Regulation of hepatic glucose metabolism by leptin in pig and rat primary hepatocyte cultures

Priya Raman, Shawn S. Donkin, and Michael E. Spurlock.

Department of Animal Sciences, Comparative Medicine Program, Purdue University, West Lafayette, Indiana 47907-1151

Submitted 20 June 2003; accepted in final form 25 August 2003

Regulation of hepatic glucose metabolism by leptin in pig and rat primary hepatocyte cultures. Am J Physiol Regul Integr Comp Physiol 286: R206–R216, 2004. First published September 25, 2003; 10.1152/ajpregu.00340.2003.—Direct effects of leptin on gluconeogenesis in pig and rat hepatocytes are equivocal, and model systems from other species have not been extensively explored in assessing the regulation of glucose metabolism by leptin. Therefore, the goal of the present study was to compare the effects of leptin on gluconeogenesis in pig and rat hepatocyte cultures as well as to investigate an underlying mechanism of action at the level of phosphoenolpyruvate carboxykinase (PEPCK). In rat hepatocytes, leptin exposure (3 h, 50 and 100 nM) attenuated glucagon-stimulated hepatic gluconeogenesis by 35 and 38% (P < 0.05), respectively. However, leptin did not produce any significant acute effect in pig hepatocytes. Leptin exposure for 24 h failed to produce any significant effect on gluconeogenesis in either rat or pig hepatocytes cultured in the presence of glucagon or dexamethasone. Mechanistically, there was a 25–35% decrease (P < 0.05) in glucagon-induced PEPCK mRNA levels in rat but not pig hepatocytes cultured with leptin. This effect on PEPCK mRNA was not due to an alteration in the relative abundance of the leptin receptor or the ability of PEPCK to respond to cAMP. The nonuniformity of the effects of leptin on gluconeogenesis in pig and rat hepatocytes indicates differences in leptin action between species. Furthermore, the unique action of leptin in porcine hepatocytes points to the utility of this model system for biomedical research and also underscores the value of comparative studies.

LEPTIN is a 16-kDa adipocyte-derived hormone that suppresses food intake, stimulates energy expenditure, increases metabolic rate, and ultimately causes loss of body fat (4, 22, 34). Although originally postulated to act largely via the central nervous system, several recent studies have demonstrated that leptin exerts a wide repertoire of peripheral effects through direct actions on target tissues. These direct effects include stimulation of fatty acid oxidation in adipocytes (4, 26, 28, 37, 49) and increased glucose uptake in skeletal muscle and brown adipose tissue in vivo (22, 47) and in a myotube cell line in vitro (3).

Leptin exerts direct effects on liver, a major site of glucose metabolism. Several lines of evidence indicate that leptin mimics some of the anabolic actions of insulin on liver. Leptin enhances the inhibitory effects of insulin on glycogenolysis and hepatic glucose production in liver in vivo (29, 32, 34), increases glycogen synthesis in perfused mouse liver (10), and causes both an inhibition and stimulation of some of the early events of insulin receptor signaling (9, 44). Available literature describing the effects of leptin on hepatic glucose metabolism in rodents is equivocal in that leptin has been shown both to increase (29, 34) as well as decrease (6) hepatic gluconeogenesis in rats. We are not aware of any published studies in which other mammalian model systems have been used to assess the effects of leptin on hepatic glucose metabolism. The pig is emerging rapidly as an important biomedical research model, and whereas genetic influences may impact the degree of similarity between pig and human systems, the regulation of some endocrine and metabolic processes in the pig may be more similar to humans than are rodents (41, 43). Therefore, the objective of the present study was to compare the effects of leptin on gluconeogenesis in pig and rat hepatocytes, using a primary cell culture system. In an attempt to delineate a possible mechanism of action of leptin, the mRNA expression of the key gluconeogenic enzyme, phosphoenolpyruvate carboxykinase (PEPCK), was also examined. Additional studies were done to investigate whether the observed effects of leptin on hepatic gluconeogenesis/PEPCK were due to an alteration in the relative abundance of the leptin receptor itself.

RESEARCH DESIGN AND METHODS

Materials. Male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 200–250 g and male pigs (2–3 wk old) weighing 5 kg were used as hepatocyte donors. All animal handling procedures were carried out in accordance with the guidelines of the Purdue University Animal Care and Use Committee. Recombinant human leptin was provided by Eli Lilly (Indianapolis, IN). Collagenase type 1 was purchased from Worthington Biochemical (Lakewood, NJ), and collagenase type 4 was obtained from Sigma Chemical (St. Louis, MO).

Isolation of hepatocytes. Rats were anesthetized by intraperitoneal injection of pentobarbital sodium (100 mg/kg). Pigs were anesthetized with pentobarbital sodium (containing 2,000 USP units of sodium heparin) via cardiac puncture. After washing of the torso and the limbs with water and 70% (vol/vol) ethanol, the animal was immobilized, sternum up, on a surgical board. Before the removal of the skin of the abdomen and thorax, these regions were thoroughly rinsed with 70% (vol/vol) ethanol.

Liver cells were isolated by the two-step collagenase perfusion technique described by Seglen (35), with slight modifications (5, 31). Briefly, the abdominal cavity was opened with a traverse incision caudal to the umbilicus, and a loose ligature was placed around the vena cava cranial to the right renal vein. The thoracic cavity was opened, and the thoracic portion of the vena cava was exposed by incision through the sternum and diaphragm. A cannula was then inserted into the thoracic vena cava ~1 cm above the diaphragm. Once the cannula was secured, perfusion of the liver was initiated with a Ca²⁺-free perfusion buffer (in g/l: 8.3 NaCl, 0.5 KCl, and 2.4 HEPES, pH 7.4) at 37°C, gassed with 95% O₂–5% CO₂, at a flow rate of 25 ml/min. The hepatic portal vein was then cannulated, which served as the outflow, and the ligature previously positioned around
the lower vena cava was tightened above the right kidney. At this point, the buffer flow rate was increased to 30–35 ml/min. Perfusion was continued in a retrograde fashion for 5–10 min; the Ca\(^{2+}\)-free buffer was then replaced with a collagenase (type 4 for pigs and type 1 for rats)-containing buffer (in g/l: 3.9 NaCl, 0.5 KCl, 2.4 HEPES, 0.7 CaCl\(_2\cdot\)2H\(_2\)O, pH 7.6), and perfusion was continued in a recirculating manner for another 15 min. The gall bladder (in case of the pig) and remaining connective tissue was removed, and hepatocytes were released by mechanical disruption of the liver capsule into 50–75 ml of collagenase-containing perfusate buffer.

Subsequently, the cells were filtered through two layers of a cotton cheesecloth and finally through a 200-μm nylon mesh (Spectrum Labs). The resulting cell suspension was then centrifuged at 50 g for 2 min, the supernatant was discarded, and the cell pellet was washed with ice-cold basal culture medium (DMEM) supplemented with 1% (wt/vol) BSA (dialyzed and fatty acid free), and gassed with 95% O\(_2\)-5% CO\(_2\). The resulting cell pellet was finally resuspended in about 75–100 ml of ice-cold basal culture medium and kept on ice until used. Hepatocyte viability was assessed after appropriate dilution and exposure to 0.2% (wt/vol) trypan blue dye using a standard Neubauer hemacytometer chamber. Cells that excluded the dye were scored as viable. Hepatocyte preparation having a viability >90% were used in all experiments.

Primary hepatocyte culture. Hepatocytes were plated on either 35-or 60-mm Primaria tissue culture dishes (Becton-Dickinson, Franklin Lakes, NJ) at a density of 1.5–2.5 \(\times\) 10\(^5\) cells in an internal plating media (DMEM) supplemented with 10% FBS, 100 nM dexamethasone, and 100 nM porcine insulin. Cells were allowed to adhere onto the culture dishes for 2–3 h and were then washed with serum-free DMEM and incubated in this media containing 100 nM dexamethasone, 1 nM porcine insulin, and 0.1% BSA. Cells were maintained in this medium overnight (18–24 h) and then utilized in subsequent experiments.

Incubation with leptin. Gluconeogenesis was measured using primary hepatocyte cultures incubated for a period of 3 or 24 h with or without leptin (10–100 nM) in the presence or absence of 10 nM glucagon or 1 μM dexamethasone in serum-free, dexamethasone-free, and insulin-free DMEM containing (in mM) 5 glucose, 2 lactate, and 0.2 pyruvate in vitro. Incubation was initiated by the addition of 2 mM \(\text{L-}[\text{U-}^{14}\text{C}]\text{lactate}\) (0.1 μCi/dish) and was continued for a period of 3 h. At endpoint, the media were removed from each dish and stored at \(-20^\circ\text{C}\), until assayed for presence of radiolabeled glucose. The total DNA was isolated from the cells by lysing the contents of each dish in a homogenization buffer and stored at \(-20^\circ\text{C}\) pending DNA quantification by the method of Labarca and Paigen (23).

For experiments requiring RNA extraction, hepatocyte cultures were incubated with or without leptin (10–100 nM) in DMEM devoid of serum, dexamethasone, and insulin, and containing (in mM) 5 glucose, 2 lactate, and 0.2 pyruvate. The incubations were continued for a period of 24 h with or without the appropriate gluconeogenic stimulants as described in the figure legends. At endpoint, total RNA was isolated from the cells using the Trizol reagent (Ambion, Austin, TX). Three 60-mm dishes were pooled together for each treatment and then frozen at \(-80^\circ\text{C}\) before RNA extraction.

In another series of experiments, hepatocyte cultures were incubated with or without leptin (100 nM) in presence or absence of 5 μM 8-(4-chlorophenyl-thio)-cAMP (8-CPT-cAMP), a cAMP analog, for a period of 24 h. At endpoint, the total RNA was isolated from the cells and stored at \(-80^\circ\text{C}\), pending RNA extraction.

Measurement of gluconeogenesis. Gluconeogenesis was measured as the rate of incorporation of \(\text{L-}[\text{U-}^{14}\text{C}]\text{lactate}\) into \([^{14}\text{C}]\text{glucose}\). Radiolabeled precursor and product were separated by ion-exchange chromatography, as outlined previously (12) using \([^{1}\text{H}]\text{glucose}\) as an internal standard. Gluconeogenic activity was determined as nanomoles of \(^{14}\text{C}\) precursor incorporated into glucose per micromgram of DNA per hour, calculated as disintegrations per minute recovered in glucose (corrected for \([^{1}\text{H}]\text{glucose}\) loss) divided by the specific activity of the precursor. Rate of gluconeogenesis was expressed as a percentage of the total gluconeogenic activity in hepatocytes incubated with glucagon or dexamethasone in the complete absence of leptin.

Extraction and analysis of cellular RNA. Total RNA was prepared using the guanidine thiocyanate method (7) with some modifications. The RNA was dissolved in Tris/EDTA, pH 8, and quantified spectrophotometrically by measuring the absorbance at 260 nm wavelength and the ribogreen assay (Molecular Probes, Eugene, OR). The purity, integrity, and equal loading of the total RNA was verified by visualization of the 18S and 28S bands with ethidium bromide after electrophoresis through a 1% agarose gel. For Northern blot analysis, 10 μg rat hepatocyte RNA, or 20–30 μg pig hepatocyte RNA, were separated by electrophoresis on 1% agarose gels containing formaldehyde and blotted overnight to GeneScreen membranes (New England Nuclear,Wilmington, DE), as previously described (21). Prehybridization was carried out using ULTRAbhyb Ultrasensitive Hybridization buffer (Ambion) for 2 h at 68°C. Riboprobes for the specific transcripts of interest were constructed from PCR products that were confirmed and validated by sequence analysis. The T7 promoter was ligated to the PCR product using the Lig'N'Scribe kit (Ambion) and the antisense riboprobe transcribed in the presence of [\(^{32}\text{P}\)]UTP. Hybridizations were performed overnight at 68°C. The membranes were then washed and exposed to film at \(-80^\circ\text{C}\) for periods ranging from 3 to 48 h. Signal intensity was quantified using a Digital Science Imaging System (V.2.0.1, Kodak, New Haven, CT).

**Fig. 1.** Expression of long form of leptin (ObRb) receptor by semiquantitative RT-PCR in freshly isolated and primary culture hepatocytes: pig (A) vs. rat (B). Freshly isolated and primary culture hepatocytes were prepared from pigs and rats as described in RESEARCH DESIGN AND METHODS. Total RNA was extracted from these hepatocyte samples, and the abundance of hepatic leptin receptor mRNA was assessed by semiquantitative RT-PCR. Total RNA was reverse transcribed using SS first-strand synthesis system for RT-PCR. The resulting cDNA was then subjected to PCR. The amplification cycle for pig and rat cDNA was 94°C for 5 min, 96°C for 1.20 min, 56°C for 1.30 min, 72°C for 1.30 min, 72°C for 10 min, 4°C on hold. Species-specific GAPDH was used as the external control for identification of the amount of mRNA; 35 and 38 cycles were used for pig and rat leptin receptor, respectively, and 22 cycles were used for both pig and rat GAPDH.
LEPTIN AND HEPATIC GLUCONEOGENESIS

Reverse transcription and amplification of cDNA. Abundance of hepatic leptin receptor mRNA was assessed by semiquantitative RT-PCR. Total RNA was reverse transcribed using the SS first-strand synthesis system for RT-PCR (Life Technologies, Rockville, MD). Two micrograms of total RNA were incubated at 65°C for 5 min with 1 μl of oligo(dT)12-18 (0.5 μg/μl), 1 μl of 10 mM dNTPs mixture, and water to a final volume of 10 μl and subsequently kept on ice. Two microliters of 10× RT buffer, 4 μl of 25 mM MgCl2, 2 μl of 0.1 M DTT, 1 μl of RNaseOUT Recombinant RNase Inhibitor, and 1 μl of Superscript II RT was added to each tube. The reaction was incubated at 42°C for 50 min and terminated at 70°C for 15 min.

The PCR amplifications were carried out in a final volume of 50 μl containing 5 μl of 10× PCR reaction buffer (160 mM ammonium sulfate, 670 mM Tris-HCl, pH 8.3, 0.1% Tween-20), 1.5 μl of 50 mM MgCl2, 1 μl of 10 mM dNTPs mixture, 1 μl of 15 μM of each primer, 0.4 U of iProof and 2 μl of the RT product. The amplification cycle for pig and rat cDNA was 94°C for 5 min, 96°C for 1.20 min, 56°C for 1.30 min, 72°C for 1.30 min, 72°C for 10 min, 4°C on hold. In addition, the species-specific gyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as the external control for identification of the amount of mRNA. For all semiquantitative RT-PCR reactions, 35 and 38 cycles were used for the pig and rat leptin receptor, respectively; 22 cycles were used for both pig and rat GAPDH. The specific sequences for the primer sets were as follows:

- pig Ob-Rb: 5′ S: GTCAGGAACCAATCAGTTCA 5′ AS: GTCAGGGAATACAGGCTGGG
- pig GAPDH: 5′ S: GCCAAGTCTTCGATGACAGTACAT 5′ AS: GCCAAGTCTTCGATGACAGTACAT
- rat Ob-Rb: 5′ S: AGAGTGTGTCGTCGGCACAAG 5′ AS: AGACTGATTCTGACAGTGGTC
- rat GAPDH: 5′ S: TGTCAAGGCGACAGTGGAA 5′ AS: GACACCGATGCAAAACAT

where S is sense and AS is antisense.

Quantification of RT-PCR products. After amplification, a 10-μl aliquot of the PCR product was electrophoresed through a 2% agarose gel containing (1%) ethidium bromide in Tris-acetate-EDTA buffer. DNA was visualized on a UV transilluminator. After photographing,
the picture was scanned and quantified using a Digital Science Imaging System (V.2.0.1, Kodak, New Haven, CT).

Statistical analyses. Data were analyzed using a randomized block design to account for the effects of species, individual animal within species, and replicate within animal. Results are expressed as means ± SE. Statistical significance was assessed using Bonferroni multiple comparison test to determine differences between treatment groups. P < 0.05 was considered as statistically significant.

RESULTS

Pig and rat hepatocytes express the long form of leptin receptor. Expression of the long form of the leptin receptor (Ob-Rb) was confirmed in both pig and rat hepatocytes (Fig. 1). Semiquantitative RT-PCR revealed bands corresponding to 369 and 400 bp in pig hepatocytes (Fig. 1A) and 302 and 240 bp in rat hepatocytes (Fig. 1B), representative of pig- and rat-specific ObRb and GAPDH, respectively. Based on these PCR images, there were no differences in the relative abundance of the receptor transcript in freshly isolated vs. primary hepatocyte cultures in either rat or pig preparations. These data confirm the presence of Ob-Rb mRNA in the culture models used and verify that its expression was maintained throughout the preparation procedure.

Glucagon dose response in the primary hepatocyte cultures. In pig hepatocyte cultures, glucagon produced a dose-dependent stimulation of hepatic gluconeogenesis using 2 mM [U-14C]lactate (0.1 µCi/dish) and 0.2 mM pyruvate as the gluconeogenic precursor. Glucagon at concentrations of 0.1–10 nM significantly elevated lactate-stimulated gluconeogenesis by 76–100% (P < 0.05, Fig. 2A). A similar effect was evident in rat hepatocytes in that a physiological concentration of glucagon (10 nM) also caused a statistically significant increase in gluconeogenesis that was of comparable magnitude (Fig. 2B).

Fig. 4. Effect of leptin on glucagon-stimulated gluconeogenesis: 24-h incubation. A: pig hepatocyte culture. B: rat hepatocyte culture. Pig and rat primary hepatocyte cultures were incubated for a period of 24 h with or without leptin (10–100 nM) in the presence or absence of glucagon (10 nM) in serum-free, dexamethasone-free, and insulin-free DMEM containing (in mM) 5 glucose, 2 lactate, and 0.2 pyruvate in vitro. Incubation was initiated by the addition of L-[U-14C]lactate (0.1 µCi/dish) and was continued for a period of 3 h. At endpoint, media were removed from each dish, and gluconeogenesis was measured as the rate of incorporation of L-[U-14C]lactate into [14C]glucose by using ion-exchange chromatography. Results are mean values (±SE) of 7 independent experiments and are expressed as % of the gluconeogenic activity in hepatocytes incubated with glucagon (10 nM) in the absence of leptin.

Fig. 5. Effect of 24-h leptin exposure on hepatic gluconeogenesis in presence of dexamethasone. A: pig hepatocyte culture. B: rat hepatocyte culture. Pig and rat primary hepatocytes were incubated with or without leptin (10–100 nM) in the presence or absence of 1 µM dexamethasone (dex) in serum-free, insulin-free DMEM containing (in mM) 5 glucose, 2 lactate, and 0.2 pyruvate in vitro. Glucagon was initiated by the addition of L-[U-14C]lactate (0.1 µCi/dish) and was continued for a period of 3 h. At endpoint, media were removed from each dish, and the rate of gluconeogenesis was measured using ion-exchange chromatography. Results are mean values (±SE) of 7 independent experiments and are expressed as % of the gluconeogenic activity in hepatocytes incubated with dexamethasone (1 µM) in the absence of leptin.
Effect of short-term leptin exposure on glucagon-stimulated gluconeogenesis in pig vs. rat hepatocyte cultures. Leptin did not produce any statistically significant effect on glucagon-stimulated gluconeogenesis in the pig primary hepatocyte cultures (Fig. 3A). However, under these conditions, and in contrast with the pig hepatocyte cultures, glucagon-stimulated gluconeogenesis was attenuated after a 3-h incubation of rat hepatocyte cultures with leptin. Specifically, leptin at concentrations of 100 and 50 nM significantly inhibited glucagon-stimulated gluconeogenesis by 38 and 35% (P < 0.05), respectively (Fig. 3B).

Effect of long-term leptin exposure in the presence of glucagon or dexamethasone on hepatic gluconeogenesis in pig and rat hepatocyte cultures. After identifying a differential response to leptin in rat and pig hepatocytes, we sought to determine if the regulation of gluconeogenesis by leptin in rat hepatocytes would be sustained and if the lack of an effect of leptin on pig hepatocytes was due to an insufficient incubation period. There was no indication of an inhibitory effect of leptin on gluconeogenesis in presence of glucagon or dexamethasone in pig hepatocytes even after 24 h of incubation (Figs. 4A and 5A). In addition, the inhibitory effect of leptin on glucagon-induced gluconeogenesis in rat cells incubated for 3 h was lost in hepatocytes cultured with leptin (10–100 nM) for 24 h (Fig. 4B). Similarly, leptin did not produce any statistically significant effect on rat hepatic gluconeogenesis in presence of dexamethasone (Fig. 5B).

PEPCK mRNA abundance in pig and rat hepatocyte cultures during long-term incubation with leptin. In both pig and rat hepatocyte cultures, glucagon and dexamethasone significantly elevated PEPCK mRNA concentrations 1.3- to 3.7-fold as expected (data not shown). As shown in Fig. 6, long-term exposure of pig hepatocytes to leptin in the presence of glucagon failed to significantly alter PEPCK mRNA concentrations. Also presented in Fig. 6, leptin (100 and 50 nM) did significantly increase the dexamethasone-induced PEPCK mRNA levels by 34 and 29% (P < 0.05) in pig hepatocytes. The effects of leptin on rat hepatocytes cultured in the presence of glucagon and dexamethasone are presented in Fig. 7. In contrast with pig hepatocytes, leptin (10–100 nM) decreased glucagon-induced PEPCK mRNA levels in rat primary hepatocytes 25–35% (P < 0.005), but there was no effect of leptin in the presence of dexamethasone.

Fig. 6. Effect of leptin on PEPCK mRNA expression in pig hepatocyte cultures. Pig primary hepatocyte cultures were incubated with or without leptin (10–100 nM) in serum-free, dexamethasone-free, and insulin-free DMEM containing (in mM) 5 glucose, 2 lactate, and 0.2 pyruvate in vitro. The incubations were continued for a period of 24 h in presence or absence of glucagon (10 nM) or dexamethasone (1 μM). At endpoint, total RNA was isolated from the cells by lysing with a denaturing solution. Three 60-mm dishes were pooled together for each treatment and then frozen at −80°C before extracting the total RNA. Total RNA was prepared using the guanidine thiocyanate method, and 20–30 μg pig hepatocyte RNA was subjected to Northern blotting, as described in RESEARCH DESIGN AND METHODS. A: representative PEPCK mRNA Northern blot in pig hepatocyte cultures. A, top: PEPCK. A, bottom: 18S. Lanes: 1) 10 nM glucagon; 2) 10 nM glucagon + 10 nM leptin; 3) 10 nM glucagon + 50 nM leptin; 4) 10 nM glucagon + 100 nM leptin; 5) 1 μM dexamethasone; 6) 1 μM dexamethasone + 10 nM leptin; 7) 1 μM dexamethasone + 50 nM leptin; 8) 1 μM dexamethasone + 100 nM leptin. The signal intensities after treatment with leptin in presence or absence of glucagon or dexamethasone from 3–4 independent experiments were quantified, and the results are shown in B. B, left: effect of leptin in presence of glucagon. B, right: effect of leptin in presence of dexamethasone.
Effect of leptin on leptin receptor expression in presence of glucagon or dexamethasone in pig and rat hepatocytes. As shown in Fig. 8, glucagon did not have any effect on the Ob-Rb mRNA expression in pig primary hepatocytes, but there was an 86% increase in the Ob-Rb mRNA after incubation with dexamethasone. Under these conditions, leptin (10 nM) significantly decreased Ob-Rb mRNA expression vs. dexamethasone alone. However, a similar reduction in receptor mRNA was not achieved by the higher concentrations (50 and 100 nM) of leptin (Fig. 8). In contrast with these results in pig hepatocytes, adding glucagon and dexamethasone to rat hepatocytes significantly decreased Ob-Rb mRNA abundance compared with the control (i.e., lactate/pyruvate-treated cells) (Fig. 9). Similar to results obtained with pig hepatocytes, leptin did not influence Ob-Rb mRNA expression in rat hepatocytes incubated with glucagon. However, in the presence of dexamethasone, leptin increased Ob-Rb mRNA expression by 88% (10 nM) and 1.4-fold (50 nM) compared with dexamethasone alone (Fig. 9).

Effect of long-term leptin exposure on PEPCK and leptin receptor mRNA in pig and rat hepatocytes. Previous studies have extensively documented that cAMP analogs, such as 8-CPT-cAMP, cause an increase in PEPCK gene transcription, acting at a site downstream from the glucagon receptor (17, 30). To verify the responsiveness of the PEPCK gene, we added 8-CPT-cAMP to our hepatocyte culture. In accordance with previous findings (24, 49), a marked increase in PEPCK mRNA level was achieved in both pig and rat hepatocyte cultures when 8-CPT-cAMP was added (Fig. 10, A and B), thus providing a link between PEPCK mRNA abundance and gluconeogenesis. However, under these conditions, leptin (with or without 8-CPT-cAMP) had no effect on the PEPCK mRNA abundance (Fig. 10, A and B), nor was there a change in the Ob-Rb mRNA expression (Figs. 10, C and D) in both pig and rat primary hepatocytes.

DISCUSSION

Pig and rat hepatocytes reveal stably expressed long forms of the leptin receptor. In this study, using RT-PCR, we have demonstrated that hepatocytes isolated from both pigs and rats under normal conditions express the long form (Ob-Rb) of the leptin receptor. Using species-specific primers to amplify...
LEPTIN AND HEPATIC GLUCONEOGENESIS

Fig. 8. Leptin receptor expression in pig hepatocyte cultures by semiquantitative RT-PCR. Pig hepatocyte cultures were incubated as described in text and Fig. 6 legend. Total RNA was reverse transcribed using SS first-strand synthesis system for RT-PCR, and the resulting cDNA was subjected to PCR as outlined in RESEARCH DESIGN AND METHODS. The amplification cycle for pig and rat cDNA was 94°C for 5 min, 96°C for 1.20 min, 56°C for 1.30 min, 72°C for 1.30 min, 72°C for 10 min, 4°C on hold. Species-specific GAPDH was used as the external control for identification of the amount of mRNA. 35 cycles were used for pig leptin receptor, and 22 cycles were used for pig GAPDH. A: representative PCR image in pig hepatocyte cultures. A: top: long form of pig-specific leptin receptor (ObRb). A: bottom: pig specific GAPDH as the housekeeping gene. Lanes: 1) 2 mM lactate/0.2 mM pyruvate (lac/pyr); 2) 10 nM glucagon; 3) 10 nM glucagon + 10 nM leptin; 4) 10 nM glucagon + 50 nM leptin; 5) 10 nM glucagon + 100 nM leptin; 6) 1 μM dexamethasone; 7) 1 μM dexamethasone + 10 nM leptin; 8) 1 μM dexamethasone + 50 nM leptin; 9) 1 μM dexamethasone + 100 nM leptin. The signal intensities of the PCR products from 4 independent experiments were quantified, and the results are shown in B as a ratio of ObRb mRNA to GAPDH mRNA.

across the unique intracellular domain of Ob-Rb, distinct bands corresponding to 369- and 302-bp products were identified in pig and rat hepatocytes, respectively. These products were consistent with the expected outcome of the PCR, and both products have been confirmed by sequence analysis previously (25, 44). Although our results are consistent with those of others (25, 44), there are conflicting reports regarding the mRNA expression of Ob-Rb in rat hepatocytes (50). This could be attributable to the conditions of hepatocyte isolation or the methods used to determine the presence of the leptin receptor. In the present study, we confirmed that freshly isolated hepatocytes and monolayer cultures express the mRNA for long form of leptin receptor (Ob-Rb) in similar abundance. These results indicate that the in vitro culture conditions used in our study did not result in a loss of the leptin receptor mRNA. However, it is possible that the receptor for mRNA expression in the liver differs from that in isolated hepatocytes.

Pig and rat primary hepatocyte cultures differ in their acute gluconeogenic response to leptin. The present study marks the first comparison of leptin actions on hepatic glucose metabolism in pig and rat hepatocyte cultures. Our findings confirm that leptin regulates gluconeogenesis in rat hepatocytes and also indicate that there is likely a significant species-dependent regulatory action of leptin on hepatic gluconeogenesis. Whereas we cannot completely rule out the possibility that physiological age influenced our results, Sumida et al. (39) have recently determined that prior plane of nutrition has a significantly greater impact on gluconeogenic activity in rat hepatocytes than does age. Previous findings show that hepatocytes isolated from 2- to 3-wk-old piglets manifest the complete gluconeogenic/glycolytic enzymatic machinery similar to that found in the larger pigs. Furthermore, it is well established that the isolation of intact, viable hepatocytes from 2- to 3-wk-old piglets is more likely than with larger pigs (13, 15, 40). Thus, while we acknowledge the potential for an age effect, we think it unlikely that species differences noted herein simply reflect the age of each animal model.

Previous findings describing the effects of leptin on glucose metabolism in the liver of rodents are conflicting (6, 9, 22, 25, 29, 50). However, the acute antigluconeogenic effect of leptin on rat hepatocytes was clearly evident in the present study and is consistent with previous work (6). The literature describing the effects of leptin on hepatic glucose metabolism has been derived primarily from rodent models. The present study underscores the differential actions of leptin across species and points to the advantages of comparative studies in biomedical research. In addition, given the similarity in some aspects of the endocrine and metabolic characteristics of the pigs and humans (41, 43), our findings pose intriguing questions regarding effects of leptin on glucose metabolism in humans and also substantiate pig hepatocytes as a useful model to evaluate the effects of leptin on hepatic metabolism.
Long-term gluconeogenic response to leptin of pig and rat hepatocyte cultures does not correlate with the PEPCK mRNA abundance. Previous studies have demonstrated an increasing concentration of glucagon and circulating corticosteroids under conditions of stress resulting in increased gluconeogenic flux (1, 20, 24). Consistent with earlier findings (24, 48), 8-CPT-cAMP, a cAMP analog, glucagon, and a synthetic corticosteroid, dexamethasone, significantly increased the PEPCK mRNA abundance in hepatocyte cultures prepared from both pigs and rats, thereby confirming the hormonal responsiveness of our hepatocyte preparation. We have demonstrated here that the effect of leptin on hepatocyte mRNA expression of PEPCK in the presence of glucagon or dexamethasone, significantly increased the PEPCK mRNA abundance in hepatocyte cultures prepared from both pigs and rats, thereby confirming the hormonal responsiveness of our hepatocyte preparation. We have demonstrated here that the effect of leptin on hepatocyte mRNA expression of PEPCK in the presence of glucagon or dexamethasone failed to correlate with the gluconeogenic response of the cells after long-term incubation. Thus, although leptin increased dexamethasone-induced PEPCK mRNA levels in pig primary hepatocytes and decreased glucagon-induced PEPCK mRNA concentrations in rat hepatocyte cultures (Figs. 6 and 7), long-term incubation with leptin under similar experimental conditions did not result in any significant effect on hepatic gluconeogenesis (Figs. 4 and 5). The rate of gluconeogenesis from lactate is known to be controlled at three points at which substrate cycling can occur. PEPCK, which regulates the phosphoenolpyruvate-pyruvate cycle, has long been thought to play a key role in the regulation of gluconeogenesis, and several lines of evidence have implicated PEPCK to be the rate-determining step in hepatic gluconeogenesis (8, 11, 16, 27, 33, 42, 45). CPT increases PEPCK gene transcription by circumventing the glucagon receptor itself. In the present study, leptin did not have any effect on PEPCK mRNA expression in either CPT-stimulated or glucagon-stimulated pig hepatocytes, suggesting that there is no interaction of leptin at the receptor or postreceptor sites in pig hepatocytes. However, in contrast, while leptin had no effect on CPT-stimulated PEPCK expression, there was a decrease in glucagon-stimulated PEPCK mRNA in rat hepatocytes. These results indicate that the effect of leptin on PEPCK mRNA in the rat hepatocytes is probably not mediated by interaction at a site downstream from the glucagon receptor itself, similar to the porcine hepatocyte study. Rather, our data suggest that in primary rat hepatocytes, leptin interferes with signaling from the glucagon receptor itself, possibly at the level of Gs protein activation.

Data from several laboratories support the concept of a multienzyme regulation of the gluconeogenic pathway (18, 19, 36). These earlier findings suggest that instead of a single dominant control point, there exist multiple enzymes (including pyruvate carboxylase, pyruvate kinase, PFK1/F1,6-P2ase) that may be responsible for regulating the gluconeogenic pathway. Indeed, in a study conducted in isolated rat hepatocytes, both pyruvate carboxylase and pyruvate kinase were shown to

Fig. 9. Leptin receptor expression in rat hepatocyte cultures by semiquantitative RT-PCR. Rat hepatocyte cultures were incubated as described in the text and Fig. 7 legend. Leptin receptor abundance was assessed as described in Fig. 8 legend and in METHODS. A: representative PCR image in rat hepatocytes. A, top: rat-specific long form of leptin receptor (Ob-Rb). A, bottom: rat-specific GAPDH as the housekeeping gene. Lanes 1–9 are as described in Fig. 8 legend. B: densitometric quantitation of leptin receptor expression in rat hepatocyte cultures. Results are mean values (±SE) of signal intensity of PCR products from 3 independent experiments.
LEPTIN AND HEPATIC GLUCONEOGENESIS

Fig. 10. PEPCK mRNA and leptin receptor expression in pig vs. rat hepatocytes. Hepatocyte cultures were incubated with or without leptin (100 nM) in the presence or absence of 5 μM 8-((4-chlorophenyl-thio)cAMP (8-CPT-cAMP) for a period of 24 h. At endpoint, total RNA was isolated from the cells and subjected to Northern blotting as described in RESEARCH DESIGN AND METHODS. A: representative pig hepatocyte Northern blot. B: representative rat hepatocyte Northern blot. A and B, top: PEPCK. A and B, bottom: GAPDH. Lanes: 1, control; L100, 100 nM leptin; CPT, 8-CPT-cAMP; CPT/L100, 100 nM leptin + 8-CPT-cAMP. Representative semiquantitative RT-PCR images in pig and rat hepatocyte cultures are shown in C and D, respectively. C and D, top: pig (C) or rat (D) specific ObRb. C and D, bottom: pig (C) or rat (D) specific GAPDH. Lanes: 1) control; 2) 100 nM leptin; 3) 8-CPT; 4) 100 nM leptin + 8-CPT.

Adiponectin, another adipose-derived hormone, has been implicated in the development of insulin resistance associated with obesity and type 2 diabetes. In primary rat hepatocytes, adiponectin increases the ability of subphysiological levels of insulin to suppress hepatic glucose production (2). Moreover, in lipoatrophic mice (46), hepatic insulin resistance is completely reversed by the combination of adiponectin and leptin, but only partially by either adiponectin or leptin alone. Preliminary studies in our laboratory demonstrate that long-term (24 h) exposure to pig recombinant adiponectin attenuates glucagon-stimulated gluconeogenesis in primary rat hepatocytes (unpublished data). Therefore, the lack an effect of chronic leptin exposure on hepatic gluconeogenesis in the present studies may reflect the absence of adiponectin, which the hepatocytes would be exposed to in vivo. We speculate that adiponectin potentiates the antigluconeogenic response of primary hepatocyte cultures to leptin. Thus, as suggested by a previous study (46), it is possible that hepatocyte cultures require the presence of adiponectin in vitro to elicit a long-term gluconeogenic response to leptin. The present study also marks the first evidence that the effect of leptin on PEPCK gene expression does not correspond with the ObRb mRNA expression in primary hepatocyte cultures. These results suggest that the differential effects of leptin on PEPCK gene transcription in pig and rat hepatocytes as observed in the present study are not due to alterations in the relative abundance of the long form of leptin receptor.

In summary, we have identified differences in the response of pig and rat hepatocytes to leptin that may be of considerable physiological importance to developing a comprehensive understanding of leptin and the regulation of glucose metabolism in animals and humans.

ACKNOWLEDGMENTS

This paper is journal article no. 17105 of the Agricultural Experiment Station, Purdue University. This study was presented in part at the annual meeting of the Endocrine Society, San Francisco, CA, 2002.
REFERENCES


