Disease and disease models in sheep produce fever, modulate reproductive and growth regulating hormones, and limit feed intake (Coleman et al., 1993; Elsasser et al., 2008; Sartin et al., 2008). Feed intake was reduced in sheep following acute lipopolysaccharide (LPS) treatment and chronic LPS treatment in cattle (McMahon et al., 1999; Sartin et al., 2008). In laboratory animals, inhibition of feed intake by LPS is mediated through proopiomelanocortin (POMC) neurons, its product (α-melanocyte stimulating hormone [α-MSH]), and its target, the melanocortin-4 receptor (MC4R; Grossberg et al., 2010). Moreover, agouti related protein (AgRP; an endogenous MC4R antagonist) and synthetic MC4R antagonists can reverse the effects of LPS to inhibit feeding in laboratory animals (Sergeyev et al., 2001) and in sheep (Sartin et al., 2008).

Interestingly, the cytokine leukemia inhibitory factor (LIF) was shown to modulate fever, POMC, and ACTH response to inflammatory disease in laboratory animals (Chesnokova and Melmed, 2000). Studies in laboratory animals indicate LIF acting through the gp-130 receptor can inhibit feed intake and BW and may be

a key link between LPS and appetite suppression (Gayle et al., 1998). Moreover, serum LIF was increased following LPS treatment in mice (Sempowski et al., 2002), and LPS was shown to increase expression of LIF in the arcuate nucleus (ARC). Additionally, LIF increased release of α-MSH from arcuate explants, and gp-130 receptors were expressed on POMC neurons (Grossberg et al., 2010). Taken together, there appears to be a role for LIF in mediating the effects of LPS to reduce feed intake in laboratory animals, and points to a role in mediating the LPS response of endocrine and metabolic factors in the acute phase inflammatory response.

This study was designed to determine whether there were similar effects of LIF in the LPS effects on appetite and on selected metabolic and endocrine changes in sheep.

**MATERIALS AND METHODS**

All animal experiments were approved by the Auburn University Institutional Animal Care and Use Committee.

**Experiment 1 – In situ Hybridization**

This study was designed to determine whether LPS would alter mRNA expression of LIF, AgRP, or POMC. Sheep (Suffolk wethers less than 1 yr old) were randomly placed in 2 groups (n = 6 per group). One group was the control group, which received saline, and the other group was the treatment group, which received intravenous LPS (1 μg/kg BW, *Escherichia coli* 055:B5; Sigma-Aldrich, St. Louis, MO). Sheep were sacrificed at 1 h after intravenous treatments were injected. Hypothalami were removed, frozen in liquid nitrogen, and used for single-label in situ hybridization to identify cells expressing LIF, AgRP, and POMC mRNA in sheep hypothalami based on previously published methods (Marks et al., 1992; Sartin et al., 2008). Coronal sections (20 μm) were cut on a cryostat and thaw-mounted onto Superfrost Plus slides (VWR Scientific, West Chester, PA). Sections of hypothalami were collected in a 1:4 series from the diagonal band of Broca caudally through the mamillary bodies. In the first study, antisense 33P-labeled bovine LIF riboprobe (corresponding to bases 323–724; GenBank accession number NM_173931.1) was denatured, dissolved in hybridization buffer at a concentration of 0.045 pmol/mL along with transfer RNA (1.7 mg/mL), and applied to slides. Slides were covered with glass cover slips, placed in a humid chamber, and incubated overnight at 55°C. The following day, slides were treated with ribonuclease A and washed under conditions of increasing stringency. The tissue was dehydrated in 100% ethanol, air-dried, and then dipped in NTB-2 liquid emulsion (Eastman Kodak Co., Rochester, NY) diluted 1:1 with distilled water. Slides were developed 5 d later and cover slipped. All slides were assigned a random 3-letter code, alphabetized, and read unilaterally under dark field illumination with custom software designed to count the total number of cells and the number of silver grains (corresponding to radiolabeled mRNA) over each cell (Rogers et al., 1988; Marks et al., 1992). Cells with a signal-to-background ratio of at least 2 or greater were considered to express LIF, AgRP, or POMC mRNA. Data were expressed as the total number of identifiable cells and grains per cell (a semiquantitative index of mRNA content/cell). Data were tested for effect of treatment by ANOVA and a t-test.

**Experiment 2 – Effects of Leukemia Inhibitory Factor on Feed Intake and Body Temperature**

This study was designed to determine whether intracerebroventricular (ICV) LIF injection would provide a dose-related decrease in feed intake as well as elevated body temperatures above 40°C (designated as a fever). Six Suffolk wethers less than 1 yr old were placed on raised floor pens, adapted to a concentrate feed (56% cracked corn, 25% cottonseed hulls, 10% soybean meal, 7% molasses, 1% limestone, 0.5% trace mineral salt, 0.2% dicalcium phosphate, 0.2% dynamite, and 0.1% vitamin A, D, and E cattle premix) providing 12% CP and 100% daily requirements, and treated with antibiotics and dewormed prior to being provided ICV canulas as previously described (Whitlock et al., 2010). Sheep were injected via ICV canulas with saline or LIF (250, 500, 1,000, and 2,500 ng/sheep; Santa Cruz Biotechnology, Dallas, TX; lot F1207) dissolved in pyrogen-free saline. The total volume injected was 100 μL. All experiments used the same lot of LIF. Feed intake was measured by taking weights of feed remaining (from a known amount) at 2, 4, 6, 8, 10, and 24 h after treatments. After each feed measurement, fresh feed was weighed and offered to the sheep. At each feed measurement, a rectal body temperature was taken. Sheep were randomly provided each treatment with at least a 1-wk interval between treatments. Cumulative feed intake at each time point was tested for effect of treatment using ANOVA with JMP Software (version
Leukemia inhibitory factor inhibits appetite

Experiment 3 – Effects of Melanocortin-4 Receptor Antagonist on Leukemia Inhibitory Factor Inhibition of Feed Intake

This study sought to block the MC4 receptor, thought to be critical to mediating the effects of LPS on feed intake in laboratory animals and sheep (Sartin et al., 2008, 2010; Grossberg et al., 2010). If the LIF inhibition of appetite is through a pathway using the MC4R, then there should be reduced or no LIF inhibition of feed intake in MC4R antagonist–treated sheep. Sheep (Suffolk wethers less than 1 yr old) were intracerebroventricularly treated with either saline or AgRP (0.5 nmol/kg BW) followed in 2 h by an ICV injection of LIF (2,500 ng/sheep) or saline, and feed intake was determined as above (see Exp. 2) at 2, 4, 6, 8, and 24 h after treatment with LIF. The treatment groups were saline/saline (n = 5), saline/LIF (n = 5), AgRP/saline (n = 4), and AgRP/LIF (n = 4). Cumulative feed intake at each time point was tested for effect of treatment using ANOVA with JMP Software (version 10). Means separation was performed using a Student’s t-test when appropriate.

Experiment 4 – Effects of Leukemia Inhibitory Factor on Selected Hormones and Metabolites

This study was designed to determine whether LIF has effects similar to acute LPS injection to increase GH concentrations, reduce LH concentrations, and elevate...
glucose and free fatty acids (FFA) in plasma. Sheep (Suffolk wethers less than 1 yr old; \( n = 6 \)) with ICV canulas were provided jugular cannulas the day before the experiment. On the day of the experiment, a blood sample was collected at −10 and 0 min. The sheep were then injected via the ICV cannula with either saline or the dose of LIF causing a maximal inhibition of feed intake (2,500 ng/sheep). Blood samples were then collected at 10-min intervals for 6 h. Sheep were randomly provided each treatment with at least a 1-wk interval between treatments. Plasma was harvested, and samples were stored frozen for later assay of LH and GH (only the first 3 h were assayed based on the typical acute response to LPS) by validated RIA (Daniel et al., 2005) and for FFA and glucose by spectrophotometric assays (McMahon et al., 1999). Minimum detectable concentration was 0.5 ng/tube for the LH assay and 1.0 ng/tube for the GH assay. The intra-assay and interassay CV for the LH assay were 6.8 and 18%, respectively; for GH were 6.9 and 10.8%, respectively; for glucose were 2.7 and 3.1%, respectively; and for FFA were 6.6 and 4.7%, respectively. Plasma concentrations of FFA, glucose, and GH were tested for effect of treatment (saline or LIF), day, time, and treatment × time interaction using procedures for repeated measures with JMP software (version 10). Means separation was performed using a Student’s \( t \)-test, when appropriate. Pulse patterns of LH were determined using cluster analysis (Veldhuis and Johnson, 1986) followed by ANOVA with JMP Software (version 10).

RESULTS

Experiment 1

In situ hybridization for LIF (Fig. 1a and 1b) determined that the number of LIF-positive cells in the ARC was increased 60 min after treatment with LPS (\( P = 0.033 \)). Additionally, the number of LIF grains per cell was increased by LPS treatment (\( P = 0.0083 \)). Analysis of POMC mRNA (Fig. 2) indicated that POMC gene expression (grains per cell) was not changed by LPS treatment (\( P = 0.15 \)) whereas AgRP expression was enhanced (\( P = 0.044 \)) at 60 min after LPS treatment (Fig. 3). The number of POMC or AgRP positive cells was not affected by LPS treatment (\( P > 0.05 \)).

Experiment 2 – Effect of Intracerebroventricular Leukemia Inhibitory Factor on Feed Intake and Body Temperature

Intracerebroventricular injection of LIF produced a fever (temperature greater than 40°C) at all doses by 4 to 6 h after injection but body temperature was not effected by treatment with saline (Fig. 4a). Temperatures of saline-treated sheep were within the normal range. The effect of LIF on feed intake was studied in the same sheep (Fig. 4b). There was a tendency for an effect of treatment on cumulative feed intake at 6 h (\( P = 0.0524 \)) and a significant effect of treatment on cumulative feed intake at 8 (\( P = 0.0173 \)) and 10 h (\( P = 0.0212 \)) after treatment. Mean separation indicated the 1,000-ng dose of LIF suppressed feed intake at 8 and 10 h after treatment and the 2,500-ng dose of LIF suppressed feed intake at 6, 8, and 10 h after treatment relative to saline-treated sheep.

Experiment 3 – Effects of Melanocortin-4 Receptor Antagonist on Leukemia Inhibitory Factor Inhibition of Feed Intake

Treatment with AgRP compared with saline did not stimulate cumulative feed intake at any time point.
Leukemia inhibitory factor inhibits appetite

Cumulative feed intake following ICV injection of the LIF group (Fig. 5) was inhibited at 6, 8, and 24 h following treatment relative to saline-treated sheep ($P < 0.05$). The cumulative feed intake in sheep treated with ICV injection of AgRP prior to LIF did not differ from saline-treated sheep at any time point, and had greater cumulative feed intake than the LIF-treated sheep at 2 ($P = 0.057$) and 24 h after LIF treatment ($P = 0.0006$).

**Experiment 4 – Effects of Leukemia Inhibitory Factor on Selected Hormones and Metabolites**

These experiments used the 2,500-ng dose of LIF. Analysis of plasma concentrations of FFA (Fig. 6a) indicated there was an effect of treatment ($P = 0.0277$), an effect of time ($P = 0.0012$), and a time × treatment interaction ($P = 0.0011$). Plasma concentrations of FFA were significantly elevated in 30-min interval samples from 3.5 to 5.5 h ($P < 0.05$) in sheep treated with LIF compared with sheep treated with saline. For plasma concentrations of glucose (Fig. 6b), there was a significant effect of treatment ($P = 0.0061$), an effect of time ($P < 0.0001$), and a time × treatment interaction ($P < 0.0001$). Plasma concentrations of glucose were significantly increased by LIF compared with saline in 30-min interval samples from 3 through 6 h after treatment. For plasma concentrations of LH (Fig. 7), there was an effect of time ($P < 0.0001$) and a treatment × time interaction ($P < 0.0001$). Plasma concentrations of LH in LIF-treated sheep did not differ from those in saline-treated sheep at the same time point. In the LIF-treated sheep compared with pre-injection plasma concentrations of LH, there were significant reductions at 10 min and 1.5 to 6 h after LIF injection ($P < 0.05$; 10-min interval samples). The LH data was also analyzed for pulse parameters (Table 1). The $P$-values for comparisons for mean LH ($P = 0.0848$), area under the curve ($P = 0.3103$), number of peaks ($P = 0.3083$), peak height ($P = 0.063$), and peak nadir ($P = 0.0514$) indicated subtle effects of LIF to reduce plasma LH concentrations. Examination of individual sheep indicated that 1 saline-treated sheep had low LH levels for the entire sampling period, which may have an effect on these data, but the sheep appeared healthy and was included in the analysis. There was only a tendency for the plasma concentrations of GH to differ between saline- and LIF-treated sheep ($P = 0.0874$; Fig. 8).

**DISCUSSION**

Feed intake suppression by disease is an important factor in considering limits to animal production. However, less is known about the mechanisms by which appetite may be suppressed in farm animals. In these experiments, we examine a possible role for LIF in appetite suppression in a disease model in sheep. Injection
of LPS via jugular cannula resulted in increased expression of LIF in the hypothalamic ARC, indicating LIF is sensitive to LPS in the region of the hypothalamus where appetite is controlled. These findings were similar to what has been observed in laboratory animals (Grossberg et al., 2010). Coincident with elevated LIF is the increase in expression of AgRP, similar to the data in laboratory animals. The AgRP neurons may express gp130 receptors, thereby providing a mechanism by which LIF could alter AgRP expression. Unlike rodents, POMC expression was unchanged at a time frame when appetite was suppressed in sheep (sheep do not eat between 1 and 6 h after LPS injection). Because POMC expression is central to the disease-associated suppression of appetite, this divergence from theory may be an important species difference. However, sheep are known to have limited changes in POMC gene expression associated with fasting (Adam et al., 2002; Archer et al., 2002; Sartin et al., 2010). Indeed, only severe feed restriction or fasting plus a long day photoperiod will reduce gene expression for POMC in sheep (McShane et al., 1993; Clarke et al., 2003). These findings suggest sheep may use a different type of regulation for the inhibition of appetite. Because sheep are grazing animals and have more consistent nutrient absorption than monogastric animals, the inhibitory pathways governing appetite may not require rapid responses to acute stimuli (Sartin et al., 2010). One possible explanation would be that sheep might maintain constant POMC expression but alter the rate of acetylation of melanocyte stimulating hormone (α-MSH), thus increasing the proportion of bioactive α-MSH without the need for altered gene expression (Guo et al., 2004; Backholer et al., 2010). It is also interesting to note that, unlike laboratory animals, plasma leptin concentrations are unchanged following LPS administration in sheep (Soliman et al., 2001; Daniel et al., 2003). These results remove the theory of a possible stimulus to POMC gene expression and require a different mechanism for appetite suppression. At this point, further speculation concerning alternate mechanisms for appetite inhibition is not warranted by our data. It is interesting to note that, although fasting and LPS did not alter POMC gene expression in sheep at 1 h after LPS, at 6 h after intravenous LPS, and coincident with initial resumption of feed intake, there is a significant decrease in POMC expression (Sartin et al., 2008). Although not conclusive, the elevated AgRP and decreased POMC expression at 6 h would provide the needed appetite stimulus to resume feeding in this model (Sartin et al., 2008).

The ICV injection of LIF, at all doses used, was capable of inducing a fever (above 40°C). Moreover, appetite was suppressed by the 2 higher doses (1,000 and 2,500 ng per sheep). These experiments confirm data in laboratory animals (Plata-Salamán, 1996; Beretta et al., 2002; Grossberg et al., 2010) and extend the results to ruminants, suggesting a role for LIF in disease-associated appetite suppression. It is important to note that LPS stimulates an increase in a number of cytokines, including tumor necrosis factor-α and IL-1β, which have been demonstrated to inhibit feed intake via action in the central nervous system (Plata-Salamán et al., 1988). However, the reduction in appetite in response to LIF was pronounced and was similar in onset and duration to the effects of LPS. Therefore, LIF may mediate LPS actions on appetite in sheep as proposed for laboratory animals (Grossberg et al., 2010).

**Table 1. Effect of leukemia inhibitory factor (LIF) on plasma LH pulse parameters**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean ± SE</th>
<th>AUC 1</th>
<th>Number of peaks</th>
<th>Peak height</th>
<th>Peak nadir</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>4.7 ± 0.25</td>
<td>1,404.8 ± 150.0</td>
<td>4.4 ± 0.8</td>
<td>5.5 ± 0.2</td>
<td>3.5 ± 0.1</td>
<td>0.0848</td>
</tr>
<tr>
<td>LIF</td>
<td>3.3 ± 0.25</td>
<td>1,059.7 ± 114.6</td>
<td>2.5 ± 0.6</td>
<td>4.3 ± 0.2</td>
<td>2.7 ± 0.1</td>
<td>0.0514</td>
</tr>
</tbody>
</table>

1AUC = Area under the curve.
In laboratory animals, the effects of LPS on appetite mechanisms are to activate POMC neurons and thus reduce stimulation of the MC4R and inhibit appetite. In previous studies, AgRP intracerebroventricularly injected into sheep was found to increase feed intake and AgRP was found to prevent the appetite suppression produced by intravenous LPS injection in sheep (Wagner et al., 2004; Sartin et al., 2008). To further examine the LIF suppression of appetite response, an experiment was performed to determine whether AgRP could inhibit the MC4R and prevent the reduction in feed intake due to LIF in sheep. Although feed intake was reduced, in this study, by LIF, sheep given AgRP followed by LIF had feed intake that did not differ from saline-treated sheep. These data would appear to support the effect of LIF mediated by the MC4R to inhibit feed intake (Grossberg et al., 2010). The AgRP appetite data suggests that the MC4R is critical in mediating the appetite suppression produced by LPS and by LIF. Although POMC gene expression is not increased by LPS (as noted earlier), the effects of AgRP to reverse LIF inhibition of appetite indicate that there is a significant impact of α-MSH to activate the MC4R. Although outside of the scope of this project, it is necessary to determine whether α-MSH acetylation is altered during LPS treatment to fully appreciate the melanocortin pathway in sheep for control of feed intake suppression after LPS.

In addition to having an impact on feed intake, LPS has effects on circulating metabolites and hormones. If LIF mediates LPS actions on appetite, it might also be a component of some of the physiological responses to LPS. Glucose and FFA represent an important metabolic change in response to LPS and their plasma concentrations can be regulated from the hypothalamus. In this experiment, a dose of LIF was selected based on its ability to maximally suppress appetite. This dose of LIF intracerebroventricularly injected increased plasma concentrations of glucose and FFA, although this effect was different for the long-term treatment with LIF as described by Beretta et al. (2002) in laboratory animals. The difference in acute versus chronic treatment as well as different species used can likely explain the differing responses. In studies using cattle (McMahon et al., 1998), the glucose response to LPS was an acute elevation followed by relative hypoglycemia (and insulin resistance). Similarly, plasma concentrations of FFA were acutely increased in cattle following LPS (McMahon et al., 1998). Therefore, physiologically, LIF injected into the lateral ventricle produces a change similar to the initial effects of LPS on these metabolites in ruminants, further suggesting a role for LIF in the mediation of at least some aspects of the animal response to disease.

A number of studies have demonstrated that ACTH secretion in disease models is mediated by LIF (Akita et al., 1995; Schwartz et al., 1999; Chesnokova and Melmed, 2000). Circulating concentrations of the hormones LH and GH are also altered in response to LPS in sheep (Coleman et al., 1993). The effect of LPS is to reduce the circulating concentration of LH and reduce various pulse parameters mediated by actions within the hypothalamus. Injection of LIF into the lateral ventricle has an effect similar to LPS to reduce plasma LH, although the inhibition is less dramatic than with LPS. Unfortunately, plasma LH concentrations in the saline-treated sheep declined slightly throughout the study and 1 saline-treated sheep had levels of LH that were half that of the other saline-treated sheep. In spite of these issues, plasma concentrations of LH were still reduced. In a pulse analysis for circulating LH, the LH mean and peak height tended to be lower, but the nadir was significantly reduced. Therefore, there is evidence for a negative impact on circulating LH concentrations. It should be noted that the dose of LIF was not adjusted to achieve maximum effects on LH but rather based on the
Therefore, a different dose of LIF may have produced a more pronounced inhibition of LH. The effects of LIF on other aspects of reproduction have been investigated. For example, LIF antagonized LH-stimulated testosterone release from cultured porcine Leydig cells (Mauduit et al., 2001). Moreover, there may also be nonpathogenic roles for LIF in normal reproductive processes in farm animals (Ptak et al., 2006). Another hormone, GH, was increased by LPS treatment in sheep (Coleman et al., 1993; Daniel et al., 2005). Although ICV injection of LIF did not significantly increase GH in this study, there was a tendency for GH to be elevated, perhaps due to LIF dose or because the primary effects of LPS to release GH are found at the pituitary and not within the hypothalamus (Daniel et al., 2005) in sheep.

These experiments provide evidence, when combined with studies in laboratory animals, to support a role for LIF in the sequence of activation events within the brain that lead to appetite suppression associated with a disease model in sheep. The effect of LIF may not be confined to appetite mechanisms but may additionally influence selected endocrine and metabolic changes that are mediated through the hypothalamus, such as glucose, FFA, and the hormones LH and GH.

**LITERATURE CITED**


Leukemia inhibitory factor inhibits appetite


