

Neuropeptide Y restores appetite and alters concentrations of GH after central administration to endotoxic sheep

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Abstract

The objective of this study was to determine whether neuropeptide Y (NPY) and recombinant human interleukin-1 receptor antagonist (IL-1ra) would: first, increase food intake; secondly, decrease concentrations of GH; thirdly, reduce GHRH-induced release of GH; and fourthly, reduce changes to concentrations of IGF-I in plasma during experimental endotoxemia in sheep.

Six treatments were given to six castrated male sheep in a 6 × 6 Latin square treatment order. Osmotic mini-pumps were implanted at 0 h and a jugular vein was cannulated. Each sheep was continuously infused with saline (0.9%) or lipopolysaccharide (LPS) (20 µg/kg per 24 h, s.c.) at 10 µl/h for 72 h via the osmotic mini-pumps. Blood samples (3 ml) were collected at 15-min intervals from 24 to 33 h. At 26 h, one of three treatments (artificial cerebrospinal fluid, NPY or IL-1ra) was injected i.c.v. within 30 s (0.3 µg/kg), then infused i.c.v. from 26 to 33 h (600 µl/h) at 0.3 µg/kg per h. GHRH was injected i.v. (0.075 µg/kg) at 32 h after which blood samples were collected at 5, 10, 15, 30, 45 and 60 min. Feed intake was reduced up to 50% for 48 h in LPS-treated compared with non-LPS-treated sheep.

NPY restored feed intake in LPS-treated sheep and induced hyperphagia in non-LPS-treated sheep from 24 to 48 h. In contrast, IL-1ra did not affect appetite. Injection of NPY increased concentrations of GH from 26 to 27 h, while IL-1ra had no effect. Infusion of NPY suppressed GHRH-induced release of GH. However, no treatment altered pulse secretion parameters of GH. Concentrations of IGF-I were 20% higher at 72 h in LPS-treated sheep given NPY than in sheep treated with LPS alone, and this may reflect increased appetite from 24 to 48 h.

We concluded that reduced appetite during endotoxemia is due to down-regulation of an NPY-mediated mechanism. Furthermore, NPY stimulates release of GH in healthy sheep, does not reduce pulse secretion parameters of GH, but does suppress GHRH-induced release of GH in endotoxic sheep. Therefore, NPY may be an important neurotransmitter linking appetite with regulation of GH during endotoxemic and healthy states in sheep.

Journal of Endocrinology (1999) **161**, 333–339

Introduction

Lipopolysaccharide (LPS), a structural component in the outer cell wall of gram-negative bacteria, stimulates cells of the immune system to release cytokines, which, in turn, affect hormones and neurotransmitters. LPS also suppresses appetite and growth (Elsasser *et al.* 1995). Of the cytokines released, interleukin-1β (IL-1β) is of particular interest because it suppresses release of neuropeptide Y (NPY) from axon terminals (McCarthy *et al.* 1995). NPY stimulates food intake (Miner *et al.* 1989, 1990) and inhibits release of growth hormone (GH) from the anterior pituitary gland (Rettori *et al.* 1990, Suzuki *et al.* 1996). In addition, i.v. injections of LPS increase concentrations of

GH for several hours and reduce concentrations of insulin-like growth factor-I (IGF-I) for days (Coleman *et al.* 1993, Elsasser *et al.* 1996, Sartin *et al.* 1998).

We hypothesize that LPS-induced chronic endotoxemia increases release of IL-1β, which suppresses release of NPY, leading to increased concentrations of GH and a concurrent decrease in food intake thereby reducing secretion of IGF-I. Our objectives were to determine whether intracerebroventricular (i.c.v.) injection, then infusion of NPY or IL-receptor antagonist (IL-1ra) (Eisenberg *et al.* 1990), into endotoxic sheep would: first, increase food intake; secondly, decrease concentrations of GH; thirdly, reduce GH-releasing hormone (GHRH)-induced release of GH; and fourthly,

reduce changes to concentrations of IGF-I in plasma of sheep.

Materials and Methods

Animals and maintenance

Six, mixed-breed, castrated male sheep were kept indoors in individual pens under conditions simulating ambient temperature and photoperiod. Sheep were 1.5 years old and weighed 64.3 ± 6.0 kg. They had *ad libitum* access to concentrate feed, which contained 12% crude protein and was calculated to meet 100% of daily requirements. After overnight fasting, sheep were anesthetized, placed in a sheep stereotaxic device (David Kopf Instruments, Tujunga, CA, USA) and maintained under anesthesia with halothane. An 18 gauge guide cannula with luer closure stylette was placed into a lateral ventricle 15 mm rostral to the interaural line, 8 mm lateral to midline, and approximately 25 mm ventral from the skull surface of each sheep. Three stainless steel screws were placed in the frontal bone and the cannula was cemented to these screws with dental acrylic, then a plastic tube with a lid was cemented around the cannula (Buxton 1988, Sartin *et al.* 1988). Cannula placement was confirmed at the beginning and the end of this study by taking a radiograph in the lateral aspect immediately after injecting 1 ml of a radio-opaque dye (Omnipaque 300; Sterling Drug Inc., New York, NY, USA) into the cannulated ventricle.

Animals were given 2 weeks to recover from i.c.v. surgery, during which time they received an analgesic, Banamine (Schering-Plough Animal Health Corp., Kenilworth, NJ, USA) i.m. for the first 3 days, an antibiotic, LA 200 (Pfizer Inc., New York, NY, USA), every other day for 2 weeks, and topical application of Nitrofurazone (Clay-Park Lab, Bronx, NY, USA) for 2 days, then 1% silver sulfadiazine (Boots Pharmaceuticals, Lincolnshire, IL, USA) for 12 days.

These studies were carried out from February to April and all experimental procedures were approved by the Institutional Animal Care and Use Committee at Auburn University.

Experimental model

Intravenous injection of LPS typically induces an acute-phase response within 30 min (Coleman *et al.* 1993, Elsasser *et al.* 1996). Lungs are particularly sensitive to LPS and respiratory distress is a common feature of endotoxemia (Martin & Silverman 1992, Domenici-Lombardo *et al.* 1995). In addition, acute i.v. injection of LPS induces fever within 30 min and we wished to avoid conducting experiments while sheep had fevers despite evidence that regulation of fever is independent of regulation of appetite (Miñano & Myers 1991, Myers *et al.* 1994). To reduce

symptoms of severe sepsis after acute injection of LPS, we developed a model of chronic endotoxemia in which osmotic mini-pumps (2ML1; Alza Corporation, Palo Alto, CA, USA) implanted s.c. delivered LPS (*E. coli* serotype 055:B5; Sigma Chemical Corporation, St Louis, MO, USA) at a dose of 20 µg/kg per 24 h and at a flow of 10 µl/h for 72 h. Osmotic-mini pumps were primed overnight in sterile saline so that they were delivering LPS when they were implanted. This model for inducing chronic endotoxemia is a modification of that described for rats by Fish & Spitzer (1984).

Treatments

Each sheep received six treatments in a 6 × 6 Latin square arrangement of treatments in which saline (SAL, 0.9%) or LPS were continuously released from osmotic mini-pumps for 72 h. Osmotic mini-pumps were surgically implanted at 0 h in accordance with the manufacturer's instructions. Twenty-six hours later one of three treatments: artificial cerebrospinal fluid (CSF), NPY (0.3 µg/kg in 600 µl), or IL-1ra (0.3 µg/kg in 600 µl) was injected i.c.v. via a syringe within 30 s. Immediately thereafter, these same doses were infused from 26 to 33 h at a flow rate of 600 µl/h via a peristaltic pump. This dose of NPY stimulates feed intake in sheep (Miner *et al.* 1989, 1990) and we infused IL-1ra at the same dose for comparison. Thus, the six treatments were: SAL/CSF, LPS/CSF, SAL/NPY, LPS/NPY, SAL/IL-1ra and LPS/IL-1ra. At 32 h (1 h before cessation of CSF, NPY or IL-1ra), GHRH (Peninsula Laboratories, Belmont, CA, USA) was injected i.v. into all sheep at a dose of 0.075 µg/kg. At 33 h sheep were returned to their rooms. At 72 h osmotic mini-pumps were removed from all sheep. Artificial CSF consisted of (in mM: NaCl 127.7, KCl 2.5, CaCl₂ 1.3, MgCl₂ 1.0, NaH₂PO₄ 1.3, NaHCO₃ 21.0, glucose 3.4); ovine NPY was purchased from Sigma and recombinant human IL-1ra (rhu-IL-1ra, lot RS05-003) was a gift from Amgen (Boulder, CO, USA).

At least 1 week separated each period of the Latin square and the order of the Latin square was balanced so that no animal was given LPS consecutively. Furthermore, each animal was exposed to LPS on three occasions with a month separating each exposure.

LPS, NPY and IL-1ra were dissolved in CSF and stored in siliconized vessels during experiments, and all tubing was siliconized (Sigmacote; Sigma) before use as previously recommended (Miner *et al.* 1990).

Body temperature was recorded at -24, 0, 4, 8, 12, 24, 48 and 72 h using a rectal thermometer. In addition, sheep were shorn every 2 weeks during the experiment to keep wool length close to 1 cm.

To facilitate infusion of CSF, NPY, IL-1ra, blood sampling and measurement of food intake, sheep were placed in wooden pens (0.5 × 1.5 m) at 24 h and removed from them at 33 h. In these pens sheep could stand and lie

down, but could not turn around. They wore a halter to which three straps were attached to prevent excess movement of their heads and to protect i.c.v. cannulae from damage. A strap was attached to each side and one underneath and attached to the bottom of the pen. The side straps were attached to an 'O' ring, which slid vertically on metal rods attached to each side at the front of the pen and they enabled 20 cm of lateral movement. The lower strap prevented sheep from lifting their heads higher than normal when standing. In addition, sheep wore a collar to which one end of a piece of elastic was attached and the other end was attached to a fitting 1.5 m above the pen. Infusion tubing and cannulae were taped to the elastic, which was kept under slight tension, to keep tubing away from the head of each sheep. Plastic feed and water containers were attached to the front of each wooden pen and were within reach of the sheep at all times. Sheep were acclimated to these pens for 2 weeks before surgery and for a further 2 weeks before the experiment for up to 7 h daily. Sheep were relaxed and voluntarily walked into these pens each day of the experiment.

Feed intake

A known weight of feed was offered to sheep at -24, 0, 24, 26, 27, 28, 30, 33, 48, 72, 96, 120 and 144 h (time 0 represents insertion of saline or LPS pumps). Feed not eaten at each time was weighed and corrected for moisture content by drying sub-samples of fresh feed and feed residue at 40 °C for 2 days. Feed residue was deducted from feed offered to yield dry matter intake (DMI) for each sheep. DMI is expressed as a percentage of body weight. DMI was measured more frequently (24 to 48 h) after injection and during infusion of CSF, NPY or IL-1ra and is presented as the cumulative DMI for that period.

Blood sampling

A jugular vein of each sheep was cannulated at 0 h. Blood samples (3 ml) were collected at 15 min intervals from 24 to 33 h. In addition, blood was collected at 5 and 10 min after injection of CSF, NPY or IL-1ra and at 5, 10, 15, 30, 45 and 60 min after injection of GHRH. Single samples of blood were collected at 0, 24, 48 and 72 h for measurement of IGF-I. Blood was collected into tubes containing 7.5 mg EDTA. Plasma was stored at -20 °C for RIA of GH and IGF-I.

Hormone analysis

GH was assayed as described previously (Sartin *et al.* 1985, 1988). Intra- and interassay coefficients of variation (C.V.)

were 8.7% and 15.5% respectively. IGF-I was assayed in one assay as described previously (Elsasser *et al.* 1988) and the intra-assay C.V. was 6.5%.

Statistical analysis

Pulse secretion parameters for GH (mean, number of peaks, peak amplitude, peak area, and inter-pulse interval) were determined for the first 8 h using Cluster analysis, version 6.0 (Veldhuis & Johnson 1986). The mean of duplicate sample concentrations was assessed using the intra-assay C.V. of 8.7% for appraisal of variance in the data. Nadir was determined from the mean of two consecutive data points, and peaks from one data point. Minimum detectable pulse was set at 1.5 ng/ml and *t*-statistics of 2 were assigned for increases and decreases in concentrations of GH.

Total area under the GH curve was calculated from 26 to 27 h (after injection of CSF, NPY or IL-1ra) and from 32 to 32.5 h (after injection of GHRH) using the trapezoid method. The area of the rectangle under the projected baseline from samples collected at 26 to 27 h and from 32 to 32.5 h, respectively, were deducted from the total areas to produce net areas under the curve for analysis (GH AUC).

Concentrations of IGF-I were different ($P < 0.05$) among treatment groups at 0 h. Therefore, concentrations of IGF-I at 0 h were used as a covariate for concentrations of IGF-I at 24, 48 and 72 h.

Data for GH pulse parameters, GH AUC, IGF-I, daily DMI, cumulative DMI, and body temperature were subjected to ANOVA using the generalized linear models procedure in SAS (1990). Factors included in the statistical model were treatment, animal, and successive periods in the Latin square. When treatments were significant, least-squares means were compared using *t*-tests.

Results

Body temperature significantly increased in LPS-treated groups for 12 h after osmotic mini-pumps were implanted but was returning to normal at 24 h (Fig. 1).

DMI was reduced up to 50% (range $P < 0.05$ to $P < 0.001$) in LPS/CSF-treated compared with SAL/CSF-treated sheep and this suppression was maintained for 48 h (Fig. 2). DMI returned to normal after removal of LPS at 72 h. NPY stimulated food intake in SAL- and LPS-treated sheep compared with SAL/CSF and LPS/CSF respectively. IL-1ra did not affect food intake (Figs 2 and 3).

Cumulative DMI was initially lower in LPS/NPY- than in SAL/CSF-treated sheep from 24 to 26 h, but then did not differ significantly from SAL/CSF-treated sheep from 26 to 48 h (Fig. 3). In addition, SAL/NPY-treated

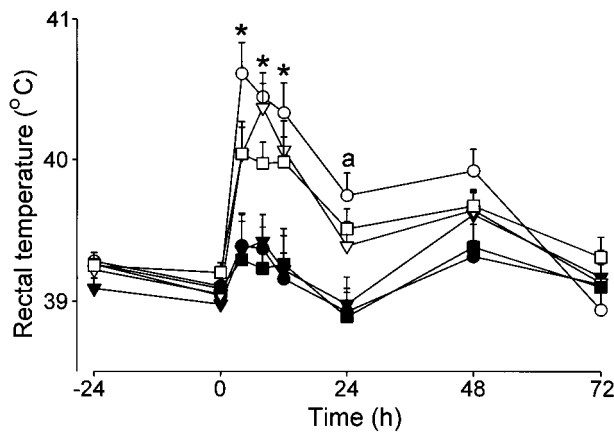


Figure 1 Least-squares mean (\pm S.E.M.) body temperature in sheep infused s.c. with either saline (0.9%) or LPS (20 μ g/kg per 24 h) between 0 and 72 h. Treatments were: SAL/CSF (●), LPS/CSF (○), SAL/NPY (▼), LPS/NPY (▽), SAL/IL-1ra (■), and LPS/IL-1ra (□). Asterisks and letters denote differences between LPS-treated and non-LPS-treated sheep (*range $P < 0.1$ to 0.01 ; ^aone LPS-treated group (LPS/CSF) had higher body temperatures than non-LPS-treated sheep at 24 h (before infusion of CSF), $P < 0.05$).

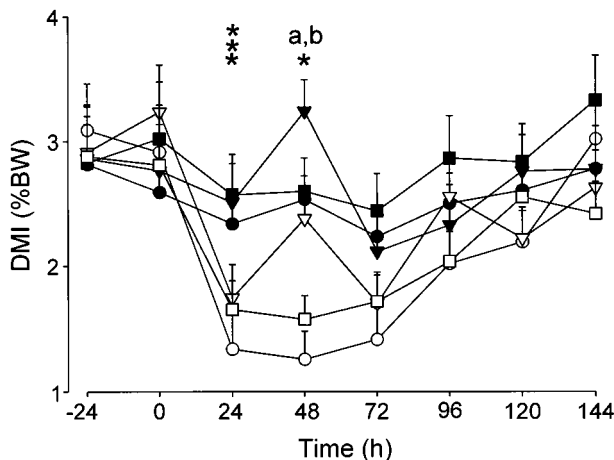


Figure 2 Least-squares mean (\pm S.E.M.) DMI for sheep infused s.c. with either saline (0.9%) or LPS (20 μ g/kg per 24 h) between 0 and 72 h. Treatments were: SAL/CSF (●), LPS/CSF (○), SAL/NPY (▼), LPS/NPY (▽), SAL/IL-1ra (■), and LPS/IL-1ra (□). Asterisks denote differences between SAL/CSF and LPS/CSF-treated sheep ($***P < 0.001$, $*P < 0.05$). Letters denote differences between LPS/CSF- and LPS/NPY-treated sheep (^a $P < 0.01$), and between SAL/CSF- and SAL/NPY-treated sheep at 48 h (^b $P < 0.05$).

sheep had significantly higher cumulative DMI than SAL/CSF-treated sheep from 30 to 48 h (Fig. 3).

Overall, GH AUC after i.c.v. administration of NPY was not different ($P = 0.18$) among treatments. However, GH AUC was greater ($P < 0.05$) in SAL/NPY-treated than SAL/CSF-treated sheep when these two groups were compared alone (Fig. 4). Furthermore, concentrations of GH increased gradually from 15 to 30 min after injection of NPY (raw data not shown).

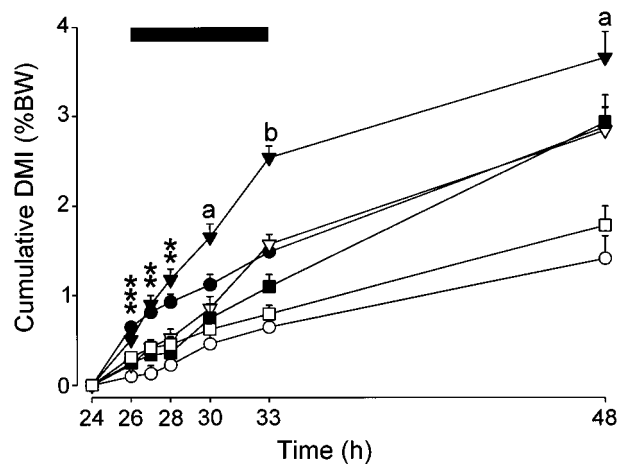


Figure 3 Least-squares mean (\pm S.E.M.) cumulative DMI from 24 to 48 h following LPS administration. Treatments were: SAL/CSF (●), LPS/CSF (○), SAL/NPY (▼), LPS/NPY (▽), SAL/IL-1ra (■), and LPS/IL-1ra (□). Asterisks denote differences between SAL/CSF- and LPS/NPY-treated sheep ($***P < 0.001$, $**P < 0.01$). Letters denote differences between SAL/CSF- and SAL/NPY-treated sheep (^a $P < 0.05$, ^b $P < 0.001$). The solid bar indicates duration of infusion of CSF, NPY or IL-1ra.

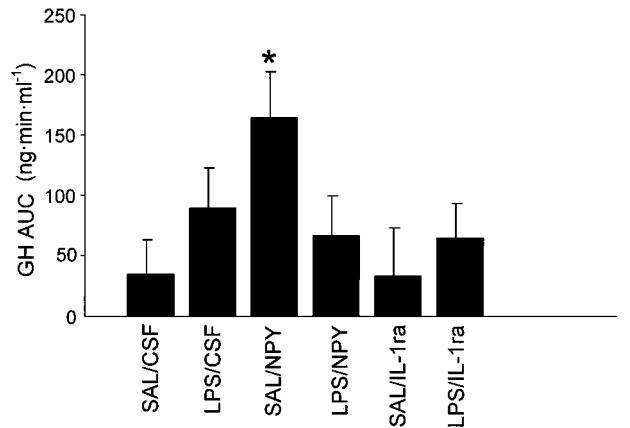


Figure 4 Least-squares mean (\pm S.E.M.) GH AUC from 26 to 27 h after i.c.v. injection and infusion of CSF, NPY or IL-1ra (commenced at 26 h). The asterisk denotes a difference between SAL/CSF- and SAL/NPY-treated sheep ($*P < 0.05$).

GHRH stimulated release of GH in all sheep with maximal concentrations of GH occurring at 5 min. Overall, GH AUC after i.v. injection of GHRH was not different ($P = 0.26$) among treatments. However, GH AUC was lower ($P < 0.05$) in LPS/NPY-treated than in LPS/CSF-treated sheep when these two groups were compared alone (Fig. 5). Treatments did not significantly alter pulse secretion parameters of GH (data not shown).

Concentrations of IGF-I were reduced in LPS-treated sheep at 24 h, but concentrations increased from 24 to 72 h (Fig. 6). At 72 h, concentrations of IGF-I were higher in LPS/NPY-treated than in LPS/CSF-treated sheep ($P < 0.05$).

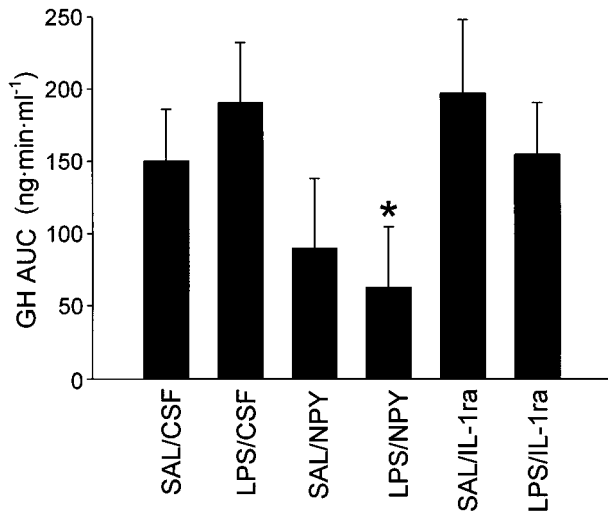


Figure 5 Least-squares mean (\pm S.E.M.) GH AUC from 32 to 32.5 h after i.v. injection of GHRH (0.075 μ g/kg) given at 32 h (after 6 h i.c.v. infusion of CSF, NPY or IL-1ra). The asterisk denotes a difference between LPS/CSF- and LPS/NPY-treated sheep (* P <0.05).

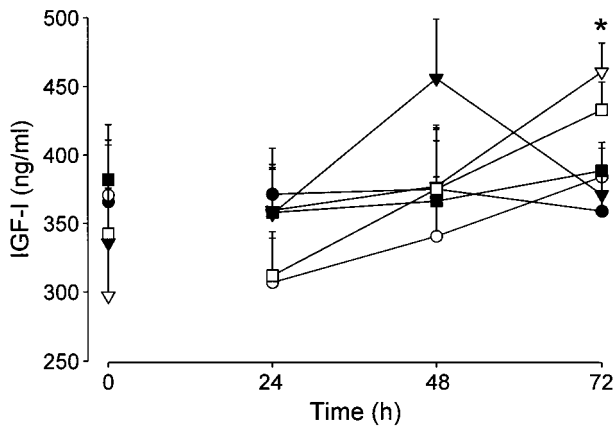


Figure 6 Least-squares mean (\pm S.E.M.) concentration of IGF-I in sheep infused s.c. with either saline (0.9%) or LPS (20 μ g/kg per 24 h) between 0 and 72 h. Treatments were: SAL/CSF (●), LPS/CSF (○), SAL/NPY (▼), LPS/NPY (▽), SAL/IL-1ra (■), and LPS/IL-1ra (□). Concentrations of IGF-I at 0 h were used as a covariate for concentrations of IGF-I from 24 to 72 h. The asterisk denotes a difference between LPS/CSF- and LPS/NPY-treated sheep (* P <0.05).

Discussion

In this study we developed a chronic model of endotoxemia in sheep in which appetite was suppressed up to 50% for 48 h. Fever had a slower onset than is typical after i.v. injection of LPS (Coleman *et al.* 1993, Elsasser *et al.* 1996) and body temperature was returning to normal at 24 h. Furthermore, sheep showed no symptoms of respiratory distress during the 72-h infusion of LPS.

In the absence of fever and respiratory distress, i.c.v. injection and subsequent 7-h infusion of NPY restored appetite in sheep that were continuously exposed to LPS for 24 h. These data support our hypothesis that endotoxin reduces appetite via suppressing release of NPY. In addition to restoring appetite in LPS/NPY-treated sheep, NPY induced hyperphagia in SAL/NPY-treated sheep. Because food intake in LPS/NPY-treated sheep remained lower than SAL/NPY-treated sheep at 48 h, we suggest that other factors, presumably other cytokines, continue to suppress appetite.

IL-1 β is a potent mediator of endotoxemia: central injection of human IL-1 β induces fever and hypercortisolemia in sheep (Vellucci *et al.* 1995) and reduces appetite in rats (Sonti *et al.* 1996). In addition, McCarthy *et al.* (1995) demonstrated that IL-1 β blocks release of NPY from axon terminals and Sonti *et al.* (1996) reversed IL-1 β -induced suppression of appetite in rats with an i.c.v. injection of NPY. The present study shows that infusion of NPY reverses suppressed appetite in sheep exposed to LPS. However, in contrast to Sonti *et al.* (1996), the present study suggests that IL-1 β does not mediate suppression of appetite in sheep because IL-1ra did not increase food intake in endotoxic sheep. In support of this argument, the dose of IL-1ra injected and infused in the current study was higher than the dose of human IL-1 β injected i.c.v. in the study of Vellucci *et al.* (1995). While it is possible that the concentration of IL-1ra used in the present study was insufficient to block endogenous IL-1 β released in the hypothalamus, others have injected similar doses i.c.v. into rats and shown IL-1ra to block central actions of IL-1 β on appetite (Plata-Salamán & Ffrench-Mullen 1992, Plata-Salamán 1994, Kent *et al.* 1996).

A number of cytokines suppress appetite; for example interleukins, tumor necrosis factor- α (TNF- α), and interferon- γ (Plata-Salamán 1996). Only TNF- α has been reliably measured in blood of sheep and cattle, and those studies show that concentrations of TNF- α increase after i.v. injection of LPS (Coleman *et al.* 1993, Elsasser *et al.* 1995). However, concentrations of TNF- α are only elevated for several hours. Moreover, central infusion of IL-1 β or IL-6 does not sustain reduced appetite in rats (Van Haasteren *et al.* 1994). Thus, reduced appetite can persist for longer than increased concentrations of individual cytokines and, paradoxically, cannot be sustained by infusion of individual cytokines. Therefore, it is probable that a combination of cytokines interacting with neurotransmitters contribute to reduced appetite during LPS-induced cachexia.

Injection of NPY increased concentrations of GH in SAL/NPY-treated sheep after a 15-min latency. We anticipated a decrease in concentrations of GH, because i.c.v. injections of NPY stimulate release of somatostatin (SS) and inhibit pulsatile release of GH from the anterior pituitary gland in rats (Rettori *et al.* 1990, Suzuki *et al.* 1996). In the present study, the latency between injection

and start of infusion of NPY and the small increase in concentrations of GH suggest that NPY may be a weak secretagogue of GH. However, McMahon *et al.* (1998) showed that NPY stimulates release of both SS and GHRH from perfused bovine hypothalamic slices. Therefore, in the current study it is possible that NPY stimulated both SS and GHRH, which delayed and diminished release of GH from the anterior pituitary gland. While injection of NPY stimulates release of GH in healthy sheep (SAL/NPY), infusion of NPY suppressed GHRH-induced release of GH in LPS/NPY-treated sheep, which supports our third objective. However, NPY did not reduce GHRH-induced release of GH in healthy sheep (SAL/NPY). Therefore, NPY does not always inhibit release of GH in sheep.

No change in pulse secretion parameters of GH were observed in the present study. Others have shown that low levels of nutrition and fasting reduced concentrations of GH (Driver & Forbes 1981, Breier *et al.* 1986, 1988). In the present study, food intake was reduced up to 50% for 24 h prior to sampling, and this may not have been a sufficient reduction in food intake or have been of long enough duration to affect regulation of GH in sheep. In addition, route and rate of delivery of LPS may be important considerations. Intravenous injections of LPS increase concentrations of GH (Coleman *et al.* 1993) while higher doses reduce concentrations of GH in cattle (Elsasser *et al.* 1995). Finally, the failure of LPS to alter pulsatile secretion of GH into blood suggests that this chronic model of endotoxemia in sheep is useful for assessing effects of LPS on appetite independent of those regulating release of GH.

Neither NPY nor IL-1ra affected changes in concentrations of IGF-I at 48 h. However, concentrations of IGF-I were higher at 72 h in LPS/NPY- than in LPS/CSF-treated sheep. Therefore, it is likely that increased concentrations of IGF-I at 72 h reflect increased food intake from 24 to 48 h in LPS/NPY-treated sheep, which, in turn, led to faster recovery from endotoxemia.

In conclusion, i.c.v. injection plus infusion of NPY, but not IL-1ra, restores appetite in endotoxic sheep, suggesting that NPY may mediate endotoxin-induced reduction in appetite. In contrast to previous studies in rats where NPY reduced secretion of GH, our data show that i.c.v. injection of NPY stimulated release of GH in healthy sheep, but not endotoxic sheep, while infusion of NPY suppressed GHRH-induced release of GH in endotoxic sheep, but not healthy sheep. Therefore, NPY may be an important neurotransmitter linking appetite with regulation of GH during endotoxemic and healthy states in sheep.

Acknowledgements

We thank Dr G Davenport for formulating the sheep diet and Ms M Johnson, Mr M Pope and Mr G Fincher for

assistance with animal feeding and care. In addition, we thank Dr D Pugh and the ambulatory crew at the College of Veterinary Medicine, Auburn University, for animal health care. Finally, we thank Amgen for the generous gift of rhu-IL-1ra.

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Received 16 April 1998

Revised manuscript received 26 November 1998

Accepted 4 January 1999