Title

Evidence That Glucose Metabolism Regulates Leptin Secretion from Cultured Rat Adipocytes*

Journal

Endocrinology, 139(2)

ISSN

0888-8809

Authors

Mueller, Wendy M

Gregoire, Francine M

Stanhope, Kimber L

et al.

Publication Date

1998-02-01

DOI

10.1210/en.139.2.551

Peer reviewed

0013-7227/98/$03.00/0
Endocrinology

Vol. 139, No. 2
Printed in U.S.A.

Evidence That Glucose Metabolism Regulates Leptin Secretion from Cultured Rat Adipocytes*

WENDY M. MUELLER, FRANCINE M. GREGOIRE, KIMBER L. STANHOPE, CHARLES V. MOBBS, TOORU M. MIZUNO, CRAIG H. WARDEN, JUDITH S. STERN, AND PETER J. HAVEL

Department of Nutrition (W.M.M., K.L.S., J.S.S., P.J.H.), and Department of Pediatrics (F.M.G., C.H.W.), School of Medicine, University of California, Davis, California 95616; and Neurobiology of Aging Laboratories, Mount Sinai School of Medicine (C.V.M., T.M.M.), New York, New York 10021

ABSTRACT

Circulating leptin secreted from adipocytes is correlated with fat mass and plasma insulin concentrations in humans and rodents. Plasma leptin, insulin, and glucose decrease during fasting and increase after refeeding; however, the underlying mechanisms regulating the changes of leptin secretion are not known. To investigate the role of insulin-stimulated glucose metabolism in the regulation of leptin secretion, we examined the effects of insulin and inhibitors of glucose transport and metabolism on leptin secretion from rat adipocytes in primary culture. Insulin (0.16–16 nM) increased leptin secretion over 96 h; however, the increase in leptin was more closely related to the amount of glucose taken up by the adipocytes ($r = 0.64$; $P < 0.0001$) than to the insulin concentration *per se* ($r = 0.20$; $P < 0.28$), suggesting a role for glucose transport and/or metabolism in regulating leptin secretion.

2-Deoxy-D-glucose (2-DG), a competitive inhibitor of glucose transport and phosphorylation, caused a concentration-dependent (2–50 mg/dl) inhibition of leptin release in the presence of 1.6 nM insulin. The inhibitory effect of 2-DG was reversed by high concentrations of glucose. Two other inhibitors of glucose transport, phloretin (0.05–0.25 mM) and cytochalasin-B (0.5–50 μM), also inhibited leptin secretion. Inhibition of leptin secretion by these agents was proportional to the inhibition of glucose uptake ($r = 0.60$ to 0.86; all $P < 0.01$). Two inhibitors of glycolysis, iodoacetate (0.005–1.0 mM) and sodium fluoride (0.1–5 mM), produced concentration-dependent inhibition of leptin secretion in the presence of 1.6 nM insulin. In addition, both 2-DG and sodium fluoride markedly decreased the leptin (*ob*) messenger RNA content of cultured adipocytes, but did not affect 18S ribosomal RNA content.

We conclude that glucose transport and metabolism are important factors in the regulation of leptin expression and secretion and that the effect of insulin to increase adipocyte glucose utilization is likely to contribute to insulin-stimulated leptin secretion. Thus, *in vivo*, decreased adipose glucose metabolism may be one mechanism by which fasting decreases circulating leptin, whereas increased adipose glucose metabolism would increase leptin after refeeding. (*Endocrinology* **139:** 551–558, 1998)

THE ADIPOCYTE hormone, leptin is implicated in the regulation of food intake, energy expenditure, and body fat stores (1). Circulating leptin decreases after fasting or caloric restriction in both humans (2–4) and rodents (5–7), and increases a number of hours after refeeding (3, 6). In humans, there is a nocturnal rise of plasma leptin (8), which has been hypothesized to be due to a delayed effect of insulin released during earlier meals. Consistent with this hypothesis, insulin increases expression of the *ob* gene in rodents (9–11) and in adipocytes *in vitro* (12, 13) after a number of hours. In humans, plasma insulin and leptin concentrations decrease in parallel after weight loss, independently of changes of adiposity (14). Furthermore, plasma leptin is negatively correlated with insulin sensitivity independently of adiposity in subjects with impaired glucose tolerance (15).

Short term insulin administration does not affect plasma leptin concentrations in human subjects (16, 17), but increases in circulating leptin have been reported after 4–6 h of high dose insulin administration (18, 19). These studies by necessity require the infusion of large amounts of glucose to prevent hypoglycemia. Similarly, prolonged hyperglycemia in response to extended glucose infusions increases plasma leptin after several hours in nonhuman primates (20) and human subjects (21); however, glucose administration also markedly increases endogenous insulin levels. Therefore, the role of insulin *per se* on the adipocyte *vs.* the effect of insulin to increase glucose flux into adipocytes was not addressed by these experiments.

Several lines of evidence have led us to hypothesize that glucose is an important regulator of leptin expression and secretion. First, increases in *ob* messenger RNA (mRNA) after glucose administration in mice are more closely related to plasma glucose concentrations than to plasma insulin concentrations (22). Second, infusion of small amounts of glucose to prevent the decline of glycemia during fasting in humans also prevents the decrease in plasma leptin (2). Third, the decrease in plasma leptin during marked caloric restriction in humans is better correlated with the decrease in plasma glucose than with changes in insulinemia (4). Fourth, we have found that low plasma leptin levels in streptozotocin diabetic rats are acutely increased by insulin administration in proportion to the degree of glucose lowering (23). Lastly, lowering plasma glucose concentrations in hyperglycemic insulin-dependent diabetic human subjects by

Received July 9, 1997.
Address all correspondence and requests for reprints to: Peter J. Havel, D.V.M., Ph.D., Department of Nutrition, University of California, Davis, California 95616. E-mail: pjhavel@ucdavis.edu.
* This work was supported by NIH Grants DK-50129, DK-18899, DK-07355, DK-35747, CA-61654, and DK-50110 and the Juvenile Diabetes Foundation.

infusing insulin at rates that produced physiological insulinemia increases circulating leptin (24).

To investigate the mechanisms by which glucose influences leptin secretion, we adapted and modified an *in vitro* system for culturing rat adipocytes in which the adipocytes are anchored in a defined mixture of extracellular matrix components (25) This matrix, Matrigel, appears to simulate normal basement membrane attachment of cells and may allow cell to cell interactions between adipocytes. Cells cultured in this system are, therefore, in an environment closer to their normal physiological milieu than in systems where adipocytes are free floating in the culture medium. Adipocytes cultured on Matrigel have been shown to maintain many of their differentiated characteristics and, in contrast with free-floating adipocytes, show no sign of dedifferentiation after 6 days of culture (25, 26). With this system we have investigated the regulation of leptin secretion by glucose and insulin and the effects of inhibitors of adipocyte glucose transport and metabolism on leptin secretion. The leptin (*ob*) mRNA content of the adipocytes after culture with insulin and inhibitors was also examined.

Materials and Methods

Materials

DMEM and FBS were purchased from Life Technologies (Grand Island, NY). The media were supplemented with 6 ml each of MEM nonessential amino acids, penicillin/streptomycin (5000 U/ml/5000 μg/ml), and nystatin (10,000 U/ml; all from Life Technologies) per 500 ml DMEM. BSA fraction V, HEPES, collagenase (*Clostridium histolyticum*; type II, Sigma Chemical Co., St. Lousi, MO; SA, 456 U/mg), insulin, D-glucose, sodium fluoride (NaFl), phloretin, iodoacetate, and fructose were purchased from Sigma Chemical Co. Matrigel matrix was purchased from Becton Dickinson (Franklin Lakes, NJ). 2-Deoxy-D-glucose (2-DG) was obtained from U.S. Biochemical Corp. (Cleveland, OH). Six-well Falcon tissue culture plates were purchased from Fisher Scientific (Pittsburgh, PA). Nylon filters were purchased from Tetko (Kansas City, MO).

Animals

Male Sprague-Dawley rats were obtained from Charles River (Wilmington, MA). Animals were housed in hanging wire cages in temperature controlled rooms (22–24 C) with a 12-h light-dark cycle and fed Purina chow diet (Ralston-Purina, St. Louis, MO) and given deionized water *ad libitum*. The study protocol was approved by the University of California-Davis animal care and use committee.

Cell isolation/preparation

Adipocytes were prepared from epididymal fat pads of male Sprague-Dawley rats (300–600 g) anesthetized with halothane. Epididymal fat depots were resected under aseptic conditions, and adipocytes were isolated by collagenase digestion according to the Rodbell procedure (27) with minor modifications as described below. The fat pads were minced into pieces in Krebs-Ringer HEPES buffer (pH 7.4; containing 5 mM D-glucose, 2% BSA, 135 mM NaCl, 2.2 mM $CaCl_2.2H_2O$, 1.25 mM $MgSO_4 \cdot 7H_2O$, 0.45 mM KH_2PO_4, 2.17 mM Na_2HPO_4, and 10 mM HEPES). Adipose tissue fragments were digested in the same buffer in the presence of type II collagenase (2.5 mg/2 ml buffer·g tissue) at 37 C with gentle shaking at 60 cycles/min for 45 min. The resulting cell suspension was diluted in 24 ml cold HEPES-phosphate buffer. Isolated adipocytes were separated from undigested tissue by filtration through a 400-μm nylon mesh and washed three times. For washing, cells were centrifuged at 500 rpm for 5 min. Each time the infranatant was discarded, and the adipocytes were resuspended in Krebs-Ringer HEPES buffer, with the final wash being in 0, 5, or 10 mM glucose culture medium supplemented with 1% or 5% FBS. The isolated adipocytes were then incubated for 30 min at 37 C before being plated in Matrigel-coated culture plates.

Adipocyte culture

Matrigel was thawed on ice to a liquid and uniformly applied to the surface of the culture dish (300 μl Matrigel/35-mm well). After the incubation, 150 μl of the adipocyte suspension (2:1 ratio of packed cells to medium) were plated on the liquid matrix. The warmth of the cells and buffer caused the Matrigel to gel around the adipocytes, effectively anchoring them to the culture dish. After a 30-min incubation at 37 C, 2 ml warm culture medium supplemented with FBS were added. The cells were maintained in an incubator at 37 C in 6% CO_2 for 96 h.

The initial medium concentration of glucose for the cultures conducted in the insulin dose-response experiment was 10.0–10.5 mM (180–190 mg/dl) to ensure that the cells would not deplete the glucose supply during the 96-h incubation when higher concentrations of insulin were used. Only 1% FBS was used in the insulin dose-response study to minimize the small amount of insulin present in the serum, which at 1% was less than 0.1 μU/ml. In the fructose study, medium made with glucose-free DMEM and 1% fetal serum was used to minimize the amount of glucose available to the adipocytes (<0.1 mmol/liter). However, it was not possible to eliminate all glucose from culture preparation because the Matrigel matrix itself contains ~4.2 mmol/liter glucose. For the fructose experiment, the Matrigel was diluted 1:2 with glucose-free medium to approximately 1.5 mmol/liter glucose.

In the other experiments with inhibitors of glucose transport, 2-DG (28), phloretin (29), and cytochalasin B (30), or with inhibitors of glycolysis, iodoacetate (31), and NaFl (32), the initial medium glucose concentration was (5.0–5.5 mM; 90–100 mg/dl) with 5% fetal serum. These agents were used at concentrations at or below those typically employed to inhibit glucose transport or glycolysis in adipocytes (28–32). Cytochalasin B was initially dissolved in ethanol and diluted to 0.5% ethanol in the well with the highest dose. Therefore, the medium in all wells in the cytochalasin B experiment was equalized to 0.5% ethanol. Aliquots of adipocytes from each animal were divided into wells with the responses to insulin, the various inhibitors, or fructose being compared with those of an appropriate control well containing adipocytes from the same animal. In a preliminary insulin dose-response study, we found that medium leptin concentrations in the presence of insulin were not increased over those in control medium (no insulin) until after 24 h of incubation. Therefore, for the remainder of the studies, 300-μl samples (15% of the medium volume) were collected at 24, 48, 72, and 96 h and replaced with 300 μl fresh medium containing the appropriate concentrations of glucose, insulin, and/or inhibitors. Cultures were observed daily with a phase contrast microscope. After 96 h, a subset of the culture plates was frozen until analyzed for leptin (*ob*) mRNA content by Northern blot.

Assays

Leptin concentrations in the medium were determined with a sensitive and specific RIA for mouse leptin as previously described (7) (Linco Research, St. Charles, MO). Leptin concentrations in medium from cultured rat adipocytes measured with this assay are very similar to those obtained with a newly developed assay specific for rat leptin. With the rat-specific assay, measured leptin concentrations in culture medium were 86 ± 3% of the mouse values and were highly correlated between the two assays ($r = 0.97$; $P < 0.0001$; unpublished data). Therefore, measurements of rat leptin made with the mouse assay provide a reliable measurement of leptin concentrations. The intra- and interassay coefficients of variation for this assay are 4.0% and 11.2%, respectively (7). The antibody used in the assay does not cross-react with insulin, proinsulin, glucagon, pancreatic polypeptide or somatostatin. Glucose and lactate were measured with a YSI glucose analyzer (model 2300, Yellow Springs Instruments, Yellow Springs, OH).

Northern blot procedure

The following procedures were performed on culture plates incubated with 5 mM glucose and 5% fetal serum alone (control), 1.6 nM insulin, and 1.6 nM insulin with 10 mg/dl 2-DG or 1 mM NaFl for 48 and 96 h. Northern blot analysis was performed as previously described (33).

In brief, 1 ml RNAzol B (Tel-Test, Friendswood, TX) was added directly to the wells containing the adipocytes and matrix. The solution was repetitively taken in and expelled from the pipette to maximize dissolution of the adipose tissue. UV absorbance and integrity gels were used to estimate RNA. To allow loading of equal mass of RNA in each well, after analysis of leptin mRNA using a single-stranded complementary DNA probe followed by quantification of bands on a phosphoimager as well as from film, the blots were reanalyzed using a probe complementary to mouse 18S ribosomal RNA. Leptin mRNA was then normalized with respect to the 18S ribosomal signal, according to the absolute signal. The 18S RNA results were virtually identical in all cases. In particular, experimental conditions did not influence the 18S ribosomal signal.

Calculations and data analysis

The uptake of glucose was assessed by measuring the concentration of glucose in the medium in each well before and after 96 h of incubation and calculating the decrease over 96 h. To examine the relationship between adipocyte carbon flux and leptin secretion in response to increased insulin-mediated glucose uptake, the amount of carbon released as lactate per amount of carbon taken up as glucose over 96 h was calculated as Δ[lactate]/Δ[glucose], where Δ is the change, and expressed as a percentage. The area under the curve for leptin concentrations in the medium between 0–96 h was calculated by the trapezoidal method. The means of two groups were compared by paired *t* test. The means of more than two groups were compared by ANOVA. To examine the relationships between the medium concentrations of insulin or inhibitors employed, the amount of glucose taken up by the adipocytes, and leptin secretion, simple and multiple linear regression analyses were performed with a statistics software package (StatView for Macintosh, Abacus Concepts, Inc., Berkeley, CA). Data are expressed as the mean ± SEM.

Results

Responses to insulin (0.16–16.0 nM)

The effects of insulin on leptin secretion, and the relationship between glucose uptake by adipocytes cultured with different concentrations of insulin and leptin secretion were examined. Insulin produced a concentration-dependent increase in glucose uptake by the cultured adipocytes ($r = 0.61$; $P < 0.0002$ *vs.* insulin concentration), as assessed by the decrease in glucose in the medium (Fig. 1A). With no added insulin, the medium glucose concentration decreased from 10.1 ± 0.1 to 8.2 ± 0.3 mmol/liter (Δ, −1.9 ± 0.3 mmol/liter; $P < 0.0001$). The addition of 0.16, 1.6, and 16.0 nM insulin increased glucose uptake (Δglucose, −2.7 ± 0.4, −3.3 ± 0.3, and −3.9 ± 0.4 mmol/liter, respectively; all $P < 0.01$ *vs.* no insulin). Insulin also produced a concentration-dependent increase in lactate production ($r = 0.70$; $P < 0.0001$), which was well correlated with the decrease in glucose in the medium over 96 h ($r = 0.61$; $P < 0.0002$), suggesting that a significant portion of the glucose entering the adipocytes was metabolized only as far as lactate and released from the cells into the medium (34, 35).

Leptin secretion was increased over the control value by all three concentrations of insulin (Fig. 1B). The production of lactate was not related to the leptin response ($r = 0.10$; $P = 0.59$). The area under the leptin concentration curve (AUC) from 0–96 h was independently related to the decrease in glucose in the medium during the incubation (Fig. 1C), but not to the insulin concentration (Table 1). Similarly, with a multiple regression model, the AUC for leptin was related to the decrease in glucose, but not to the insulin concentration. In addition, the percentage of carbon released as lactate per amount of carbon taken up as glucose was calculated. Over-

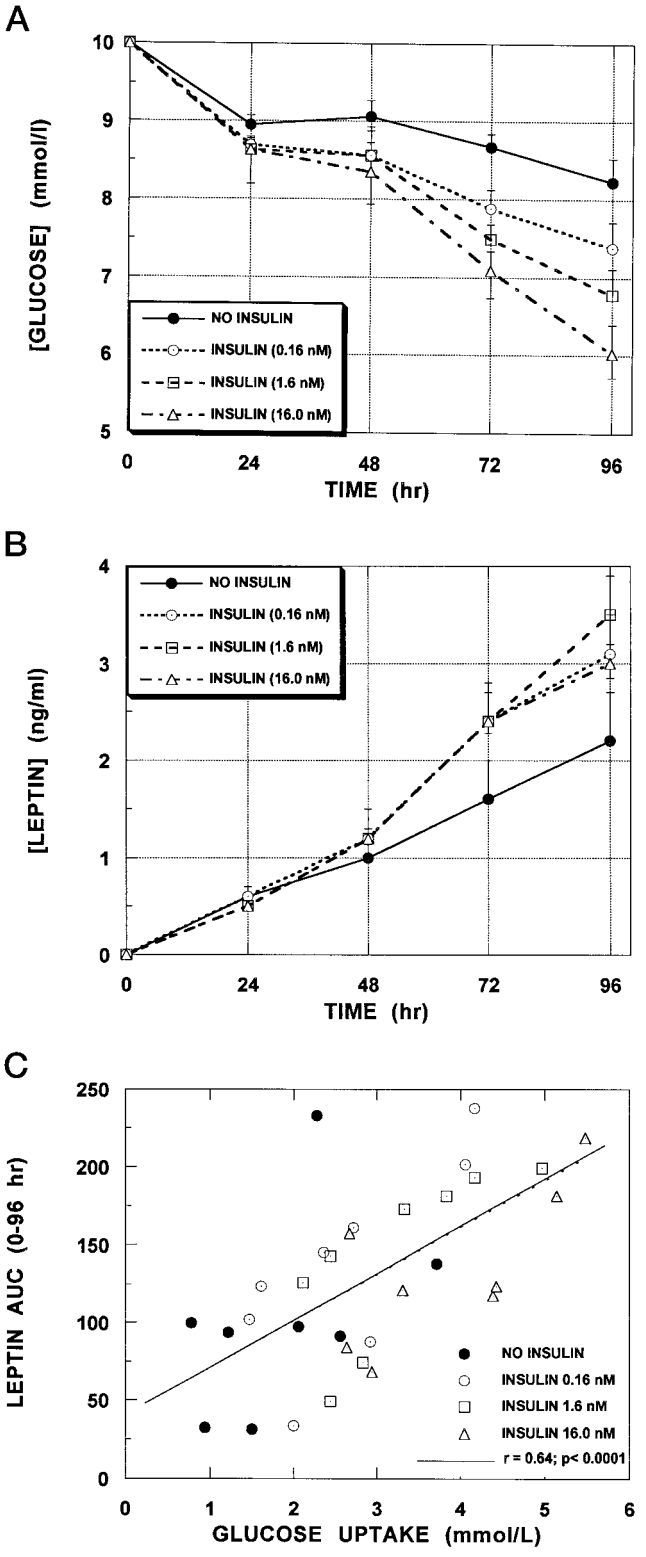

FIG. 1. A, Glucose concentrations in medium from 0–96 h from isolated rat adipocytes in primary culture with insulin concentrations from 0–16 nM (n = 8/treatment). B, Leptin concentrations from 0–96 h from isolated rat adipocytes in primary culture with insulin concentrations from 0–16 nM (n = 8/treatment). C, Relationship between glucose uptake, as assessed by the decrease in glucose in the culture medium, and leptin secretion, expressed as the AUC from 0–96 h, during incubation of adipocytes with 0–16 nM insulin (n = 32).

TABLE 1. Relationship between glucose uptake (ΔGluc), leptin secretion (ΔLept) and the concentration of insulin or inhibitors of glucose uptake and metabolism after 96-h incubation of adipocytes with insulin and insulin plus inhibitors (2-DG, phloretin, cytochalasin-B, iodocetate, or sodium fluoride at varying concentrations; see *Materials and Methods*)

Insulin or inhibitor (n)	Simple Regression				Multiple regression	
	ΔLept *vs.* ΔGluc		ΔLept *vs.* [inhibitor]		ΔLept *vs.* ΔGluc (*P*)	ΔLept *vs.* [inhibitor] (*P*)
	r	*P*	r	*P*		
Insulin (32)	0.64	0.0001	0.20	0.28	0.0001	0.09
2-DG (38)	0.67	0.0001	0.51	0.001	0.001	0.29
Phloretin (38)	0.86	0.0001	0.78	0.0001	0.0012	0.75
Cytochalasin-B (19)	0.60	0.01	0.58	0.02	0.22	0.25
Iodoacetate (34)	0.83	0.0001	0.74	0.0001	0.0001	0.17
Sodium fluoride (28)	0.85	0.0001	0.60	0.001	0.0001	0.73

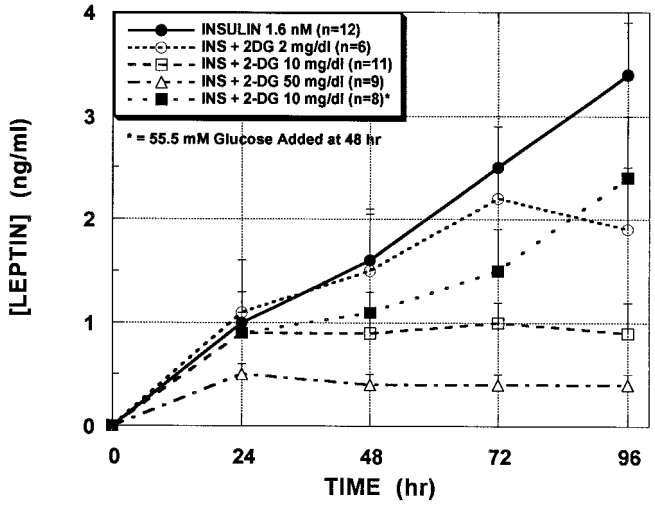

FIG. 2. Effects of inhibiting glucose transport and metabolism with 2-DG on leptin concentrations from 0–96 h in medium from isolated rat adipocytes in primary culture for 96 h with 1.6 nM insulin and the effect of adding glucose (55 mM) at 48 h on the inhibition of leptin secretion produced by 10 mg/dl 2-DG.

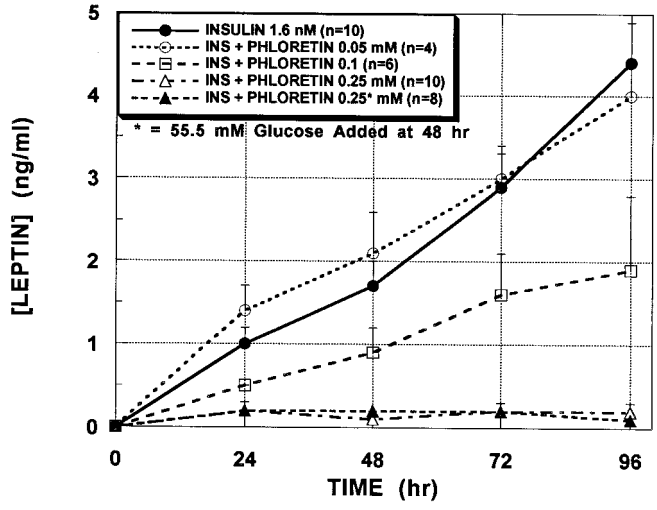

FIG. 3. Effects of inhibiting glucose transport with phloretin on leptin concentrations from 0–96 h in medium from isolated rat adipocytes in primary culture for 96 h with 1.6 nM insulin and the effect of adding glucose (55 mM) at 48 h on the inhibition of leptin secretion produced by 0.25 mM phloretin.

all, in the insulin experiment between 10–68% of the amount of carbon taken up as glucose was released as lactate (mean, 34 ± 2%). There was no direct relationship between this parameter and the insulin concentration; however, it was inversely proportional to the amount of leptin secreted, as expressed by the 0–96 h leptin AUC (r = 0.64; $P < 0.0001$). By multiple regression analysis, the relationship between glucose conversion to lactate and leptin secretion was not significantly related to lactate production ($P = 0.06$), but leptin secretion was equally related to both the change in glucose and the amount of glucose carbon released as lactate (both $P < 0.001$).

Effects of 2-DG (2–50 mg/dl)

The effect of inhibiting glucose uptake and metabolism with 2-DG on leptin secretion and its relationship to adipocyte glucose uptake were examined. 2-DG at a concentration of 50 mg/dl completely inhibited glucose uptake (Δ, 0.1 ± 0.3 mmol/liter) in the presence of 1.6 nM insulin (Δglucose, −4.0 ± 0.6 mmol/liter) and inhibited the leptin response (AUC 0–96 h) by 69 ± 4% ($P < 0.0001$) compared with insulin alone (Fig. 2A). At a lower concentration of 2-DG (10 mg/dl), glucose uptake was still markedly inhibited (Δ, −0.1 ± 0.4 mmol/liter) and leptin secretion was inhibited by 47 ± 5% ($P < 0.0001$). The lowest concentration of 2-DG (2 mg/dl) produced less of an inhibition of glucose uptake (Δ, −1.5 ± 0.9 mmol/liter; $P < 0.01$ *vs.* insulin alone). At this concentration, the leptin response was not significantly inhibited until the 96 h point ($P < 0.02$ *vs.* insulin alone; Fig. 2A).

Overall, the change in leptin at 96 h was related to the concentration of 2-DG and was well correlated with the decrease in medium glucose (Table 1). By multiple regression, the leptin concentration in the medium at 96 h was significantly correlated with the change in glucose, but not to the 2-DG concentration (Table 1). The addition of glucose (55.5 mM) at 48 h reversed the inhibition of leptin secretion produced by 2-DG at 10 mg/dl by 96 h ($P < 0.01$ *vs.* 2-DG; NS *vs.* insulin alone; Fig. 2).

Effects of phloretin (0.05–0.25 mM)

The effect of inhibiting glucose uptake with phloretin on leptin secretion was examined. Phloretin at a concentration of 0.25 mM completely inhibited leptin secretion (Fig. 3). The 0–96 h AUC for leptin was inhibited by 91 ± 2% of insulin alone ($P < 0.0001$). This higher concentration of phloretin (0.25 mM) also completely blocked glucose uptake in the

presence of 1.6 nM insulin (Δglucose, 0.7 ± 0.1 mmol/liter). Overall, the leptin response was inversely related to the concentration of phloretin and was highly correlated with the decrease in glucose in the medium (Table 1). However, by multiple regression, the leptin response was correlated with the decrease in glucose, but not with the concentration of phloretin (Table 1). The addition of 55.5 mM glucose at 48 h did not reverse the inhibition of leptin secretion by phloretin (Fig. 3).

Effects of cytochalasin B

The effect of inhibiting glucose uptake with cytochalasin B on leptin secretion was examined. Cytochalasin B produced a concentration-dependent inhibition of glucose uptake and leptin secretion (Fig. 4). The leptin response was significantly correlated with glucose uptake by simple regression (Table 1), but was not significantly correlated with glucose uptake (as observed with the other inhibitors; Table 1) by multiple regression, perhaps due to the smaller number of replicates (n = 19) in this experiment.

Effects of iodoacetate (0.005–1.0 mM)

The effect of inhibiting glycolysis with iodoacetate on leptin secretion was examined. Iodoacetate at 1.0, 0.1, and 0.01 mM markedly inhibited glucose uptake (Δglucose, −0.1 ± 1.1, 0.5 ± 0.2, and 0.3 ± 0.2 mmol/liter, respectively) and leptin secretion. The 0–96 h AUC for leptin was inhibited by −95 ± 2%, −91 ± 2%, and −87 ± 3%, respectively, compared with insulin alone; (all $P < 0.0001$). The lowest concentration of iodoacetate (0.005 mM) produced less of an inhibition of glucose uptake (Δglucose, −1.8 ± 0.8 mmol/liter) and less of an inhibition of leptin secretion (−51.0 ± 16%) than insulin alone ($P < 0.02$; Fig. 5). By simple regression, the release of leptin was related to the concentration of iodoacetate and was highly correlated with the change in glucose in the medium (Table 1). However, by multiple regression, the leptin secreted at 96 h was related to the change in glucose, but not to the concentration of iodoacetate (Table 1).

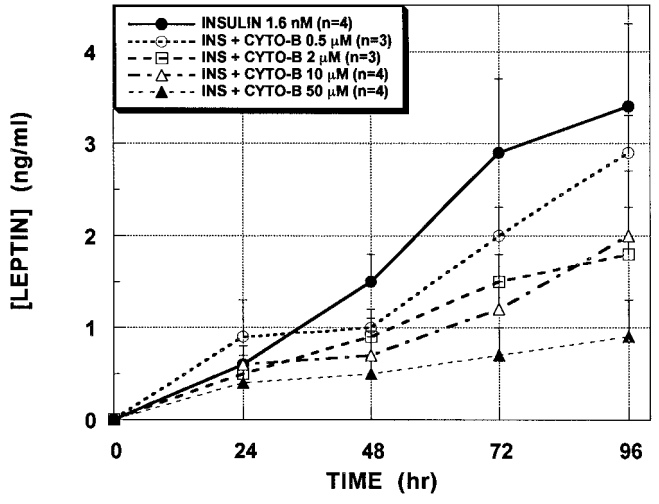

FIG. 4. Effects of inhibiting glucose transport with cytochalasin B on leptin concentrations from 0–96 h in medium from isolated rat adipocytes in primary culture for 96 h with 1.6 nM insulin.

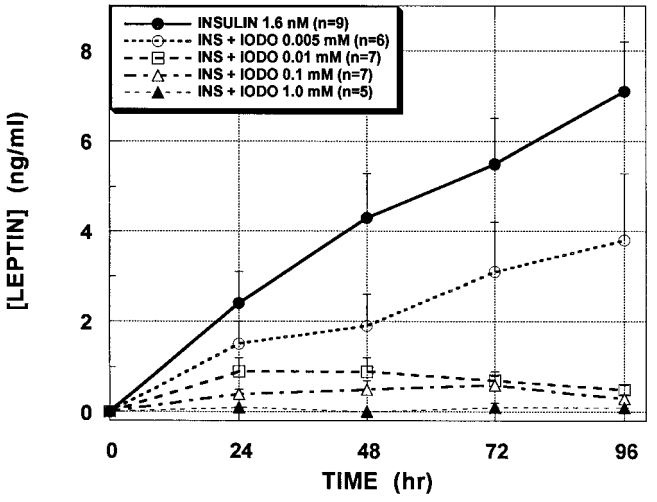

FIG. 5. Effects of inhibiting glycolysis with iodoacetate on leptin concentrations from 0–96 h in medium from isolated rat adipocytes in primary culture for 96 h with 1.6 nM insulin.

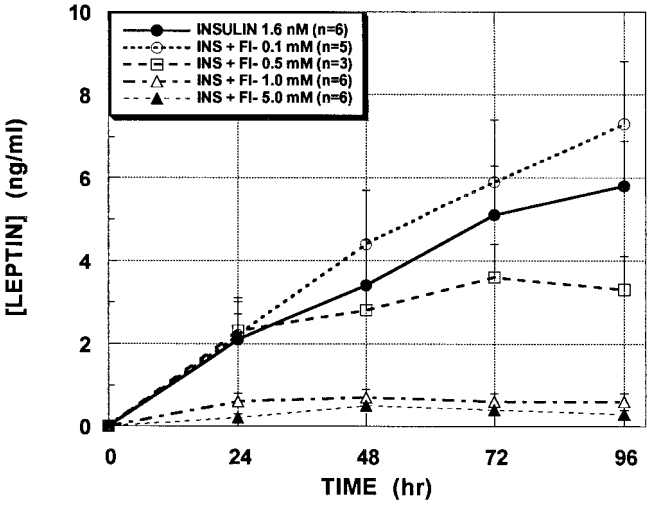

FIG. 6. Effects of inhibiting glycolysis with NaFl on leptin concentrations from 0–96 h in medium from isolated rat adipocytes in primary culture for 96 h with 1.6 nM insulin.

Effects of NaFl (0.1–5.0 mM)

The effect of inhibiting glycolysis with NaFl was examined. The two highest concentrations of NaFl (5.0 and 1.0 mM) completely inhibited glucose uptake (Δglucose, 0.2 ± 0.1 and 0.0 ± 0.3 mmol/liter, respectively). The 0.5 mM concentration of NaFl produced less of an inhibition of glucose uptake (Δ, −2.1 ± 0.6 mmol/liter), and the lowest concentration (0.1 mM) of NaFl did not inhibit glucose uptake (Δglucose, −3.9 ± 0.5 mmol/liter) compared with the effect of insulin alone. The two highest concentrations of NaFl (5.0 and 1.0 mM) markedly inhibited leptin secretion (−81 ± 6% *vs.* insulin alone; $P < 0.0001$). The next concentration of NaFl (0.5 mM) produced an intermediate inhibition of leptin secretion (−47 ± 15% of insulin alone; $P < 0.05$). The 0.1-mM concentration of NaFl did not inhibit leptin secretion (−4 ± 15% *vs.* insulin alone; P = NS; Fig. 6).

Overall, the decline in medium glucose was significantly

correlated with the concentration of NaFl and highly correlated with the 96 h leptin concentration (Table 1). By multiple regression, the amount of leptin secreted at 96 h was strongly correlated with the change in glucose in the medium ($P < 0.0001$), but not to the NaFl concentration (Table 1).

Effects of insulin, 2-DG, and NaFl on leptin (ob) mRNA and 18S ribosomal RNA

The effects of inhibiting glucose uptake and metabolism with 2-DG or NaFl on leptin gene expression and ribosomal 18S RNA were examined. As shown in Fig. 7A, leptin (*ob*) mRNA was detectable in adipocytes incubated for 48 h either with 1.6 nM insulin or without insulin (control). However, the leptin mRNA signal was reduced to near undetectable levels when adipocytes were incubated with 1.6 nM insulin and either 2-DG (10 mg/ml) or 1.0 mM NaFl (Fig. 7A). The effect of 2-DG and NaFl was specific, because in the same samples there was no effect of these concentrations of 2-DG or NaFl on 18S ribosomal RNA (Fig. 7B) or on nonspecific RNA bands (with a different mol wt than leptin mRNA) that could be detected on the Northern blots after long exposures (data not shown). Leptin mRNA was significantly reduced by 2-DG or NaFl regardless of whether the signal was normalized for 18S ribosomal signal ($P = 0.0174$). Qualitatively similar effects of 2-DG or NaFl were observed in cultures incubated for 96 h ($P = 0.0228$; data not shown).

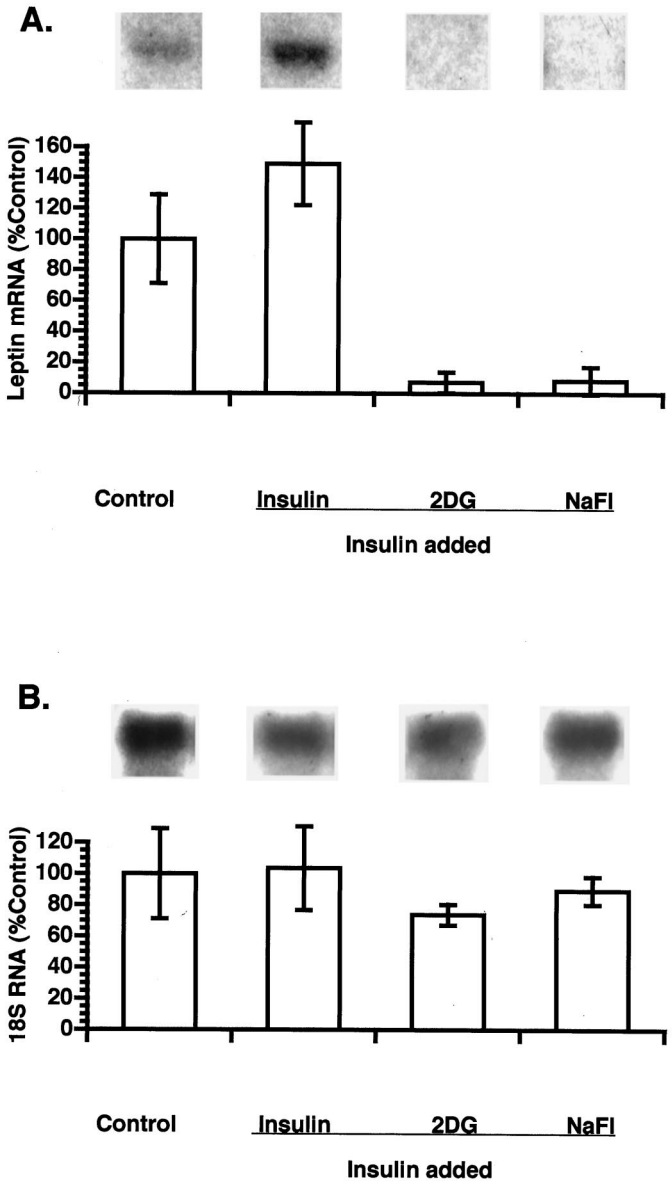

FIG. 7. A, Effects of no insulin (control), 1.6 nM insulin, and 1.6 nM insulin plus 10 mg/dl 2-DG or 1.0 mM NaFl on leptin (*ob*) mRNA after 48 h of incubation, as assessed by Northern blots. The *inset above each bar* is representative of the signal obtained for each condition. B, Effects of control (no insulin), 1.6 nM insulin, and 1.6 nM insulin plus 10 mg/dl 2-DG or 1.0 mM NaFl on 18S ribosomal RNA after 48 h of incubation, as assessed by Northern blots. The *inset above each bar* is representative of the signal obtained for each condition.

Effects of fructose (5 mM)

The addition of 5 mM fructose to medium of cultures in which the glucose concentration was minimized by diluting the Matrigel 1:2 and using glucose-free DMEM with 1% serum augmented leptin secretion after 48 h. The initial response in the control wells was probably due to the residual glucose (~1.5 mmol/L) in the diluted Matrigel. However, both the integrated AUC from 0–96 h ($P < 0.02$) and the leptin concentration at 96 h ($P < 0.01$) were increased by fructose (Fig. 8).

Discussion

In the present study we found that addition of physiological concentrations of insulin stimulates leptin secretion from isolated rat adipocytes in primary culture. In this *in vitro* system we did not see an acute effect of insulin on leptin secretion. This is in agreement with previous reports that have demonstrated that the expression of *ob* gene and leptin protein release are not acutely regulated by insulin *in vivo* and *in vitro* (17, 36). The strong correlation between adipocyte glucose uptake measured by the decrease in glucose in the media during incubation with insulin and the amount of

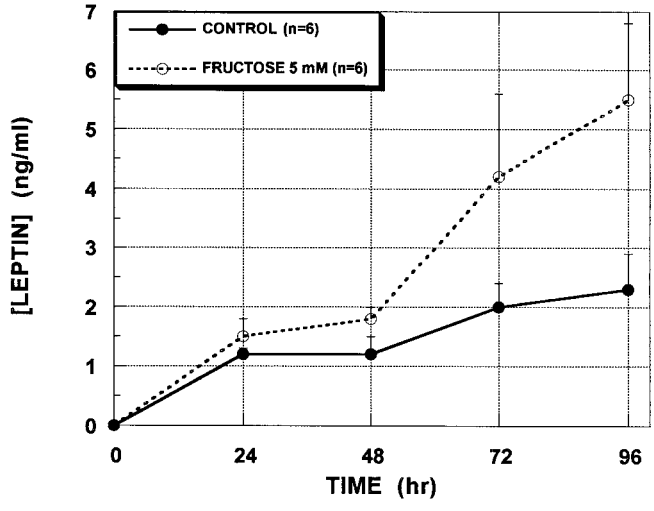

FIG. 8. Effect of fructose (5 mM) on leptin concentrations from 0–96 h in medium from isolated rat adipocytes in primary culture with a low (~1.5 mmol/liter) initial glucose concentration (n = 6/treatment).

leptin secreted from isolated adipocytes is consistent with the hypothesis that the rate of glucose metabolism is a determinant of leptin secretion. In addition, the absolute insulin concentration was not related to the leptin response, independently of the effect of insulin to increase glucose uptake.

Blockade of glucose transport with 2-DG, phloretin, or cytochalasin B at concentrations at or below those typically used in adipocytes (29, 30) produced a dose-dependent decrease in leptin secretion in the presence of high physiological concentrations of insulin. The competitive inhibition produced by 2-DG could be reversed by the addition of a high concentration of glucose, suggesting that 2-DG did not inhibit leptin secretion via a nonspecific toxic effect on the adipocytes. As expected, the inhibition by phloretin was not reversed by glucose, as phloretin is not a competitive inhibitor and, therefore, produces an irreversible inhibition of glucose transport that is not readily overcome by high glucose concentrations. These experiments provide evidence that glucose uptake is required to increase leptin secretion from isolated adipocytes despite the presence of high physiological insulin concentrations.

Inhibition of glycolysis with either iodoacetate or NaFl at low concentrations (31, 32) also produced concentration-dependent inhibition of leptin secretion in the presence of insulin. When glycolysis is inhibited, glycolytic intermediates accumulate, resulting in a secondary impairment of glucose uptake. As with primary blockade of glucose uptake, during inhibition of glucose metabolism by either glycolytic inhibitor, the amount of glucose taken up over 96 h of incubation was highly correlated with the amount of leptin secreted despite the presence of insulin. These results suggest that the stimulation of leptin secretion by insulin is unlikely to be due to a direct effect of insulin *per se*, but is secondary to the effect of insulin to stimulate glucose uptake and metabolism in adipocytes.

We also found that inhibition of glucose transport and metabolism with 2-DG or glycolysis with NaFl markedly inhibited leptin (*ob*) gene expression, as assessed by Northern blot analysis of leptin mRNA. In the same cultures, 18S ribosomal RNA levels were unaffected by either 2-DG or NaFl, suggesting that the decrease in leptin gene expression was not due to a nonspecific overall effect of these inhibitors to impair adipocyte RNA synthesis. In addition, we examined the amount of heparin-released lipoprotein lipase (LPL) from adipocytes cultured with the various inhibitors (data not presented). Although LPL was modestly decreased by the inhibitors (~25–50% of insulin-stimulated levels), the suppression of leptin secretion was significantly greater (80–90%), suggesting a relative specificity of blocking glucose uptake and metabolism on leptin secretion *vs.* that on another protein (LPL) produced by adipose tissue. Lastly, the effects of the blockers to inhibit leptin expression and secretion are unlikely to be due to a depletion of adipocyte energy stores, as it is known that adipocytes can generate energy (ATP) by oxidizing fatty acids via mitochondrial β-oxidation (37, 38).

Taken together, these data suggest a physiological role for glucose in the regulation of leptin expression and secretion by adipocytes. Accordingly, we hypothesize that during fasting, when circulating insulin and glucose concentrations are low and glucocorticoids are elevated, leptin secretion declines secondary to decreased glucose transport into adipose tissue. Upon refeeding, increases in circulating insulin and glucose and the resulting increases in adipose glucose uptake and metabolism stimulate leptin secretion and restores circulating leptin concentrations to prefasting levels. This model, therefore, can explain the effects of fasting and refeeding on circulating leptin in humans (2–4) and rodents (5–7). In addition, the nocturnal increase in plasma leptin observed in humans could potentially arise as a delayed consequence of increased insulin-stimulated glucose metabolism following meals (8). The effect of glucose infusions to prevent the fall of plasma leptin during fasting in human subjects may be similarly mediated (2).

Thus, leptin secretion appears to reflect the amount of glucose transported and metabolized by adipose tissue. There is convincing evidence that suggests that a significant portion of glucose entering adipose tissue is metabolized to lactate and released (34, 35). This lactate may contribute to the pool of gluconeogenic precursors during fasting. Our results show that when a smaller proportion of glucose carbon taken up by adipocytes is released as lactate, more leptin is secreted. These data are consistent with the changes in leptin secretion observed during fasting and refeeding. In addition, fructose, in the presence of low glucose concentrations, stimulates leptin secretion, demonstrating that a non-glucose substrate can induce the adipocyte to secrete leptin and suggesting that stimulation of leptin secretion by glucose metabolism occurs downstream of phosphofructokinase.

In summary, blockade of glucose transport or inhibition of glycolysis inhibits leptin secretion from and gene expression in isolated cultured adipocytes. The secretion of leptin is directly proportional to the amount of glucose taken up by the adipocytes. These results suggest that leptin secretion is linked to glucose transport and metabolism and help to explain the known effects of feeding/fasting and long term glucose and insulin administration on circulating leptin concentrations.

Acknowledgments

We acknowledge the expert technical assistance of Ms. Debbie Porter, and thank Drs. Richard Freedland, Michael Schwartz, and Barbara Kahn for their helpful advice and discussion of the results.

References

1. **Caro JR, Sinha MK, Kolaczynski JW, Zhang PL, Considine RV** 1996 Leptin: the tale of an obesity gene. Diabetes 45:1455–1461
2. **Boden G, Chen X, Mozzoli M, Ryan I** 1996 Effect of fasting on serum leptin in normal human subjects. J Clin Endocrinol Metab 81:3419–3423
3. **Weigle DS, Duell PB, Connor WE, Steiner RA, Soules MR, Kuijper JL** 1997 Effect of fasting, refeeding, and dietary fat restriction on plasma leptin levels. J Clin Endocrinol Metab 82:561–565
4. **Dubuc GR, Phinney SD, Stern JS, Havel PJ,** Changes of serum leptin and endocrine and metabolic parameters after 7 days energy restriction in men and women. Metabolism, in press
5. **Ahima SR, Prabakaran D, Mantzoros C, Qu D, Lowell B, Maratos-Flier E, Flier JS** 1996 Role of leptin in the neuroendocrine response to fasting. Nature 382:250–252
6. **Hardie LJ, Rayner DV, Holmes S, Trayhurn P** 1996 Circulating leptin levels are modulated by fasting, cold exposure and insulin administration in lean but not Zucker (*fa/fa*) rats as measured by ELISA. Biochem Biophys Res Commun 223:660–665
7. **Ahren B, Mansson S, Gingerich RL, Havel PJ** 1997 Regulation of plasma leptin in mice: influence of age, high-fat diet and fasting. Am J Physiol 273:R113–R120
8. **Sinha MK, Ohannesian JP, Heiman ML, Kriauciunas A, Stephens TW,**

Magosin S, Marco C, Caro JF 1996 Nocturnal rise of leptin in lean, obese, and non-insulin-dependent diabetes mellitus subjects. J Clin Invest 97:1344–1347
9. **Becker DJ, Ongemba LN, Brichard V, Henquin J-C, Brichard SM** 1995 Diet- and diabetes-induced changes of *ob* gene expression in rat adipose tissue. FEBS Lett 371:324–328
10. **Cusin R, Sainsbury A, Doyle P, Rohner-Jeanrenaud F, Jeanrenaud B** 1995 The *ob* gene and insulin: a relationship leading to clues to the understanding of obesity. Diabetes 44:1467–1470
11. **Saladin R, De Vos P, Guerre-Millo M, Leturque A, Girard J, Staels B, Auwerx J** 1995 Transient increase in obese gene expression after food intake or insulin administration. Nature 377:527–529
12. **Leroy P, Dessolin S, Villageois P, Moon BC, Friedman JM, Ailhaud G, Dani C** 1996 Expression of *ob* gene in adipose cells. J Biol Chem 271:2365–2368
13. **Yoshida T, Hayashi M, Monkawa T, Saruta T** 1996 Regulation of obese gene expression by hormonal factors in primary cultures of rat adipocytes. Eur J Endocrinol 135:619–625
14. **Havel PJ, Kasim-Karakas S, Mueller W, Johnson PR, Gingerich RL, Stern JS** 1996 Relationship of plasma leptin to plasma insulin and adiposity in normal weight and overweight women: effects of dietary fat content and sustained weight loss. J Clin Endocrinol Metab 81:4406–4413
15. **Larrson H, Elmstahl S, Ahren B** 1996 Plasma leptin levels correlate to islet function independently of body fat in postmenopausal women. Diabetes 45:1580–1584
16. **Dagogo-Jack S, Fanelli C, Paramore D, Brothers J, Landt M** 1996 Plasma leptin and insulin relationships in obese and nonobese human. Diabetes 45:695–698
17. **Kolaczynski JW, Nyce MR, Considine RV, Boden G, Nolan JJ, Henry R, Mudaliar SR, Olefsky J, Caro JF** 1996 Acute and chronic effect of insulin on leptin production in humans: studies *in vivo* and *in vitro*. Diabetes 45:699–701
18. **Havel PJ, Aoki TT, Grecu EO, Stern JS, Kasim-Karakas S** 1996 Leptin/adiposity relationships in intensively treated IDDM and NIDDM and increased plasma leptin after 6 hours of high dose insulin infusion. Obesity Res 4:15S (Abstract)
19. **Utriainen R, Malmstrom R, Makimattila S, Yki-Jarvinen H.** 1996 Supraphysiological hyperinsulinemia increases plasma leptin concentrations after 4 h in normal subjects. Diabetes 45:1364–1366
20. **Havel PJ** 1997 Glucose infusion increases circulating leptin in proportion to adipose stores in rhesus monkeys. J Endocrinol Diabetes [Suppl 3] 105:37–38
21. **Sonnenberg GE, Krakower GR, Hoffmann RG, Maas DL, Hennes MMI, Kissebah AH** 1996 Plasma leptin concentrations: effects of extended fasting and stepwise increases in glucose infusions. Obesity Res 4:13S (Abstract)
22. **Mizuno T, Bergen H, Kleopoulos S, Bauman WA, Mobbs CV** 1996 Effects of nutritional status and aging on leptin gene expression in mice: importance of glucose. Horm Metab. Res 28:679–684
23. **Havel PJ, Uriu-Hare JY, Stanhope KL, Stern JS, Keen CL** 1997 Marked and rapid decrease of plasma leptin in insulin deficient diabetes: reversal by insulin. Diabetologia [Suppl 1] 40:A270 (Abstract)
24. **Havel PJ, Connors MH, Acerini CL, Crowne ED, Dunger DB** 1997 Plasma leptin is increased proportionally to adiposity by physiologic insulinemia in insulin-dependent diabetes. Diabetologia [Suppl 1] 40:A268 (Abstract)
25. **Hazen SA, Rowe WA, Lynch CJ** 1995 Monolayer cell culture of freshly isolated adipocytes using extracellular basement membrane components. J Lipid Res 36:868–878
26. **Hajduck EJ, Guerre-Millo M, Hainault IA, Guichard CM, Lavau MM** 1992 Expression of glucose transporters (GLUT1 and GLUT4) in primary cultured rat adipocytes: differential evolution with time and chronic insulin effect. J Cell Biochem 49:251–258
27. **Rodbell M** 1964 Metabolism of isolated fat cells. I. Effects of hormones on glucose metabolism and lipolysis. J Biol Chem 239:375–380
28. **Brown J** 1962 Effects of 2-deoxyglucose on carbohydrate metabolism: review of the literature and studies in the rat. Metabolism 11:1098–1112
29. **Wierlinga T, Van Putten JP, Krans HM** 1981 Rapid phloretin-induced dephosphorylation of 2-deoxyglucose-6-phosphate in rat adipocytes. Biochem Biophys Res Commun 103:841–847
30. **Marette A, Bukowiecki LJ** 1991 Noradrenaline stimulates glucose transport in rat brown adipocytes by activating thermogenesis. Evidence that fatty acid activation of mitochondrial respiration enhances glucose transport. Biochem J 277:119–124
31. **Nimmo GA, Nimmo HG** 1984 Studies of rat adipose tissue glycerol phosphate acyltransferase. Biochem J 224:101–108
32. **Holland RI** 1977 Effect of fluoride on glycerol production in rat adipocytes *in vitro*. Acta Pharmacol Toxicol 41:97–102.29
33. **Mizuno TM, Bergen H, Funabashi T, Kleopoulos SP, Zhong YG, Bauman WA, Mobbs CB** 1996 Obese gene expression: reduction by fasting and stimulation by insulin and glucose in lean mice, and persistent elevation in acquired (diet-induced) and genetic (yellow agouti) obesity. Proc Natl Acad Sci USA 93:3434–3438
34. **Digirolamo M, Newby FD, Lovejoy J** 1992 Lactate production in adipose tissue: a regulated function with extra-adipose implications. FASEB J 6:2405–2412
35. **Heckler BK, Carey GB** 1997 Lactate production by swine adipocytes: effects of age, nutritional status, glucose concentration, and insulin. Am J Physiol 272:E957–E966
36. **Vidal H, Auboeuf D, De Vos P, Staels B, Riou JP, Auwerx J, Laville M** 1996 The expression of *ob* gene is not acutely regulated by insulin and fasting in human abdominal subcutaneous adipose tissue. J Clin Invest 98:251–255
37. **Mayers PA** 1993 Lipid transport and storage. In: Murray RK, Granner DK, Mayes PA, Rodwell VW (eds) Harper's Biochemistry. Appleton and Lange, Norwalk, pp 250–265
38. **Moore KH, Tsatsos P, Staudacher DM, Kiechle FL** 1996 Counter modulation of adipocyte mitochondrial processes by insulin and *S*-oxalylglutathione. Int J Biochem Cell Biol 28:183–191