cannot be discounted and, as described for LR, changes in SOCS-3 gene expression within individual cell types were not determined in the current experiments.

Results of the present studies demonstrate that, although leptin is effective in stimulating the release of LH in fasted cows, changes in the expression of LR and SOCS-3 mRNA at the adenohipophyseal level do not appear to account for their increased responsiveness to leptin. Whether leptin activates STAT-3 and/or other signaling mechanisms in the adenohipophysis remains to be determined.

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