Leptin alters antibody isotype in the pig in vivo, but does not regulate cytokine expression or stimulate STAT3 signaling in peripheral blood monocytes in vitro

T. E. Weber and M. E. Spurlock

Department of Animal Sciences, Purdue University, West Lafayette, IN 47907

ABSTRACT: Although leptin modulates immunological pathways in some species, the role of leptin as a regulator of immunocyte function in the pig has not been studied. Therefore, the primary objective of this study was to determine whether leptin influences specific immunocyte response variables in the pig in vivo or in vitro. Fifteen pigs (five pigs per treatment) were 1) injected with recombinant human leptin and allowed to consume feed ad libitum, 2) injected with vehicle and allowed to consume feed ad libitum, or 3) injected with vehicle and limit-fed to the intake of the leptin-injected group. All the pigs also were injected with the antigen, Limulus hemocyanin, on d 0 and 15 of the experiment. Exogenous leptin decreased (P < 0.05) daily feed intake and antigen-specific immunoglobulin (Ig) G1, but had no effect on lymphocyte proliferation or antigen-specific IgG2. In a second series of experiments, peripheral blood mononuclear cells (PBMC) were isolated from venous blood to determine the effect of stimulation with the polyclonal mitogen, concanavalin A (ConA), on the long form of the leptin receptor (Ob-Rl) mRNA abundance, and to determine whether leptin altered mitogen-induced proliferation, cytokine production, or signal transducer and activator of transcription 3 (STAT3) activation. Leptin had no effect on the proliferation of PBMC or on cytokine mRNA abundance or secretion. The abundance of Ob-Rl mRNA was decreased (P < 0.05) in response to stimulation with ConA. Constitutive STAT3 DNA binding was evident in mobility shift assays, but was not altered by either leptin or serum deprivation. These data indicate that leptin modifies antibody isotypes in the pig, and that Ob-Rl expression is downregulated in response to polyclonal mitogens in porcine PBMC. The constitutive activation of STAT3, coupled with the absence of leptin-inducible binding, indicates an alternative signaling pathway for leptin in pig PBMC.

Key Words: Antibody Isotypes, Immune System, Leptin, Pigs

Introduction

Leptin, a 16-kDa protein secreted by adipocytes, has been shown to regulate multiple physiological systems in several species (Houseknecht et al., 1998), and data obtained with rodent models indicate a substantial role for leptin in the immune system. Leptin-deficient mice have lymphoid atrophy and decreased thymic cellularity, both of which are reversed with exogenous leptin (Howard et al., 1999). They also have greater mortality rates when challenged with the pathogen Klebsiella pneumoniae (Mancuso et al., 2002). Leptin increases T-cell proliferation and skew cytokine production to that of a Th helper type 1 (Th1) milieu (Lord et al., 1998). However, species differences are indicated in that circulating leptin is increased in response to bacterial endotoxin and tumor necrosis factor-α (TNF-α) in rodents and primates (Grunfeld et al., 1996; Landman et al., 2003), but not in cattle or sheep (Soliman et al., 2001, 2002; Daniel et al., 2003). Likewise, endotoxin increases adipose expression of leptin mRNA in rodents (Grunfeld et al., 1996), but does not alter expression in the pig (Spurlock et al., 1998). Nonetheless, the potential regulation of immune response pathways by leptin in the pig is indicated in that the mRNA for the leptin receptor has been identified in the spleen and bone marrow (Lin et al., 2000). To date, there are no published data as to the effects of exogenous leptin on immunological variables in the pig, nor is it known whether leptin signals through the signal transducer and activator of tran-
Leptin and the immune system of the pig

Leptin and the immune system of the pig 1631

scription 3 (STAT3) pathway in pig lymphocytes, as has been shown in the human lymphocytes (Sanchez-Margalet et al., 2001). Therefore, the objectives of this study were to determine whether leptin could modify specific immunological responses in the pig, and whether leptin activates STAT3 binding in pig lymphocytes.

Materials and Methods

Animals and Experimental Design

All animal care and handling procedures were approved by the Purdue University Animal Care and Use Committee. For the in vitro studies, blood was collected from healthy 3- to 5-mo-old crossbred barrows (Yorkshire-Landrace × PIC 357) that were allowed ad libitum access to a standard corn-soybean meal diet and water. Blood (approximately 40 mL) was aseptically collected from the jugular vein into Vacutainers containing sodium heparin (Becton Dickinson, Franklin Lakes, NJ). For each in vitro experiment, a minimum of five pigs was used (i.e., each treatment was replicated a minimum of five times with cells from different pigs, n ≥ 5). The exact number of pigs used in each experiment is indicated in the figure legends.

For the in vivo experiment, 15 crossbred barrows (Yorkshire-Landrace × PIC 357; initially 63.4 kg BW) were stratified by ancestry and BW, and allotted at random to one of three experimental treatments (five pigs per treatment). Treatments consisted of vehicle or recombinant human leptin (Eli Lilly and Co., Indianapolis, IN) injected i.m. at a dose of 0.025 mg of leptin/kg BW twice daily (0600 and 1800; 0.05 mg of leptin/kg BW total daily dose). This injection regimen increases serum leptin concentrations by approximately 300% and decreases feed intake by 30% in growing pigs (Ajunw et al., 2003). The recombinant leptin protein was reconstituted in a sterile glycerol-based phosphate buffer (pH 7.5). The pigs in these two treatment groups were allowed ad libitum access to a corn-soybean meal diet that was formulated to meet or exceed the requirements described by the NRC (1998). A third treatment group received vehicle injections and was pair-fed the average amount of feed consumed by the leptin-injected group of pigs on a daily basis. All pigs were immunized with antigen on d 0 and 15 of the experiment. Immunization consisted of 1 mg of Limulus hemocyanin emulsified in incomplete Freund’s adjuvant (both obtained from Sigma Chemical Co., St. Louis, MO) injected i.m. The pigs were housed in an environmentally controlled facility in individual pens and had free access to water via a nipple waterer. Body weights and real-time ultrasound (Aloka 500; Aloka Inc., Tokyo, Japan) measurements of 10th-rib loin muscle and backfat depths were collected on each pig at the beginning and end of the experiment. These measurements were taken approximately 5 cm from the midline. Heparinized blood (20 mL) was collected from each pig on d 0, 7, 14, and 21, and blood (10 mL) for serum recovery was collected on d 0, 7, 14, 21, and 35.

Isolation of Peripheral Blood Mononuclear Cells

Heparinized blood was diluted (1:3) in sterile PBS, pH 7.4, and subsequently layered over Ficoll-Hypaque 1.077 (Sigma Chemical Co.). After centrifugation at 400 × g for 30 min at room temperature, the mononuclear cell band at the interface was removed and washed twice by centrifugation in basal medium at 200 × g for 10 min at room temperature. Basal medium consisted of RPMI-1640 (pH 7.4) media containing 2 g/L of sodium bicarbonate, 10 mM HEPES buffer, 100 U/mL of penicillin, and 100 μg/mL of streptomycin (Sigma Chemical Co.). Residual erythrocytes were lysed in a buffer containing 0.83% (wt/vol) ammonium chloride for 1 min before the second wash with basal medium. The peripheral blood mononuclear cells (PBMC) were then stained with trypan blue (Sigma Chemical Co.) to assess viability, enumerated with a hemocytometer, and diluted in complete medium (basal medium + 10% fetal bovine serum; Sigma Chemical Co.) to the appropriate concentration for each assay. Pooled samples of fetal bovine serum were found to contain 3.55 ng/mL of leptin via validated RIA procedures (courtesy of D. H. Keisler, University of Missouri, Columbia). The viability of the PBMC utilized for these experiments exceeded 95%, as assessed by the exclusion of trypan blue.

In Vitro Lymphocyte Proliferation Assay

One hundred microliters of PBMC, at a density of 4 × 10⁶ cells/mL in complete medium was added to each well of a 96-well tissue culture plate (Corning Glass Works, Corning, NY). To the PBMC, 100 μL of the respective treatments was added in triplicate. Treatments consisted of 1) complete medium, 2) leptin (100 nM), 3) concanavalin A (ConA; 5 μg/mL; Sigma Chemical Co.), 4) pokeweed (Phytolacca Americana) mitogen (PWM; 10 μg/mL; Sigma Chemical Co.), 5) leptin + ConA, 6) leptin + PWM, 7) dexamethasone (Dex; 300 nM; Sigma Chemical Co.), 8) Dex + ConA, and 9) Dex + ConA + leptin. Leptin at a dose of 100 nM has been shown to alter variables in porcine hepatocytes (Raman et al., 2003) and adrenal chromaffin cells (Takekoshi et al., 2001) cultured in vitro. Cultures were incubated for 42 h at 37°C and 5% CO₂. The cells were pulsed with 0.5 μCi ³H-thymidine in 50 μL of media for the final 18 h of culture. Cells were harvested onto filter paper with deionized water by an automated cell harvester (Biomedical Research and Development Laboratories, Inc., Gaithersburg, MD). The filter papers were then transferred to scintillation vials containing scintillation fluid (Cytoscinct, ICN, Costa Mesa, CA), and the vials were counted (5 min) in a liquid scintillation counter (Packard, Meridian, CT).
Whole Blood Lymphocyte Proliferation Assay for the In Vivo Leptin Injection Experiment

Wells of 96-well tissue culture plates (Nunc, Wiesbaden, Germany) were seeded with 100 μL of heparinized whole blood, and treatments in 100 μL of complete medium were added to the blood. Treatments consisted of ConA (5 μg/mL), Limulus hemocyanin (10 μg/mL), or complete medium only. The plates were subsequently incubated for 90 h at 37°C in a 5% CO₂ humidified environment, and then pulsed with 0.5 μCi ³²P-thymidine in 50 μL of media for the final 18 h of culture. Stimulation indices (Rebelatto et al., 2001) were calculated by dividing the mean counts per minute (cpm) of the triplicate wells containing ConA or Limulus hemocyanin by the mean cpm of the wells containing complete medium only.

Enzyme-Linked Immunosorbent Assay for Antigen-Specific Antibodies and Cortisol

Serum samples were analyzed for Limulus hemocyanin-specific IgG1 and IgG2 according to the method of Rebelatto et al. (2001) with some modifications. Individual wells of 96-well plates were coated with 100 μL/well of Limulus hemocyanin (10 μg/mL) suspended in carbonate buffer (pH 9.6) for 24 h at 4°C. The plates were then washed three times with PBS containing 0.05% Tween 20 (PBST, Sigma Chemical Co.) and blocked with 0.5% (wt/vol) BSA in PBST for 1 h at room temperature. Serum samples diluted 1:10,000 in PBST were added and the plates were incubated for 1 h at room temperature. After four washings with PBST, 100 μL/well of mouse anti-porcine immunoglobulin (Ig) G1 or G2 (Serotec, Kidlington, Oxford, U.K.) was added at a 1:1000 dilution and incubated for 1 h at room temperature. The plates were then washed four times with PBST, after which alkaline-phosphatase-goat anti-mouse IgG (Sigma Chemical Co.), diluted at 1:400 in PBST, was added at 100 μL/well. After four additional rinses, 100 μL/well of p-nitrophenol phosphate (1 mg/mL; Sigma Chemical Co.) was added and the plates were incubated for 1 h at room temperature. After 1 h, 100 μL/well of 2 M NaOH was added to stop the color development and the optical density (OD) at 405 nm was determined in a microtiter plate spectrophotometer (Bio-Tek Instruments, Inc., Winooski, VT). The average OD of triplicate wells was calculated and an index was obtained by dividing the average OD of a sample postimmunization by the OD of the sample from the same pig on d 0 of the experiment. Serum cortisol was determined using a commercially available kit (Active Cortisol EIA, Diagnostic Systems Laboratories, Inc., Webster, TX). The samples were run in duplicate in a single assay using the manufacturer’s protocol. The assay was validated for pig serum by spiking a pooled serum sample with known quantities of standard, and by serial dilution of the pooled sample. Based on two assays, the intra- and interassay CV were less than 12%.

In Vitro Cytokine Secretion and mRNA Expression by Porcine PBMC

To test the effects of recombinant leptin on ConA-induced interferon-γ (IFN-γ) and interleukin-4 (IL-4) secretion and cytokine mRNA relative abundance, PBMC were cultured at a concentration of 4 × 10⁶ cells/mL in 60-mm tissue culture dishes (BD Falcon, Franklin Lakes, NJ) in a total volume of 5 mL of complete medium. Treatments consisted of basal complete medium, leptin (100 nM), ConA (5 μg/mL), and leptin + ConA. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂. Supernatants were collected at 24 h poststimulation for the analysis of cytokine release into the media, and cells were harvested at 12 h after stimulation for cytokine mRNA analysis. Supernatants were stored at −80°C until analysis, and the cell pellets were resuspended in Trizol (Invitrogen, Inc., Carlsbad, CA) and stored at −80°C pending RNA extraction. These times were chosen to correspond to the time of maximal IFN-γ and IL-4 protein and mRNA production by porcine PBMC, as found by other investigators (Dozois et al., 1997; Verfaillie et al., 2001).

Culture supernatants were analyzed for IFN-γ and IL-4 via commercially available ELISA kits, which are porcine-specific. The IFN-γ ELISA (Endogen, Inc., Woburn, MA) has a range of detection from 2 to 1,000 pg/mL. The IL-4 ELISA (Biosource Int., Inc., Camarillo, CA) has a range of detection from 2 to 1,000 pg/mL. The ELISA were conducted according to the manufacturers’ recommendations.

RNA Extraction and Purification

The PBMC pellets were thawed, and total RNA was isolated using the Trizol reagent (Invitrogen, Inc., Carlsbad, CA) per the manufacturer’s recommended protocol, and then treated with DNase I to remove residual genomic DNA. Total RNA was quantified by spectrophotometry (Beckman, Fullerton, CA) at 260 nm, and purity was assessed by determining the ratio of the absorbance at 260 and 280 nm. All samples had 260-/280-nm absorbance ratios above 1.8, indicating a relatively pure nucleic acid preparation. Additionally, the integrity of the RNA preparations was verified by visualization of the 18S and 28S ribosomal bands stained with ethidium bromide after electrophoresis on 1.0% agarose gels.

Multiprobe Ribonuclease Protection Assay

A commercially available porcine-specific DNA template set (pCK-1, BD Biosciences) was used to make [α-³²P]-labeled anti-sense probes that could hybridize with mRNA encoding IL-4, IL-10, IL-15, IL-2, IL-6, and IFN-γ, and the housekeeping genes, L32 and GAPDH. The DNA template set was transcribed in vitro with a T7 RNA polymerase. The reaction was performed with 50 μCi [α-³²P] uridine triphosphate (800 Ci/mmol) using a commercially available in vitro transcription kit.
(Maxiscript T7, Ambion, Inc., Austin, TX). The ribonuclease protection assay was conducted using a commercially available kit according to the manufacturer’s instructions (Ribonuclease Protection Assay III, Ambion, Inc.). Autoradiographs were quantified using digital imaging software (Kodak Digital Science Imaging System V.2.0.1, Kodak, Inc., New Haven, CT).

**Preparation of Nuclear Extracts and Gel Mobility Shift Assay for STAT3 DNA Binding**

Nuclear extracts were prepared from PBMC to determine the effects of culture with leptin (625 nM) for 15 min and serum starvation for 24 h on STAT3 DNA binding activity. The PBMC were cultured at a density of 5 x 10^6 cells/mL in 60-mm culture dishes (Falcon, Lincoln Park, NJ) in a total volume of 5 mL. The treated PBMC were harvested by centrifugation at 1,850 x g for 10 min, and the cells were washed two times in PBS for 10 min at 1,850 x g. The pellet was then resuspended in a hypotonic lysis buffer (10 mM KCl, 10 mM HEpes [pH 7.9], 1 mM dithiothreitol [DTT], 0.1 mM EDTA, 0.1 mM ethylene glycol bis(β-aminoethyl ether) N, N', N'-tetraacetic acid [EGTA], 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/mL of leupeptin, and 2 μg/mL of aprotinin; Sigma Chemical Co.). The cells were allowed to swell on ice for 15 min with intermittent vortexing, after which 25 μL of 1% NP40 (Sigma Chemical Co.) was added. The tubes were vortexed vigorously, and the homogenate was centrifuged at 3,300 x g for 15 min at 4°C. The supernatant was aspirated, and the resulting pellet was resuspended in a cold hypertonic buffer (20 mM HEpes [pH 7.9], 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 20% glycerol, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 2 μg/mL of leupeptin, and 2 μg/mL of aprotinin; Sigma Chemical Co.). The tubes were kept on ice for 30 min with intermittent vortexing and then centrifuged for 30 min at 20,000 x g. An aliquot of the resulting supernatant was used to determine protein concentration, and the remainder was stored at −80°C until it was needed for the gel mobility shift assay. Protein concentrations were determined using the bicinchoninic acid assay (Smith et al., 1985), which was purchased as a kit (Pierce, Rockford, IL).

The double-stranded STAT3 consensus oligonucleotide (5'-GAT CCT TCT GGG AAT TCC TAG ATC-3'; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was end-labeled with [γ-32P]-ATP using polynucleotide kinase. Five micrograms of nuclear extract protein from the PBMC was incubated with 50,000 cpm of the labeled probe and 2 μg of poly (dI-C) in binding buffer (50 mM KCl, 20 mM HEpes, 20% glycerol, 0.05% NP-40, and 10 mM β-mercaptoethanol [pH 7.5]) in a total volume of 25 μL. The binding reaction mixture was incubated at room temperature for 30 min, and the samples were analyzed on a 5% acrylamide gel in 1×TBE (44.5 mM Tris, 44.5 mM boric acid, and 1 mM EDTA [pH 8]) for 75 min at 300 V. The gels were then dried and subjected to autoradiography. Specificity of extract binding to the probe was confirmed in preliminary experiments in which nonradiolabeled probe was shown to displace the signal generated with the radiolabeled probe.

**Reverse Transcriptase-PCR for the Leptin Receptor in Porcine PBMC**

The effect of stimulation with ConA on leptin receptor mRNA abundance was tested by culturing the PBMC with either basal complete medium or ConA (5 μg/mL) for 24 h. The PBMC were cultured and the total RNA was isolated and purified as previously described. Two micrograms of total RNA was reverse-transcribed into cDNA using a commercially available kit (Superscript, Invitrogen; Carlsbad, CA), and 2 μL of the cDNA reaction was subjected to PCR following a standard protocol (Sambrook et al., 2001). The up- and downstream primers for PCR amplification were 5'-TCGGAAGATAT-CAGTGTT-3' and 5'-TTGGATGTCATCTGATGAA-3' for the leptin receptor (Ob-R1; 382-bp product; 36 cycles; Gen-Bank accession AF036908); and 5'-GACTTC-GAGCAGAGATGG-3' and 5'-GCACGTTGAGCGTAGGG-3' for β-actin (233-bp product; 28 cycles; Gen-Bank accession U07786). The cycle number used with each primer set was designed to amplify the product within the linear range of amplification as determined by PCR on cDNA dilutions. The temperature cycling consisted of 1 cycle of PCR for 7 min at 94°C, 45 s at 54°C, and 45 s at 72°C. The remaining cycles consisted of 45 s at 94°C, 45 s at 54°C, and 45 s at 72°C. The PCR products were incubated at 72°C for 10 min upon completion of the last cycle. An aliquot of each PCR reaction sample was separated in 2% TAE (an electrophoresis buffer)-agarose gels, stained with ethidium bromide, and photographed. The density of the bands was quantified using the Kodak digital imaging software noted above. To compare the relative mRNA abundance the values are presented as a ratio of the band intensities of the reverse transcriptase (RT)-PCR product to the corresponding β-actin RT-PCR product. Aliquots of the PCR products were sequenced (Lark Technologies, Houston, TX) to confirm the specificity of the oligonucleotide primers.

**Statistical Analyses**

All data were analyzed by ANOVA using the GLM procedure of the SAS (SAS Inst., Inc., Cary, NC). For the in vivo experiment, the serum antibody, cortisol, and lymphocyte proliferation data were analyzed as a repeated measures experiment. The lymphocyte proliferation data and real-time ultrasound data from the in vivo leptin injection study were analyzed using the d-0 data as a covariate. When ANOVA indicated a significant (P < 0.05) difference, means were separated using the Student-Newman-Keuls multiple-range test, where applicable, based on covariate usage. Otherwise, if co-
Table 1. Feed intake and growth characteristics of pigs injected with recombinant leptin for 35 d

<table>
<thead>
<tr>
<th>Item</th>
<th>Ad libitum</th>
<th>Leptin</th>
<th>Pair-fed</th>
<th>SEM</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial BW, kg</td>
<td>62.91</td>
<td>64.00</td>
<td>63.27</td>
<td>0.9</td>
<td>0.70</td>
</tr>
<tr>
<td>Final BW, kg</td>
<td>96.18(x)</td>
<td>87.45(y)</td>
<td>89.55(y)</td>
<td>2.1</td>
<td>0.03</td>
</tr>
<tr>
<td>ADG, kg</td>
<td>0.95(x)</td>
<td>0.67(y)</td>
<td>0.75(y)</td>
<td>0.06</td>
<td>0.03</td>
</tr>
<tr>
<td>ADFI, kg(^b)</td>
<td>3.00(x)</td>
<td>2.38(y)</td>
<td>2.41(y)</td>
<td>0.13</td>
<td>0.007</td>
</tr>
<tr>
<td>Gain:feed</td>
<td>0.32</td>
<td>0.28</td>
<td>0.31</td>
<td>0.02</td>
<td>0.58</td>
</tr>
<tr>
<td>Loin depth, cm(^c)</td>
<td>5.58(x)</td>
<td>5.25(y)</td>
<td>4.99(y)</td>
<td>0.17</td>
<td>0.08</td>
</tr>
<tr>
<td>Backfat, mm</td>
<td>13.90(x)</td>
<td>9.46(y)</td>
<td>9.00(y)</td>
<td>1.2</td>
<td>0.02</td>
</tr>
</tbody>
</table>

\(^a\)Pigs (n = five pigs per treatment) were allowed ad libitum feed intake and injected with vehicle, allowed ad libitum feed intake and injected with recombinant human leptin, or pair-fed to the average feed intake level of leptin-injected pigs and injected with vehicle.

\(^b\)As-fed basis.

\(^c\)Depth of the loin muscle at the 10th rib approximately 5 cm from the midline.

\(^x,y\)Means with different superscripts within each row differ, \(P < 0.05\).

Variates were used, the means were separated using the PDiff option in SAS.

Results

Growth and Feed Intake by Pigs Injected with Leptin

Exogenous leptin decreased \((P < 0.05)\) feed intake by 26% over the entire course of the experiment compared with vehicle-injected pigs fed ad libitum (Table 1). Pigs injected with leptin and pair-fed pigs had decreased BW gains and lower final BW \((P < 0.05)\) compared with the ad libitum group. Both leptin injection and limit feeding decreased \((P < 0.05)\) the 10th-rib backfat thickness measured at the completion of the experiment. Only the limit-fed pigs had decreased \((P < 0.05)\) loin depths at the completion of the experiment, as compared with the ad libitum control group.

Lymphocyte Proliferation

Leptin, in vitro, had no effect on lymphocyte proliferation in response to ConA or PWM (Figure 1), nor did it alleviate the suppression of ConA-induced proliferation by Dex, as measured via \(^3\)H-thymidine incorporation (Figure 2). Likewise, exogenous leptin had no effect on the whole-blood lymphocyte proliferative response to ConA at any time point measured (Figure 3). As expected, there was no notable proliferative response to Limulus hemocyanin on d 0, but by d 7, pigs in all treatments showed a proliferative response to the antigen that was maintained throughout the course of the experiment (Figure 4). However, leptin administration did not alter the proliferative response to the antigen at any time point.

Serum Antigen-Specific IgG1, IgG2, and Cortisol

Pigs in each treatment group developed antigen-specific IgG1 by d 14 of the experiment (Figure 5A). However, at d 21, pigs receiving exogenous leptin had lower \((P < 0.05)\) serum concentrations of antigen-specific IgG1 than did control or limit-fed pigs. For antigen-specific IgG2, there was an increase \((P < 0.05)\) over time in all treatment groups, but there was no effect of exogenous leptin at any time point measured (Figure 5B). There was no significant effect of time or treatment on serum cortisol concentrations (Figure 6).

In Vitro Regulation of Cytokine mRNA Abundance and Protein Secretion

As expected, exposing PBMC to ConA increased \((P < 0.05; 88.93 \pm 4.3 \text{ pg/mL})\) for ConA vs. \(6.21 \pm 4.3 \text{ pg/mL}\) for basal) the secretion of IFN-\(\gamma\) into the media. However, leptin had no effect on IFN-\(\gamma\) secretion in the basal state \((6.58 \pm 4.3 \text{ pg/mL} \text{ for leptin alone})\) or in those stimulated with ConA \((93.14 \pm 4.3 \text{ pg/mL} \text{ for leptin})\).

![Figure 1](image-url). Effect of recombinant leptin on porcine peripheral blood mononuclear cells (PBMC) proliferation in vitro. The PBMC were incubated in the presence of the specific treatments for a total of 42 h and \(^{3}\)H-thymidine was added for the final 18 h of culture. Treatments included basal medium (B), leptin at 100 nM (L), concanavalin A (ConA) at 5 \(\mu\)g/mL (C), the combination of leptin and ConA (C + L), pokeweed mitogen at 10 \(\mu\)g/mL (P), and the combination of leptin and pokeweed mitogen (P + L). n = 9. Treatment means that do not have a common letter are differ \((P < 0.05)\).
Leptin and the immune system of the pig

Figure 2. Effect of dexamethasone and leptin on mitogen-induced porcine peripheral blood mononuclear cell (PBMC) proliferation, in vitro. The PBMC were incubated in the presence of the specific treatments for a total of 42 h and [3H]-thymidine was added for the final 18 h of culture. Treatments included basal medium (B), concanavalin A (ConA) at 5 μg/mL (C), dexamethasone at 300 nM and ConA (D), and the combination leptin at 100 nM, dexamethasone, and ConA. n = 7 pigs. Treatments that do not have common letters differ (P < 0.05).

+ ConA). There was no detectable IL-4 in the media harvested from PBMC treated in vitro. However, ConA increased (P < 0.05) the relative abundance of the mRNA for IL-10, IL-2, and IFN-γ (Figure 7). Leptin (alone or in combination with ConA) had no effect on the relative mRNA abundance for these cytokines.

Detection of Ob-Rl mRNA and STAT3 Activation

Porcine PBMC express the mRNA for Ob-Rl (Figure 8), and stimulation with ConA for 24 h decreased (P < 0.05) the relative abundance of this transcript. The STAT3 DNA binding activity of nuclear extracts prepared from porcine PBMC was clearly evident in the basal state (Figure 9), and was not altered by leptin. Furthermore, serum starvation for 24 h did not affect STAT3 DNA binding activity, nor did leptin induce the activation of STAT3 in serum-deprived PBMC (Figure 10).

Discussion

As in previous studies with pigs (Ajuwon et al., 2003; Barb et al., 1998), exogenous leptin decreased feed intake in the present experiment, and thus confirmed the activity of the human leptin analog used in this experiment. Leptin injection led to a sustained decrease (26%) in feed intake for the 35 d experiment. The reduced feed intake was also reflected in a corresponding reduction in back fat depth in the leptin and limit-fed groups. However, only the limit-fed pigs tended to have decreased loin depths at the end of the experiment vs. control pigs. This is perhaps indicative of an improvement in nitrogen retention attributable to leptin, despite the lower feed intake, and is consistent with the earlier finding that exogenous leptin decreases serum urea nitrogen vs. pair-fed pigs (Ajuwon et al., 2003). Furthermore, the decrease in feed intake and backfat indicates that the pigs used in the current study were physiologically responsive to the recombinant leptin at the dosage used (0.05 mg/[kg·d]).

In the in vitro experiments, leptin did not influence lymphocyte proliferation nor did it enhance the proliferative response to polyclonal mitogens. Likewise, in vivo administration of leptin did not change the magnitude of the proliferative response to Limulus hemocyanin.
Figure 5. Effect of exogenous leptin on the index of Limulus hemocyanin-specific IgG1 and IgG2. Pigs (n = 15; five pigs per treatment) were allowed ad libitum feed intake and injected with vehicle (ad-lib), allowed ad libitum feed intake and injected with recombinant human leptin (leptin; 0.05 mg/[kg·d]), or pair-fed to the feed intake of leptin injected pigs and injected with vehicle (pair-fed). All pigs received 1 mg of Limulus hemocyanin i.m. on d 0 and 15 of the experiment. A) Serum Limulus hemocyanin-specific IgG1. Significance: \( P = 0.11 \) for the effect of leptin; \( P < 0.001 \) for day; and \( P < 0.09 \) for the interaction. Means within day with different letters differ \((P < 0.05)\). B) Serum Limulus hemocyanin-specific IgG2. Significance: \( P = 0.50 \) for the effect of leptin; \( P < 0.002 \) for day; and \( P = 0.27 \) for the interaction.

These data are in contrast with those obtained in mouse and human models. In mice, leptin in a dose-dependent manner increased the allogenerative response of CD4+ T-cells in a mixed lymphocyte reaction (Lord et al., 1998). In humans, leptin stimulated proliferation of human PBMC, and augmented the proliferative response in mixed lymphocyte reactions (Lord et al., 1998; Zarkesh-Esfahani et al., 2001). However, the mitogenic stimuli used in our in vitro experiments were different from those used by other investigators (ConA and PWM vs. MHC-mismatched lymphocytes), and it is possible that this difference explains the disparate results, at least in part.

Concanavalin A increased IFN-\( \gamma \) mRNA abundance and secretion, and increased the relative abundance of IL-2 and IL-10 mRNA; however, leptin did not influence cytokine mRNA abundance or secretion in the stimulated cells or those in the basal state. These findings are also contrary with what has been reported for mice and humans in that recombinant leptin profoundly increased the secretion of IFN-\( \gamma \) in PBMC and purified T-cells subjected to a mixed lymphocyte reaction (Lord et al., 1998; 2002). Leptin also decreased IL-4 secretion by mouse T-cells and human PBMC, indicative of a Th1 response (Lord et al., 1998). However, we did not detect IL-4 in the media harvested from stimulated porcine PBMC, nor were we able to detect the mRNA using a ribonuclease protection assay. Other investigators have detected IL-4 expression in porcine PBMC using the more sensitive RT-PCR procedure and have determined that IL-4 mRNA abundance is increased in response to ConA (Dozois et al., 1997; Verfaille et al., 2001), albeit there are currently no reports of IL-4 secretion into the media collected from porcine PBMC stimulated with this mitogen. Although it is possible that we missed the peak production of IL-4 mRNA and protein, other investigators have found that IL-4 mRNA abundance peaks at 12 h post-stimulation, and is sustained for 72 h (Dozois et al., 1997). Thus, it seems more likely that IL-4 production is not significantly increased in response to ConA in the pig as it is in humans (Gonzalez et al., 1994). Collectively, these data indicate that leptin exerts little influence on ConA-induced lymphocyte proliferation in the pig and it does not skew cytokine production to that of a Th1 response.
In the pig, the IgG1:IgG2 has been positively correlated with hemolysin neutralization ability (r = 0.60) and negatively correlated (r = −0.67) with lung lesion scores in animals challenged with *Actinobacillus pleuropneumoniae* (Furesz et al., 1998). In the series of experiments reported herein, leptin decreased the abundance of serum *Limulus* hemocyanin-specific IgG1, but had no effect on antigen-specific IgG2. This is the first report of a direct modulation of antibody isotypes by exogenous leptin in any species. In mice, exogenous leptin given i.v. did not alter the abundance of serum IgE, IgG1, or IgG2a specific for ovalbumin (Hetland et al., 2001). These mice were only injected with leptin on d 0, 14, and 17 of the experiment, which may have been insufficient to modify antibody isotypes. Consistent with our results, the leptin-deficient (ob/ob)
ob) mouse produces less of the various antigen-specific antibodies (including IgG1) as compared with wild-type mice (Busso et al., 2002). However, it is also possible that the decreased antibody production in ob/ob mice simply reflects T-cell lymphopenia (Howard et al., 1999; Farooqi et al., 2002) because several cytokines produced by T-cells are required for efficient antibody isotype switching (Janeway et al., 2001). Recent data have shown that T\textsubscript{h}2 cytokines may increase the IgG1:IgG2 in the pig (Crawley et al., 2002), suggesting that Ig isotype production in the pig may indeed follow the T\textsubscript{h}1/T\textsubscript{h}2 paradigm observed in rodents (Snapper and Paul, 1987). Therefore, our finding that leptin decreases antigen-specific IgG1 may indicate that artificially increasing leptin concentrations does skew the antibody response to that of a T\textsubscript{h}1.

Increased circulating corticosterone is a hallmark of leptin deficiency in the rodent and can be reversed by leptin replacement (Howard et al., 1999). This increase in glucocorticoids may be partially responsible for the immunological deficits noted in ob/ob mice. Leptin protects PBMC (and more specifically, T-cells) against apoptosis induced by dexamethasone and serum starvation (Howard et al., 1999; Fujita et al., 2002; Sanchez-Margalet et al., 2002). Although we did not measure cell death due to apoptosis or necrosis in the present study, leptin did not alter serum cortisol in vivo, nor did it not protect porcine PBMC from the inhibitory effect of Dex on ConA-induced proliferation. Consequently, we confirmed the presence of the leptin receptor mRNA in porcine PBMC, as has been reported for mouse and human PBMC (Lord et al., 1998; Tsiotra et al., 2000). The presence of the receptor RNA was clearly indicated, and stimulation of pig cells with ConA for 24 h decreased the relative abundance of Ob-RI mRNA. This is also contrary with previous data obtained in human PBMC (Sanchez-Margalet et al., 2002), and is perhaps indicative of a mechanism in the pig by which stimulated lymphocytes become refractory to leptin. This may partially explain the lack of response to leptin by porcine PBMC, in vitro. Given the length of time required to achieve the decrease in Ob-RI mRNA abundance, it is possible that the down regulation of Ob-RI in response to ConA is a secondary effect of stimulation.
that is driven by the cytokine milieu produced by stimulated cells.

Leptin signaling in immunocytes is controversial. In purified human T-cells, leptin treatment led to the activation of STAT3, but not mitogen-activated protein kinase (MAPK) (Maccarrone et al., 2003), whereas in PBMC leptin activates both the MAPK and STAT3 pathways (van den Brink et al., 2000). We found that activation of STAT3 is constitutive in porcine PBMC. In contrast with human PBMC and T-cells (Sanchez-Margalet et al., 2001; Maccarrone et al., 2003), leptin did not increase STAT3 activation in pig cells, even at supraphysiological concentrations, nor did serum deprivation for 24 h reduce STAT3 binding or render porcine PBMC sensitive to STAT3 activation by leptin. Collectively, our findings indicate that any effect of leptin on pig PBMC is likely independent of an induction of STAT3 binding.

**Implications**

We presented evidence in the pig that exogenous leptin modifies antibody isotypes to a profile indicative of a cellular immune response. However, leptin does not influence several other aspects of the immune system in the pig, but it does in rodent models and humans. Collectively, these data imply that leptin does not play as substantial a role in the regulation of the adaptive immune response of the pig as in these other species, and that any effects of leptin are independent of signaling pathways activated in lymphocytes of other mammalian species. Thus, the relevance of leptin to commercial pork production may pertain largely to the role of this cytokine in the regulation of metabolism and feed intake, rather than as a means of influencing immune response pathways.

**Literature Cited**


