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BARD Final Scientific Report
(Cover Page)

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Evaluating Panel: Animal Production

הספריה המרכזית
למדעי החקלאות
בית דגן

Project Title: Regulation and Action of Leptin in Pregnant and Lactating Dairy Cows.

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Agricultural Research Organization

Continuation of (Related to) Previous BARD Project:

☐ Yes ☒ No Number:

Keywords *not* appearing in the title and in order of importance. Avoid abbreviations. Radioimmunoassay, bovine, recombinant protein, body composition.

Abbreviations used in the report, in alphabetical order: rbLeptin = recombinant bovine leptin;
RIA = radioimmunoassay; WAT = white adipose tissue.

Budget: IS: \$ 10,000

US: \$ 55,000

Total: \$ 65,000

Signature
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Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted)		1		1
Submitted, in review, in preparation				
Invited review papers				
Book chapters				
Books				
Master theses				
Ph.D. theses				
Abstracts	1			1
Not refereed (proceedings, reports, etc.)				

Patent Summary (numbers)

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
Submitted		0		0
Issued (allowed)				0
Licensed				0

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Visits/Meetings				0
Sabbaticals				0
Postdoctorates				0

- **Cooperation**, briefly explain whether synergistic, complementary or supportive.

Cooperation was supportive. Efforts at Cornell were entirely devoted to development of the bovine RIA. Efforts in Israel were devoted to exploratory work to support a future grant application to BARD.

Abstract

The original project had four specific objectives: (1) To complete the development of a radioimmunoassay for bovine leptin; (2) To characterize the leptin system in lactating dairy cows during the transition from pregnancy to lactation; (3) To identify endocrine factors regulating the production of leptin by bovine adipose tissue; (4) To study the actions of leptin on bovine adipose and mammary tissues in vitro. However, BARD funded only the development of the bovine leptin RIA (Objective 1) for a single year. This report describes our work in completing this objective.

Leptin, a protein hormone secreted predominantly by white adipose tissue, plays a critical role in the regulation of energy metabolism. In rodents and humans, leptin informs the central nervous system of the size of the energy reserves, coordinates adaptations to periods of nutrient insufficiency, and regulates the metabolism of key tissues involved in the storage and dissipation of energy. However, almost nothing is known on the biology of leptin in cattle, in part because of the absence of a valid assay to measure bovine leptin. To remediate this situation, we have developed a radioimmunoassay capable of measuring bovine leptin with a high degree of sensitivity, accuracy and precision.

First, we produced recombinant bovine leptin and used it to immunize rabbits, and to prepare bovine leptin trace and standards. A single antiserum with sufficient affinity and titer was identified. Using this antiserum, binding of ^{125}I bovine leptin was displaced in a dose dependent manner by the addition of bovine or ovine leptin. Serial dilution of bovine and ovine plasma gave displacement curves that were parallel to that of bovine or ovine leptin. Recoveries of external addition of bovine leptin in ewe and cow plasma ranged between 94 and 104%. Plasma leptin concentration measured by this assay was increased by the plane of nutrition in growing calves and lambs. Finally, plasma leptin concentration was linearly related to the fat content of the empty carcass in growing cattle. We conclude that circulating leptin in sheep and cattle is increased by fatness and plane of nutrition, consistent with results in humans and rodents. This assay provides an important tool to investigate mechanisms that regulate plasma leptin in cattle and sheep.

Achievement

Research on the biology of leptin in domestic animals has been hampered seriously by the lack of homologous leptin radioimmunoassays (RIAs). Prior to our work, estimates of plasma leptin had been reported in pigs, sheep, and cattle using a commercial assay originally designed to measure human leptin (Multi-Species leptin RIA by Linco Research, Inc). In preliminary work, we found that this RIA provided estimates of plasma leptin in sheep and cattle that were low and unresponsive to changes in nutrition or adiposity.

In this context, our successful development of a RIA that works equally well with bovine and ovine samples is significant. This will allow rapid progress in understanding how factors such as nutrition, hormones, and physiological states regulate circulating leptin in domestic ruminants. Areas of obvious interest include changes in circulating leptin in growing animals as they approached puberty, and as they transit from pregnancy to lactation. An added benefit of this research is that we have developed expertise in producing bioactive recombinant bovine leptin. This recombinant material will be useful in probing the biological actions of leptin in ruminants using in vivo and in vitro approaches.

The benefits of this research are long term. They will come from the use of this RIA to understand the role of leptin in coordinating complex, energy-dependent processes such as the onset of puberty in growing animals, and the transition from pregnancy to lactation in mature animals. While the role of leptin in regulating these processes remains to be characterized, we believe that a better understanding of leptin biology will allow the design of management practices and therapies to optimize fertility and the periparturient period in ruminants.

As described in the original proposal submitted to BARD, development of this RIA was done entirely by the US team. In addition, the original proposal

had objectives that required the specific expertise of the Israeli team. However, these objectives were not funded by BARD. Nevertheless, the Cornell and Israeli team jointly decided to allocate some of the funds to support exploratory work in Israel devoted to the acquisition of data for a future grant application on leptin. This work has established that antral follicles of the bovine ovary, but not corpora lutea or early developing follicles, express the leptin receptor. Therefore, the work of the US and Israeli teams can be described as supportive.

Report

Background

Leptin is a protein secreted predominantly by white adipose tissue (WAT) (Flier 1997, Houseknecht & Portocarrero 1998). Leptin acts on the central nervous system and perhaps on peripheral tissues to regulate food intake, the storage and dissipation of energy, and the coordination of metabolism during periods of suboptimal nutrition (Flier 1997, Houseknecht & Portocarrero 1998). Leptin also plays important roles in regulating processes critically dependent on energy supply such as reproductive and immune functions (Cunningham *et al.* 1999).

Thus far, actions and regulation of leptin have been studied most extensively in rodents and humans, and very little information exists in domestic animals (Flier 1997, Houseknecht & Portocarrero 1998). Research on the biology of leptin in domestic animals has been hampered by the lack of homologous leptin radioimmunoassays (RIAs). Estimates of plasma leptin have been reported in pigs (Qian *et al.* 1999), sheep (Bocquier *et al.* 1998) and cattle (Chilliard *et al.* 1998) using a commercial assay originally designed to measure human leptin. In preliminary work, we found that this commercial RIA provided estimates of plasma leptin in sheep and cattle that were low and unresponsive to changes in nutrition or adiposity. Therefore the major aim of the present work was to develop a specific RIA for bovine leptin and to provide evidence for regulation of circulating leptin by nutrition and fatness in cattle and sheep.

Leptin has been postulated to regulate the onset of puberty and fertility (Cunningham *et al.* 1999). Therefore, a secondary exploratory objective was to document the presence of the leptin receptor in the bovine ovary.

Methodologies and materials

A partial cDNA encoding bovine leptin was amplified by reverse transcription-PCR from WAT and subcloned into the expression plasmid pQE31 (Qiagen, Inc) downstream of a histidine purification tag. This plasmid was transformed into *E. coli*, and expression induced by isopropyl- β -D-thiogalactopyranoside. After lysis of the bacteria, recombinant bovine leptin (rbLeptin) was purified by affinity chromatography refolded exactly as described by Fawzi *et al.* (1996). rbLeptin was repeatedly injected into rabbits to obtain antisera against bovine leptin.

For use in the RIA, rbLeptin was iodinated with ^{125}I iodine using Iodo-gen. The iodinated rbLeptin was purified by Sephadex G-50 chromatography before use in a disequilibrium, double antibody RIA. Briefly, unknown samples or rbLeptin standards were incubated at 4°C with the primary antisera. After 16 h of incubation, ^{125}I -labeled rbLeptin was added. Bound rbLeptin was precipitated by the addition of secondary antibody and polyethylene glycol and the radioactivity present in the precipitated material was measured by γ -counting.

Leptin receptor mRNA expression in the bovine ovary was determined by RT-PCR using the house keeping gene GAPDH as an internal standard.

Results

In the presence of isopropyl- β -D-thiogalactopyranoside, *E. coli* cells transformed with the leptin expression vector overexpressed a protein having an apparent molecular mass of 16 kDa. After purification, this material was > 95% pure and bioactive when injected in mice devoid of leptin (i.e., ob/ob mouse).

After 4 rounds of immunization with rbLeptin, an antisera of acceptable characteristics for use in a RIA was obtained in a single rabbit. At a final dilution of 1:1800, this antisera bound 35-40% of ^{125}I -rbLeptin in the absence of unlabelled

rbLeptin. Addition of increasing amounts of unlabelled rbLeptin or recombinant ovine leptin inhibited the binding of ^{125}I -rbLeptin in a dose-dependent manner between 0.1 and 2.0 ng/tube (corresponding to concentrations of 1 to 20 ng/mL).

Serial dilution of bovine plasma gave displacement curves that were parallel to that of rbLeptin. Recoveries of external addition of rbLeptin to pregnant or non pregnant cow plasma ranged between 94 and 104%. Recovery of rbLeptin was also evaluated in plasma pools obtained from non-pregnant ewes. Again, quantitative recoveries, ranging from 96% to 98% were obtained. Method of sample preparation (i.e., serum vs plasma) did not affect the estimation of circulating leptin in cattle and sheep.

Under routine use, the sensitivity of this assay, defined as the lowest mass distinguishable from the zero standard, was 50 pg or 0.5 ng/mL. Half-maximal displacement of tracer binding to the antiserum occurred at 0.61 ng or 6.1 ng/mL. Finally, intra- and inter- assay coefficients of variation in bovine plasma were less than 4% and 10%, respectively and less than 4% and 8% in ovine plasma, respectively.

Data obtained with this assay indicate that plasma leptin concentrations are increased by the plane of nutrition and fatness in growing calves and sheep. Holstein bull calves were fed a specially formulated milk replacer to grow at target rates of 500 or 1400 g/d from day 3 of postnatal life until they reached 105 kg of body weight. Plasma leptin concentration was elevated by 46% in the fast-growing calves compared to the slower-growing calves (6.7 vs. 4.6 ng/mL, $P < 0.01$). In these animals, plasma leptin was linearly related to the fat content of the empty carcass ($r = 0.91$, $P < 0.001$). In contrast, when plasma from these same growing calves was analyzed by the Multi-Species Leptin assay (from Linco Research, Inc), estimates were reduced by 50%, and effects of nutrition and fatness were not detected. Finally, effects of nutrition were also studied in weaned 11-14 week old Finn x Dorset lambs offered similar amounts of a moderate quality diet or a high quality diet. Lambs receiving the higher quality

diet grew faster and had plasma leptin concentrations that were elevated by 51% ($P < 0.001$).

Finally, we explored whether bovine ovary contains leptin receptor. Ovaries were collected at a local slaughterhouse, follicles were isolated and divided into three groups small ($< 5\text{mm}$ $n=14$) medium ($5-9\text{mm}$ $n=10$) and large follicles ($> 9\text{mm}$ $n=8$). In addition corpora lutea, estimated to be from early luteal phase and ovarian cortex devoid of visible follicles were collected. The results showed that leptin receptors mRNA was present in follicles of all sizes, but not in corpora lutea and ovarian cortex containing only follicles at early stages of development.

Discussion

The primary objective of this work was to develop an RIA for measuring leptin in bovine plasma. This required production of rbLeptin as a source of antigen and for the preparation of radioiodinated trace and standards. Using one of many antisera that we produced, an RIA with sufficient sensitivity to detect concentrations of circulating leptin in plasma was established. Serial dilution of bovine plasma and rbLeptin produced parallel displacement curves indicating that the antiserum recognized recombinant and plasma leptin in an identical manner. Moreover, additions of rbLeptin were quantitatively recovered in plasma. Both of these validations were successful with plasma originating from pregnant and non-pregnant dairy cows. This assay was also valid in the sheep as recombinant ovine leptin and ovine plasma yielded displacement curves that were essentially identical to those of bovine materials.

Using this RIA, we showed that plasma leptin concentration was elevated in growing calves and lambs on a high plane of nutrition. Moreover, in growing calves, the concentration of leptin in plasma was found to be positively

correlated with body fatness. Because plasma leptin was measured after an extended period of treatment in these growing animals, the changes in plasma leptin concentration were probably influenced by both level of nutrition and body composition. Studies in which plasma leptin is measured before body composition is significantly altered are needed to resolve this issue.

Leptin concentrations measured by the Multi-Species assay in adult sheep and growing cattle plasma were considerably lower than those obtained by the bovine RIA. This discrepancy may be explained by the limited ability of the antisera used in the Multi-Species assay to bind rbLeptin. Moreover, when plasma from growing cattle were analyzed for leptin concentration by the Multi-Species assay, effects of nutrition were not detected, and circulating leptin was not related to body fat (results not shown).

Scientific implication

In summary, these results demonstrate that we have developed a sensitive RIA for the measurement of leptin in both cattle and sheep. This assay measures plasma leptin concentration as low as 0.5 ng/mL and is able to detect differences in plasma leptin caused by nutrition and adiposity. This RIA provides an important tool to study factors and mechanisms regulating leptin in sheep and cattle. The identification of leptin receptor in the bovine ovary suggests that investigating the actions of leptin in follicles is warranted.

Impact on agriculture

Regulation of energy use is critical to animal productivity. Leptin is likely to be a central player in the regulation of energy metabolism. We will use this RIA to understand the role of leptin in complex, energy-dependent, processes such as the onset of puberty and the transition from pregnancy to lactation. While the role of leptin in regulating puberty and transition from

pregnancy to lactation remains unknown, we believe that a better understanding of leptin biology will allow the design of management practices and therapies to optimize these processes, and to maximize productivity of the US and Israeli dairy industry.

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Publication

- Ehrhardt, R.A., R. Slepatis, M.E. Van Amburgh, J. Siegal-Willot, A.W. Bell and Y.R. Boisclair. Development of a specific RIA to measure physiological changes of circulating leptin in cattle and sheep. *J. Endocrinology* (In press).
- Boisclair, Y.R., R.A. Ehrhardt, S.S. Block, R.M. Slepatis, M.E. Van Amburgh and A.W. Bell. Regulation of leptin mRNA and plasma leptin during fetal life and during the transition from pregnancy to lactation in ruminants. 2000 meeting of the European Association of Animal Production, The Hague, The Netherlands. (Abstract, In press).

Shamay, A., J.D. Feurmann, Y.R. Boisclair, and R. Braw-Tal. 1999. Expression of the leptin receptor in the bovine ovary. 1999 meeting of the Society for the Study of Reproduction, Aberystwyth, United Kingdom. J. Reprod. Fert. 23 (Abstract series):28.

Patents

None.