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Hypothalamic Leptin Signaling Regulates Hepatic Insulin Sensitivity via a Neurocircuit Involving the Vagus Nerve

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1 **Abstract**

2 Recent evidence suggests that hormones such as insulin and leptin act in the
3 hypothalamus to regulate energy balance and glucose metabolism. Here, we show that in
4 leptin receptor-deficient Koletsky (fa^k/fa^k) rats, adenovirally-induced expression of leptin
5 receptors in the area of the hypothalamic arcuate nucleus improved peripheral insulin
6 sensitivity via enhanced suppression of hepatic glucose production, with no change of
7 insulin-stimulated glucose utilization. This effect was associated with increased insulin
8 signal transduction via phosphatidylinositol-3-OH kinase (as measured by pY-IRS-1 and
9 pS-PKB/Akt) in liver, but not skeletal muscle, and with reduced hepatic expression of the
10 gluconeogenic genes, glucose-6-phosphatase and phosphoenolpyruvate kinase.
11 Moreover, the beneficial effects of hypothalamic leptin signaling on hepatic insulin
12 sensitivity were blocked by selective hepatic vagotomy. We conclude that hypothalamic
13 leptin action increases peripheral insulin sensitivity primarily via effects on the liver, and
14 that the mechanism underlying this effect is dependent on the hepatic branch of the vagus
15 nerve.

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1 **Introduction**

2 Growing evidence suggests that the brain receives input from both nutrient-related and
3 hormonal signals such as insulin and leptin that convey information regarding levels of
4 circulating energy substrates as well as fuel stored in the form of fat. In response to this
5 input, key brain areas such as the hypothalamus activate pathways that regulate food
6 intake, energy expenditure, autonomic function and glucose metabolism to maintain both
7 energy and glucose homeostasis (1). Consequently, conditions associated with reduced
8 or defective “adiposity” signaling are predicted to favor both increased food intake and
9 insulin resistance in peripheral tissues. Although several studies have investigated the
10 control of glucose metabolism by hypothalamic insulin action, less is known about how
11 leptin affects insulin sensitivity.

12

13 In addition to its well known effects in peripheral tissues, recent studies support a role for
14 brain insulin action in the regulation of glucose homeostasis. For example, neuron-
15 specific insulin receptor (IR)- and insulin receptor substrate-2 (IRS-2)-deficient mice are
16 characterized by mild obesity and insulin resistance that is at least partially independent
17 of increased body fat (2-4). Further, inducible inactivation of the IR in both the brain and
18 peripheral tissues in mice causes a more pronounced hyperglycemia compared to
19 peripheral tissues alone (5). Consistent with these studies, both intrahypothalamic
20 administration of antisense oligonucleotides to reduce IR signaling and
21 intracerebroventricular (icv) infusion of an inhibitor of phosphatidylinositol-3-OH kinase
22 (PI3K, a major intracellular mediator in insulin action) cause insulin resistance in rats (6),
23 while conversely, infusion of insulin either into ventricular cerebrospinal fluid (CSF) or

1 directly into the hypothalamic arcuate nucleus (ARC) improved insulin sensitivity via
2 enhanced insulin suppression of hepatic glucose production (HGP), rather than increased
3 glucose uptake (7).

4

5 Like insulin, leptin signaling in the brain is also implicated in the control of insulin
6 sensitivity in peripheral tissues. Models of genetic leptin deficiency (*ob/ob* or
7 lipodystrophic mice) are characterized by severe insulin resistance and type 2 diabetes (8,
8 9), and leptin treatment ameliorates these conditions via a mechanism that involves the
9 central nervous system (CNS) and cannot be explained by changes of food intake (9-11).

10 These observations are further supported by our recently published evidence that
11 restoring leptin signaling to the hypothalamic ARC of leptin receptor-deficient Koletsky
12 (*fa^k/fa^k*) rats improves their insulin sensitivity via a mechanism that is, at least in part,
13 independent of food intake and body weight (12). Similarly, Elmquist and colleagues
14 reported a dramatic improvement in glucose homeostasis in leptin receptor-deficient mice
15 in which leptin signaling was selectively restored to the ARC (13). Thus, intact neuronal
16 signaling by both insulin and leptin appear to be important for maintenance of normal
17 insulin sensitivity.

18

19 One hypothesis forwarded to explain how these circulating signals act in the CNS to
20 regulate insulin sensitivity proposes that key subsets of hypothalamic neurons regulate
21 HGP via descending projections to hindbrain areas that control autonomic outflow to the
22 liver via the vagus nerve. Consistent with this, the ability of hypothalamic insulin and

1 free fatty acids (FFAs) to suppress HGP is blocked by hepatic branch vagotomy, but
2 unaffected by selective vagal deafferentiation (14, 15).

3

4 Based on these observations, we hypothesized that the effect of ARC-directed leptin
5 receptor gene therapy to increase insulin sensitivity in obese Koletsky rats is mediated via
6 enhanced insulin-induced suppression of HGP, rather than by increased glucose uptake.

7 To test this hypothesis, we directed expression of either the signaling form of the leptin
8 receptor or a reporter gene to the ARC of obese Koletsky rats and measured insulin
9 sensitivity using the euglycemic-hyperinsulinemic clamp technique. Our results

10 demonstrate that restored hypothalamic leptin signaling enhanced insulin-induced
11 suppression of HGP and that this effect was blocked by hepatic vagotomy (HV). The
12 mechanism underlying this effect appears to involve increased hepatic insulin signal
13 transduction via PI3K, resulting in reduced gluconeogenic gene expression. Taken
14 together, our findings support the existence of a brain-liver neurocircuit that plays an
15 important role in the regulation of glucose metabolism by leptin.

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1 **Materials and Methods**

2 **Experimental animals**

3 Adult male obese (fa^k/fa^k) Koletsky rats (Vassar College, Poughkeepsie, NY) were
4 generated from serial backcrosses (N10 equivalent) of the fa^k mutation to the inbred rat
5 strain, LA/N. Additional obese Koletsky rats were obtained from Charles River
6 Laboratories (Wilmington, MA). All animals were housed individually in a specific
7 pathogen-free environment, maintained in a temperature-controlled room with a 12-12h
8 light:dark cycle and provided with *ad libitum* access to water and standard laboratory
9 chow (PMI Nutrition International Inc., Brentwood, MO), unless otherwise stated. All
10 study protocols were approved by the Animal Care and Use Committee at the University
11 of Washington and conducted in accordance with NIH guidelines for the care and use of
12 laboratory animals.

13

14 **Adenovirus microinjection**

15 Adenovirus (Ad) expressing either green fluorescent protein (Ad-GFP; 2.7×10^{12} pfu/ml)
16 or a construct that contains the mouse $lepr^b$ and also expresses GFP (Ad-LEPR-B;
17 4.7×10^{12} pfu/ml), were microinjected bilaterally into the ARC of obese Koletsky rats
18 under isoflurane anesthesia as previously described (12, 16-18) (n=6-8/group). Bilateral
19 microinjection of adenovirus and implantation of an indwelling catheter in both the right
20 internal jugular vein and the left carotid artery were performed during the same surgical
21 session 7d prior to testing via the euglycemic-hyperinsulinemic clamp technique.
22 Buprenorphine hydrochloride (0.3 mg/kg; Rickett Colman Pharmaceuticals, VA) was
23 administered at the completion of the surgery. Upon study completion, anatomical

1 distribution of adenoviral gene expression was assessed by visualization of GFP in
2 coronal brain sections by fluorescent microscopy, which permits detection of either
3 adenoviral construct (Ad-GFP or Ad-LEPR-B-GFP). Animals in which the ARC was not
4 successfully targeted (<10%) were removed from the study.

5

6 **Selective hepatic branch vagotomy**

7 Prior to adenoviral injection and implantation of intravenous catheters, a subgroup of
8 obese Koletsky rats were subjected to selective hepatic vagotomy (HV) or a sham
9 operation (Sham) to generate 4 treatment groups: 1) Ad-GFP-Sham, 2) Ad-GFP-HV, 3)
10 Ad-LEPR-B-Sham and 4) Ad-LEPR-B-HV (n=4-6/group). Briefly, a laparotomy
11 incision was made on the ventral midline and the abdominal muscle wall opened,
12 revealing the gastrointestinal tract in the peritoneum. The gastrohepatic ligament was
13 severed, and the stomach was gently retracted onto sterile saline soaked cotton gauze,
14 revealing the descending ventral esophagus and the ventral subdiaphragmatic vagal
15 trunk. The hepatic branch of this vagal trunk was visualized using a neurosurgical
16 dissecting scope under 10-20X, and the hepatic branch of the vagus was ligated using two
17 6-0 silk ties. The hepatic nerve trunk was then transected by microcautery in between the
18 two sutures, severing and cauterizing the hepatic vagus, thereby minimizing the
19 possibility of regeneration. The abdominal muscle wall incision was closed, and the skin
20 incision was closed with stainless steel wound clips.

21

22 **Body composition analysis**

1 Determinations of body lean mass and fat mass were made in conscious rats both the day
2 prior to adenoviral microinjection and euglycemic-hyperinsulinemic clamp studies using
3 quantitative magnetic resonance (QMR) (EchoMRI Body Composition Analyzer; Echo
4 Medical Systems, Houston, TX).

5

6 **Euglycemic-hyperinsulinemic clamps.**

7 Six days following adenoviral microinjections, 24-wk old obese Koletsky rats were
8 provided with 6g of food at dark cycle onset and then fasted overnight. This early time
9 point for metabolic studies was selected as it preceded the effect of ARC-directed leptin
10 receptor gene therapy to reduce food intake and body weight. Animals were placed into a
11 clear animal enclosure with bedding and connected to a rat infusion system (Instech
12 Solomon, Plymouth Meeting, PA) to allow simultaneous sampling from the artery and
13 infusion into the vein in a conscious, unrestrained animal. The clamp protocol consisted
14 of a 120-min tracer equilibration period ($t=-120$ to 0 min) followed by a 120 min
15 experimental period ($t=0$ to 120 min). A blood sample was obtained at $t=-120$ min for
16 determination of fasting plasma glucose, insulin, leptin and free fatty acids (FFA). A 24
17 μCi prime of [$3\text{-}^3\text{H}$] glucose was given at $t=-120$ min for 3 min followed by a continuous
18 $0.2 \mu\text{Ci}/\text{min}$ infusion for 2-h. At $t=-30, -20, -10, 0$ min, blood samples of $80 \mu\text{L}$ were
19 taken for determination of basal glucose turnover. Two hours after the basal period, a
20 primed continuous infusion of regular human insulin ($60 \text{ mU}/\text{kg}$ bolus followed by 5
21 $\text{mU}/\text{kg}/\text{min}$ Humulin[®]R, Eli Lilly, Indianapolis, IN) was administered at $t=0$ min. In
22 studies determining if vagal outflow to the liver is required for the effect of hypothalamic
23 leptin signaling on peripheral insulin sensitivity, a primed continuous infusion of human

1 insulin at 2.5 mU/kg/min was used instead, as these studies were performed in 12-wk,
2 relatively more insulin sensitive Koletsky rats (mean BW~350g). The [3-³H] glucose
3 infusion was increased to 0.3 μCi/min (at t=0 min) for the remainder of the experiment
4 (t=120 min) to keep specific activity (SA) constant. During the clamp, glucose levels
5 were determined every 10 min using a hand held glucometer (Accu-Chek, Roche,
6 Indianapolis, IN) and maintained at ~110 mg/dl by infusion of a 50% dextrose solution,
7 as needed. Plasma insulin levels and clamp glucose turnover rates were calculated from
8 80 μL blood samples drawn at 10 min intervals during the last 30 minutes of the clamp.

9

10 **Processing of plasma samples**

11 Plasma for [3-³H] glucose determinations was deproteinized with Ba(OH)₂ and ZnSO₄,
12 then dried overnight at 60°C. Plasma glucose levels were measured using a GM9D
13 glucose direct analyzer (Analox Instruments, Ltd., United Kingdom). Plasma
14 immunoreactive insulin and leptin levels were determined by ELISA (Crystal Chem,
15 Chicago, IL). FFAs were measured using a colorimetric assay kit that relies on fatty acid
16 as substrate for enzymatic acylation of CoA (WAKO Chemicals, Richmond, VI) while
17 plasma corticosterone levels were measured using EIA (Diagnostics Systems
18 Laboratories, Webster, TX).

19

20 **Tissue processing and biochemical analysis**

21 We have previously demonstrated that hypothalamic leptin signaling improves peripheral
22 insulin sensitivity, as measured by an insulin tolerance test (12). In this same group of
23 animals, sixteen days following adenoviral microinjection, animals were fasted overnight

1 and received an intraperitoneal (ip) injection of either insulin (10U/kg; Humulin®R, Eli
2 Lilly, Indianapolis, IN) or vehicle. Twenty minutes later, animals were euthanized and
3 liver and skeletal muscle (gastrocnemius) were immediately excised and snap frozen for
4 subsequent analysis.

5

6 Muscle and liver were homogenized in T-Per lysis buffer (10µl/mg tissue) (Pierce,
7 Rockford, IL) supplemented with protease and phosphatase inhibitor cocktails (Roche
8 Diagnostics Corporation, Indianapolis, IN). Homogenates were centrifuged, pellets
9 discarded and supernatants retained for determination of protein content using a Micro
10 BCA protein assay kit (Pierce, Rockford, IL) and equal amounts of protein were used for
11 each condition in each assay. Tyrosine phosphorylation (pY) of insulin-receptor-
12 substrate-1 (IRS-1) was assessed by Western blot using a monoclonal anti-
13 phosphotyrosine antibody (Cell Signaling Technology, Beverly, MA) following
14 immunoprecipitation with an anti-IRS-1 antibody (Cell Signaling Technology, Beverly,
15 MA) and SDS-PAGE of liver and muscle extracts. The membranes were stripped and re-
16 probed with an anti-IRS-1 antibody to verify equal amounts of total IRS-1 protein. IRS-1
17 tyrosine phosphorylation protein bands were quantified by densitometry using Image J
18 software, while activation of PI3K signal transduction was assessed by measuring serine
19 phosphorylation of Akt (residue 473), respectively, using an ELISA assay (Invitrogen,
20 Camarillo, CA).

21

22 **RT-PCR**

1 Total RNA was extracted from liver and muscle using TRIzol B according to
2 manufacturers' instructions (MRC, Cincinnati, OH), quantitated by spectrophotometry at
3 260nm, reverse-transcribed (1µg) with AMV reverse transcriptase (Promega, Madison,
4 WI) and real-time PCR performed on a ABI Prism 7900 HT (Applied Biosystems, Foster
5 City, CA) as previously described (16). Expression levels of each gene were normalized
6 to a house-keeping gene (18S RNA) and expressed as a % of controls. Non template
7 controls were incorporated into each PCR run.

8

9 **Triglyceride content**

10 Liver and muscle tissue triglyceride content was measured in frozen tissues samples
11 collected as described above using the Folch method (19) for lipid extraction followed by
12 spectrophotometric measurement of triglyceride content (Thermo Electron, Louisville,
13 CO). In some animals, liver triglyceride levels were also determined using the Echo 3-in-
14 1 MRI analyzer (Echo Medical Systems, Houston, TX).

15

16 **Calculations**

17 After deproteinization with and $ZnSO_4$ and $Ba(OH)_2$ and dried 12 hours at 60°C, plasma
18 [$3\text{-}^3\text{H}$] glucose radioactivity was determined by liquid scintillation on a Beckman Tri-
19 Carb 2810 (20). Sample radioactivity divided by plasma glucose concentration gives the
20 plasma glucose specific activity. Glucose rate of appearance (Ra) and rate of disposal
21 (Rd) were calculated by using (Steele's) non-steady-state equations. Endogenous Ra
22 (Endo Ra) was determined by subtracting the glucose infusion rate (GIR) from the Ra.

23

1 **Statistical analysis**

2 All results are expressed as mean \pm SEM. Statistical analyses were performed using
3 Statistica (Version 7.1; StatSoft, Inc., Tulsa, OK). A one-way analysis of variance with a
4 LSD post-hoc test was used to compare mean values between multiple groups and a two-
5 sample unpaired student's t-test was used for two-group comparisons. In all instances,
6 probability values of <0.05 were considered significant.

1 **Results**

2 **Effect of ARC-directed leptin receptor gene therapy on insulin sensitivity**

3 To determine the effect of hypothalamic leptin action on peripheral insulin sensitivity, we
4 performed euglycemic-hyperinsulinemic clamp experiments in obese Koletsky rats (mean
5 BW=715g) 7d following bilateral microinjection of adenovirus expressing either GFP or
6 LEPR-B directed to the ARC, at a time there was no significant differences between
7 groups with respect to body weight, food intake or body composition (Table 1).
8 Consistent with our previously reported results, basal measures of plasma glucose,
9 insulin, leptin and FFA were similar between groups following an overnight fast (Table
10 1). During the clamp procedure, arterial glucose (Fig. 1A) and plasma insulin levels were
11 similar between the two groups ($p=ns$) (Table 1). However, the GIR required to maintain
12 euglycemia was increased by 43% in animals that received Ad-LEPR-B compared with
13 those receiving Ad-GFP (Fig. 1B). These data confirm our previous findings that
14 restored hypothalamic leptin signaling improves peripheral insulin sensitivity (12).

15

16 During both the basal and the clamp periods, differences in the rate of glucose utilization
17 measured using tracer dilution techniques were not detected between animals that
18 received Ad-LEPR-B compared to Ad-GFP (Fig. 1C). In contrast, while basal levels of
19 endogenous Ra were similar, the rate of endogenous Ra was significantly reduced during
20 the clamp in animals that received Ad-LEPR-B compared with Ad-GFP (Fig. 1D), such
21 that insulin-mediated suppression of GP was enhanced ($p<0.05$) (Fig. 1F). These data
22 suggest that restoration of leptin receptor signaling to the area of the ARC improves
23 insulin sensitivity of Koletsky rats by increasing insulin-mediated suppression of HGP.

1 To assess the extent to which clamp values were affected by stress associated with the
2 procedure, we measured plasma corticosterone levels throughout the clamp. Since mean
3 corticosterone levels were not different between the groups (Table 1), procedure-related
4 stress was unlikely to have influenced outcomes.

5

6 To gain further insight into the mechanism by which leptin acts in the hypothalamus to
7 suppress HGP, we measured expression of two key gluconeogenic genes, glucose-6-
8 phosphatase (*G6Pase*) and phosphoenolpyruvate kinase (*Pepck*) in liver samples obtained
9 at the completion of the clamp. Relative to Ad-GFP-treated animals, the hepatic
10 expression of *G6Pase* and *Pepck* were significantly lower (by 80% and 51%,
11 respectively) in Ad-LEPR-B-treated compared with Ad-GFP-treated rats ($p < 0.05$ for
12 each) (Fig. 1G,H). Thus, the effect of restored hypothalamic leptin signaling in Koletsky
13 rats to increase insulin-mediated suppression of HGP is associated with reduced
14 gluconeogenic gene expression.

15

16 **Role of vagal outflow in effect of ARC-directed leptin receptor gene therapy to** 17 **suppress GP**

18 We next sought to determine whether the effect of ARC-directed leptin receptor gene
19 therapy to suppress GP involves vagal innervation of the liver. To accomplish this,
20 groups of obese Koletsky rats (mean BW=342g) were subjected to either selective HV or
21 a sham operation prior to ARC-directed microinjection of adenovirus expressing either
22 GFP or LEPR-B, followed 7d later by performance of clamps as described above. The
23 baseline metabolic characteristics of the experimental rats were similar in each group

1 (Table 2). During the clamp procedure, arterial glucose (Fig. 2A), plasma insulin, FFA
2 and corticosterone concentrations were similar in all groups (Table 2). Consistent with
3 our previous experiment, the GIR required to maintain euglycemia was significantly
4 increased in sham animals that received Ad-LEPR-B compared to Ad-GFP directed to the
5 ARC (Fig. 2B). While HV had no effect on GIR in Ad-GFP-treated animals, HV blocked
6 the increase of GIR in Ad-LEPR-B-treated sham controls (Fig. 2B). We next examined
7 whether the vagus-dependent increase in GIR induced by hypothalamic leptin signaling
8 was due to effects on glucose utilization or HGP. Consistent with our earlier
9 observations, insulin-induced suppression of HGP was increased in sham-treated animals
10 that received Ad-LEPR-B compared to Ad-GFP ($p < 0.05$) (Fig. 2F). While the
11 suppression of HGP was similar in Ad-GFP-sham and -HV-treated animals, the increase
12 of HGP suppression induced by hypothalamic leptin receptor gene therapy was blocked
13 by HV ($p < 0.05$) (Fig. 2F). In contrast, the rate of glucose utilization was not affected by
14 either Ad-LEPR-B-treatment or HV ($p = ns$) (Fig. 2E). Moreover, hepatic expression of
15 *G6Pase* and *Pepck* mRNA was reduced by hypothalamic leptin receptor gene therapy in
16 sham-operated animals ($p < 0.05$ for each), but not in animals subjected to HV (Fig.
17 2G,H).

18

19 **Effect of hypothalamic leptin signaling on hepatic insulin signal transduction**

20 The results from the clamp studies led us to ask whether hypothalamic leptin signaling
21 enhances insulin signal transduction selectively in liver and not skeletal muscle. This
22 study was conducted in an experiment designed to examine whether indirect effects may
23 contribute to the effect of hypothalamic leptin signaling to improve peripheral insulin

1 sensitivity and therefore was carried out over a time frame where restored hypothalamic
2 leptin signaling reduces food intake and body weight. Consequently, an additional group
3 of obese Koletsky rats was included that received Ad-GFP and were pair-fed (Ad-GFP-
4 PF) to the intake of Ad-LEPR-B-treated animals (n=8), as previously described (12).
5 Following IP saline, there were no differences in levels of either tyrosine-phosphorylated
6 IRS-1 (pY-IRS-1) or serine-phosphorylated Akt [pS473] (a downstream marker of PI3K
7 activation) in liver tissue from animals that received Ad-LEPR-B, or the reporter gene,
8 regardless of whether they were fed ad libitum or pair-fed (Fig. 3). As expected,
9 systemic insulin injection significantly increased hepatic levels of both pY-IRS-1 and
10 pS473-Akt in all groups compared to saline vehicle ($p<0.05$) (Fig. 3A, B). Importantly,
11 the ability of insulin to increase hepatic levels of both pY-IRS-1 and pS473-Akt was
12 significantly enhanced in animals that received Ad-LEPR-B compared to Ad-GFP
13 ($p<0.05$). This effect cannot be explained by reduced food intake or body weight, as
14 hepatic content of both pY-IRS-1 and pS473-Akt were also significantly greater in Ad-
15 LEPR-B compared to Ad-GFP-PF-treated animals (Fig. 3A). In contrast, the effects of
16 insulin to increase pY-IRS-1 and Akt [pS473] (Fig. 3C,D) in skeletal muscle were not
17 affected by leptin receptor gene therapy.

18

19 To investigate whether changes of tissue lipid accumulation might contribute to centrally-
20 mediated effects of leptin on hepatic glucose metabolism, we measured triglyceride
21 content in both liver and skeletal muscle in this same group of animals. In liver,
22 triglyceride content was significantly reduced in Ad-LEPR-B-treated animals compared
23 with those that received Ad-GFP (20.3 ± 1.3 vs. 25.8 ± 1.3 $\mu\text{mol/g}$ wet tissue; $p<0.05$).

1 However, this effect was likely due to reduced food intake and body weight, as Ad-GFP-
2 PF animals exhibited similar decreases of liver TG content (Fig. 3E). In contrast, neither
3 Ad-LEPR-B-treatment nor pair-feeding had a significant affect on triglyceride content in
4 muscle (Fig. 3F). Moreover, liver TG levels were similar in Ad-GFP and Ad-LEPR-B-
5 treated animals that had either HV or a sham operation (data not shown).

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1 **Discussion**

2 Recent studies suggest that hypothalamic input from humoral signals such as insulin and
3 FFA improve insulin sensitivity via increased suppression of HGP (7, 14, 15). We and
4 others have provided evidence that leptin also plays an important role in the regulation of
5 glucose metabolism through actions in the hypothalamus (12, 13). To investigate the
6 mechanisms mediating the insulin-sensitizing effect of leptin, we used the euglycemic-
7 hyperinsulinemic clamp technique to determine the effect of restored hypothalamic leptin
8 signaling on insulin sensitivity of muscle and liver in obese Koletsky rats. We found that
9 the insulin-sensitizing effect of hypothalamic leptin receptor gene therapy in these
10 animals is mediated by enhanced insulin-mediated inhibition of HGP via a mechanism
11 associated with inhibition of liver gluconeogenic gene expression, and that both effects
12 were blocked by selective hepatic vagotomy. Taken together, these findings suggest that
13 like insulin, leptin activates a neural circuit that exists between the brain and liver that
14 regulates hepatic insulin action.

15

16 Our finding that restoration of hypothalamic leptin signaling to obese Koletsky rats
17 increases insulin inhibition of HGP would appear at odds with published evidence that in
18 normal rats, icv administration of pharmacological doses of leptin fails to affect HGP (21,
19 22). Rather than having no effect on hepatic glucose metabolism, however, central leptin
20 administration stimulates gluconeogenesis (via activation of the central melanocortin
21 pathway) while simultaneously decreasing glycogenolysis (via a melanocortin-
22 independent pathway) (22), an effect that requires central signaling through STAT3 (23).
23 Furthermore, icv leptin infusion reversed the insulin resistance induced by short-term

1 exposure of rats to a high-fat diet by reducing both glycogenolysis and gluconeogenesis
2 (24). Our rat model also differs in that our goal was to restore functional leptin signaling
3 to discrete hypothalamic nuclei of animals that otherwise lack the leptin receptor, rather
4 than inject a pharmacological dose of leptin delivered into the 3rd ventricle. Thus, the
5 source of the ligand in our model is circulating leptin, and the site of leptin action in our
6 model is restricted to the area of the ARC whereas icv leptin can simultaneously act at
7 multiple leptin-sensitive sites within the hypothalamus (25, 26), as well as
8 extrahypothalamic sites including the ventral tegmental area (27, 28) and the nucleus of
9 the solitary tract (NTS) in the hindbrain (29).

10

11 Recent studies suggest that the effect of hypothalamic insulin and FFA signaling to
12 suppress GP requires intact vagal input to the liver (14, 15). Consistent with the
13 hypothesis that a similar mechanism underlies the hepatic response to leptin action in the
14 ARC, we found that the effect of restored hypothalamic leptin signaling to improve
15 peripheral insulin sensitivity in obese Koletsky rats was attributable to increased insulin-
16 induced suppression of HGP, and that this effect was blocked by selective HV.
17 Previously published data suggests that vagal efferent fibers that supply the liver are the
18 critical vagal population, as the hypothalamic effects of both insulin and FFA to suppress
19 HGP are blocked by selective HV, but not by selective vagal deafferentiation (14, 15).
20 By comparison, the rate of HGP was not affected by HV in Koletsky rats receiving the
21 control adenovirus, suggesting that although the parasympathetic nervous system is a key
22 mediator of hypothalamic leptin action on hepatic insulin sensitivity (as is also true for
23 insulin and FFA), it is not a key determinant in the absence of a leptin signal. One

1 potential explanation for this observation is the possibility that vagal tone to the liver is
2 reduced or absent in leptin receptor-deficient rats. Since previous studies have shown
3 that selective HV in genetically normal rats is also without effect on hepatic glucose
4 metabolism (14, 15), however, vagal input to the liver may play a redundant role in the
5 control of hepatic insulin action in the absence of a change of key CNS control systems.
6 A relevant parallel may be found in the observation that whereas vagal afferents are well
7 known to play a crucial role in the perception of satiety and hence control of meal size,
8 total or selective vagotomies fail to affect overall energy balance (30), owing to
9 compensatory responses elsewhere in the system. Additional studies are warranted to
10 better understand the role of the hepatic vagus in the control of hepatic glucose
11 metabolism.

12

13 The potential importance of the role played by vagal innervation of the liver in our
14 studies is highlighted by our inability to find other, indirect mediators of the effect of
15 hypothalamic leptin on insulin sensitivity. Previous studies suggest that increased central
16 leptin signaling has wide-ranging effects in multiple tissues including activation of
17 AMPK in muscle (31), reduced TG content in liver (10), increased mitochondrial activity
18 in BAT (32), and changes in plasma hormone levels, that may in turn, affect peripheral-
19 tissue insulin action. We have previously reported that ARC-directed leptin receptor
20 gene therapy does not reliably alter fasting plasma levels of leptin, FFA, TG or
21 corticosterone in Koletsky rats (12). Our findings are also unlikely to result from
22 decreased liver TG content, as there was no difference in hepatic fat content between
23 treatment groups at the completion of the clamps, and studies examining biochemical

1 hepatic insulin sensitivity showed that the modest effect of hypothalamic leptin receptor
2 gene therapy to reduce liver TG content was likely explained by reduced food intake.
3 Since altered hepatocellular fatty acid metabolism can clearly influence insulin signal
4 transduction (33), however, it is interesting to speculate that changes in vagal outflow to
5 the liver influence hepatic lipid handling, and that this effect in turn explains our findings.
6 Moreover, central leptin signaling stimulates fatty acid oxidation in WAT (34) and
7 controls adipose tissue lipogenesis (35), an affect implicated in the ability of
8 hypothalamic leptin to improve peripheral insulin sensitivity. However, recent data
9 suggest that leptin regulation of peripheral lipid metabolism is predominantly explained
10 by effects on food intake (36).

11

12 To identify cellular mechanisms whereby hypothalamic leptin signaling increases hepatic
13 insulin sensitivity, we examined whether restored hypothalamic leptin signaling increases
14 insulin receptor signal transduction in either liver or skeletal muscle. Here, we found that
15 hypothalamic leptin receptor gene therapy increased insulin signaling via the IRS-PI3K
16 pathway selectively in liver, but not in muscle, among obese Koletsky rats. As insulin
17 stimulation of PI3K signaling inhibits gluconeogenesis in hepatocytes, we also measured
18 expression levels of PEPCK and G6Pase, in liver samples obtained at the completion of
19 the hyperinsulinemic clamp and found them to be reduced in Ad-LEPR-B- compared to
20 Ad-GFP-treated controls. However, the question of whether restored hypothalamic leptin
21 signaling in our studies reduced gluconeogenesis, glycogenolysis, or both awaits further
22 investigation.

23

1 One mechanism proposed to explain how leptin reduces food intake is by enhancing the
2 response to gut-derived satiety peptides, such as cholecystinin (CCK), that are released
3 upon food ingestion and activate vagal afferent fibers that terminate in the NTS in the
4 hindbrain (37-39). Specifically, leptin signaling in the ARC has been shown to activate a
5 descending projection to the NTS that enhances the response to input from CCK (17, 40).
6 Our current findings raise the possibility that a parallel neurocircuit may link
7 hypothalamic leptin signaling to control of hepatic insulin sensitivity, and that a leptin-
8 sensitive neuronal pathway conveys input to the liver by modulating the activity of
9 neurons in the hindbrain. Consistent with this hypothesis, inhibition of fat oxidation in
10 the ARC increases hepatic insulin sensitivity via a mechanism that requires intact hepatic
11 vagal signaling and also activates hindbrain neurons (15).

12

13 Because insulin resistance was only partially reversed by rescue of leptin receptor
14 signaling in the area of the ARC, an important role for leptin action in other brain areas in
15 the control of glucose homeostasis is suggested. Leptin receptors are expressed in several
16 additional hypothalamic areas as well as extrahypothalamic sites (29, 41, 42), and several
17 of these are also important in the control of glucose metabolism and involve signaling
18 mechanisms that may differ from those involved in the ARC. For example, hypothalamic
19 leptin action increases AMPK activity in muscle (31), and this effect may be mediated by
20 leptin action in the VMH, as microinjection of leptin to this brain area increases glucose
21 uptake in muscle via the sympathetic nervous system (43, 44). Consistent with this,
22 selective deletion of leptin receptors from SF1 neurons in the VMH causes obesity, as
23 well as insulin resistance (45).

1

2 One limitation of these experiments is that since we are unable to direct the adenovirus to
3 specific neuronal cell types, we cannot identify the neuronal cell groups that mediate the
4 observed effects. In this regard, the recent finding that expression of insulin receptors by
5 hypothalamic NPY/Agrp neurons is required for the full effect of circulating insulin to
6 suppress HGP (46), combined with evidence that leptin, like insulin, inhibits the firing of
7 these neurons (47), suggests that this neuronal subset might contribute to the actions we
8 observed following ARC-directed leptin receptor gene therapy. Indeed, we previously
9 reported inhibition of hypothalamic NPY gene expression following this intervention
10 (16), but whether leptin signaling in these neurons is required for its hepatic effects
11 awaits further study. Another caveat is that our approach does not selectively target
12 neurons that normally express leptin receptors, nor can we verify that normal expression
13 levels were attained in individual neurons, however, this is unlikely to affect
14 experimental outcomes via a nonspecific mechanism as neither Ad-LEPR-B-GFP nor
15 Ad-GFP has any detectable metabolic effect when injected into the ARC of wild-type
16 animals (12, 16, 17).

17

18 In conclusion, we report that restoring leptin receptor signaling to the area of the ARC of
19 leptin receptor-deficient Koletsky rats improves peripheral insulin sensitivity by
20 enhanced suppression of HGP, rather than increased glucose uptake, and that these
21 effects are blocked by selective hepatic vagotomy. Taken together, these findings
22 support the existence of a neurocircuit linking hypothalamic leptin signaling with
23 autonomic innervation of the liver that plays a physiological role in the control of insulin

1 sensitivity. Moreover, these data raise the possibility that pharmaceutical approaches to
2 increase central leptin sensitivity in conditions of insulin resistance resulting from diet-
3 induced obesity may improve both energy and glucose homeostasis.

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1 **Table 1**
 2 **Basal and clamp characteristics in 24-wk old obese Koletsky rats 7 days following**
 3 **bilateral microinjection of an adenovirus expressing either Ad-LEPR-B or Ad-GFP**
 4 **to the area of the ARC.**

5

	Ad-GFP	Ad-LEPR-B
<i>n</i>	5	5
<i>Basal</i>		
Δ Body weight (g)	-18.9 ± 3.3	-22.9 ± 5.2
Food intake (g/day)	18.2 ± 1.1	16.7 ± 1.1
Fat (%)	41.2 ± 1.0	42.2 ± 0.7
Arterial plasma glucose (mg/dl)	122.0 ± 3.9	120.1 ± 3.4
Plasma insulin (ng/ml)	14.2 ± 4.7	11.7 ± 2.3
Plasma leptin (ng/ml)	161.3 ± 11.5	144.7 ± 7.4
Plasma FFA (mmol/l)	1.00 ± 0.14	0.90 ± 0.10
Plasma corticosterone (ng/ml)	806 ± 163	677 ± 147
<i>Clamp</i>		
Arterial plasma glucose (mg/dl)	114.9 ± 3.2	124.0 ± 4.7
Plasma insulin (ng/ml)	27.9 ± 4.5	22.9 ± 6.4
Plasma FFA (mmol/l)	0.72 ± 0.10	0.51 ± 0.06
Plasma corticosterone (ng/ml)	619 ± 125	597 ± 89
Glucose infusion rate (mg/kg/min)	4.08 ± 0.38	5.84 ± 0.57*

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1 **Table 2**
2 **Basal and clamp characteristics in 12-wk old obese Koletsky rats 7 days following**
3 **bilateral microinjection of an adenovirus expressing either Ad-LEPR-B or Ad-GFP**
4 **to the area of the ARC.**

5

	Ad-GFP- SHAM	Ad-GFP- HV	Ad-LEPR-B- SHAM	Ad-LEPR-B- HV
<i>n</i>	5	5	5	6
<i>Basal</i>				
Δ Body weight (g)	3.2 ± 12.0	3.3 ± 6.2	-8.7 ± 2.6	1.3 ± 2.5
Food intake (g/day)	28.0 ± 2.4	27.8 ± 0.8	24.5 ± 1.9	27.6 ± 0.9
Fat (%)	38.0 ± 1.1	36.8 ± 2.0	36.7 ± 2.9	37.7 ± 1.6
Arterial plasma glucose (mg/dl)	115.6 ± 8.1	110.3 ± 4.9	112.0 ± 9.1	121.0 ± 7.3
Plasma insulin (ng/ml)	11.6 ± 2.4	11.4 ± 1.5	9.7 ± 2.6	8.2 ± 1.3
Plasma leptin (ng/ml)	80.5 ± 6.5	67.6 ± 8.9	69.4 ± 20.5	60.1 ± 5.7
Plasma FFA (mmol/l)	1.45 ± 0.38	1.26 ± 0.08	1.36 ± 0.16	1.63 ± 0.24
Plasma corticosterone (ng/ml)	695 ± 102	807 ± 69	591 ± 182	773 ± 172
<i>Clamp</i>				
Arterial plasma glucose (mg/dl)	116.4 ± 3.9	115.4 ± 1.6	121.4 ± 5.5	122.4 ± 1.5
Plasma insulin (ng/ml)	14.7 ± 2.6	13.5 ± 1.4	13.3 ± 3.4	11.4 ± 1.3
Plasma FFA (mmol/l)	1.04 ± 0.38	0.87 ± 0.16	0.90 ± 0.17	1.01 ± 0.12
Plasma corticosterone (ng/ml)	636 ± 51	674 ± 254	673 ± 249	869 ± 210
Glucose infusion rate (mg/kg/min)	7.08 ± 0.34	6.57 ± 0.66	9.04 ± 0.32*	6.25 ± 0.77

6

1 **Figure Legends**

2

3 **FIG. 1.** ARC-directed leptin receptor gene therapy improves insulin sensitivity via
4 increased suppression of endogenous glucose production. A) Arterial glucose and B)
5 glucose infusion rate required to maintain euglycemia during the euglycemic-
6 hyperinsulinemic clamp in obese Koletsky rats that received Ad-GFP or Ad-LEPR-B
7 directed to the ARC. C) Glucose utilization (Rd) and the D) endogenous rate of glucose
8 appearance (Endo Ra) in Ad-GFP and Ad-LEPR-B-treated animals during the basal
9 (open squares) and the clamp (closed squares) period. Effect of ARC-directed leptin
10 receptor gene therapy on E) % increase in glucose utilization, F) % suppression of
11 hepatic glucose production and hepatic mRNA levels of the gluconeogenic genes G)
12 *G6Pase* and H) *Pepck*. n=5/group. *p<0.05 vs. Ad-GFP.

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1 **FIG. 2.** Restoring hypothalamic ARC leptin receptor signaling improves hepatic insulin
2 action via the hepatic branch of the vagus nerve. A) Arterial glucose and B) glucose
3 infusion rate required to maintain euglycemia during the euglycemic-hyperinsulinemic
4 clamp in obese Koletsky rats that underwent either sham surgery or selective hepatic
5 vagotomy (HV) and then received Ad-GFP or Ad-LEPR-B directed to the ARC. C)
6 Glucose utilization (Rd) and the D) endogenous rate of glucose appearance (Endo Ra) in
7 Ad-GFP and Ad-LEPR-B-treated animals during the basal (shaded squares) and the
8 clamp period in animals that received sham surgery (open squares) or HV (closed
9 squares). Effect of selective HV compared to sham surgery in animals that received
10 either Ad-GFP or Ad-LEPR-B on E) % increase in glucose utilization, F) % suppression
11 of hepatic glucose production and hepatic mRNA levels of the gluconeogenic genes G)
12 *G6Pase* and H) *Pepck*. n=5-6/group. *p<0.05 vs. Ad-GFP-Sham.

13

14 **FIG. 3.** ARC-directed leptin receptor gene therapy increases hepatic insulin signal
15 transduction. Effect of intraperitoneal (10U/kg) insulin (closed bars)-induced activation
16 of tyrosine phosphorylation of IRS-1 (A and C) and serine phosphorylation of PKB/Akt
17 (B and D) compared to vehicle (open bars) in liver and muscle (gastrocnemius),
18 respectively in obese Koletsky rats 16 days following microinjection of an adenovirus
19 expressing Ad-LEPR-B or the reporter gene, Ad-GFP that were either fed ad libitum or
20 pair-fed to the Ad-LEPR-B-treated animals. E) Hepatic and F) muscle triglyceride
21 content in this same group of animals. *p<0.05 vs. Ad-GFP; # p<0.05 vs. Ad-GFP-PF.

22

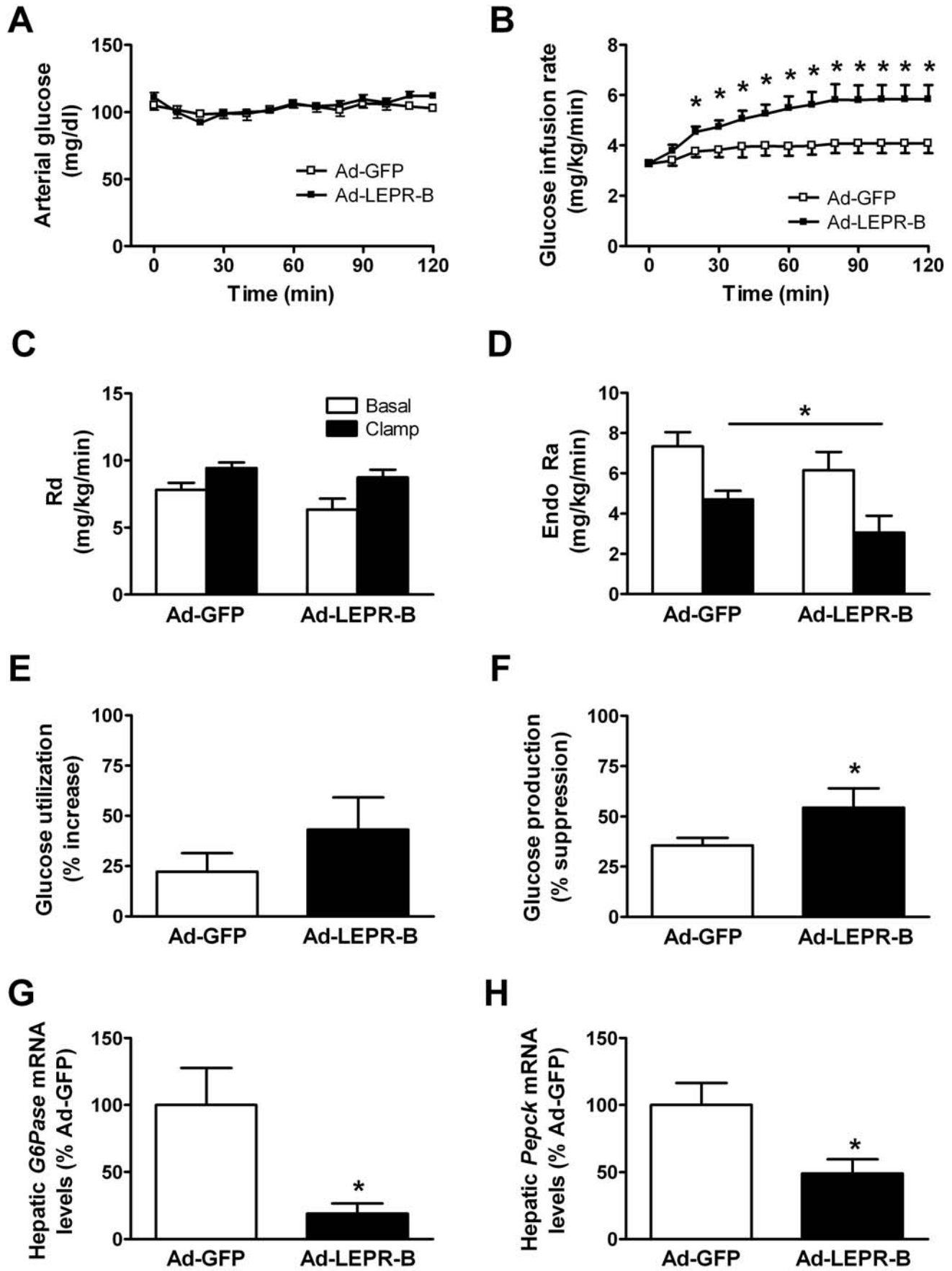
FIG. 1

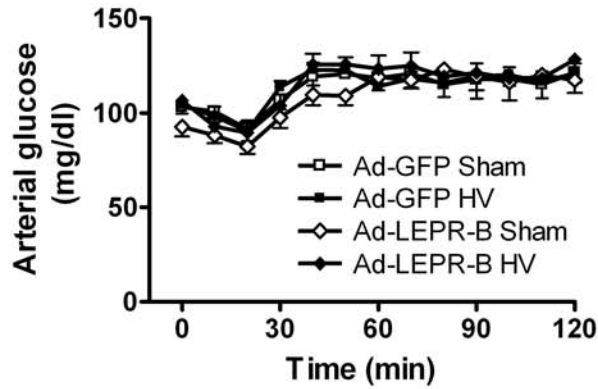
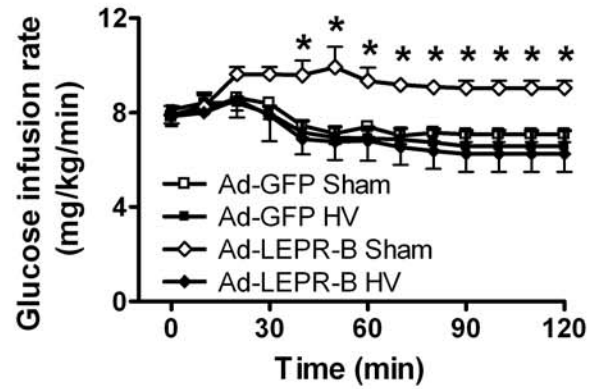
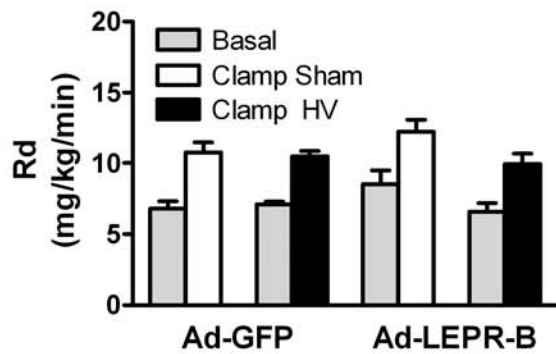
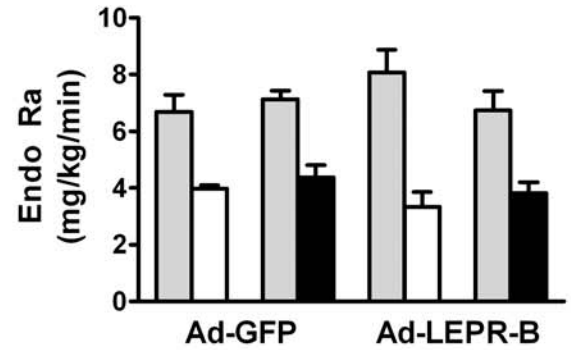
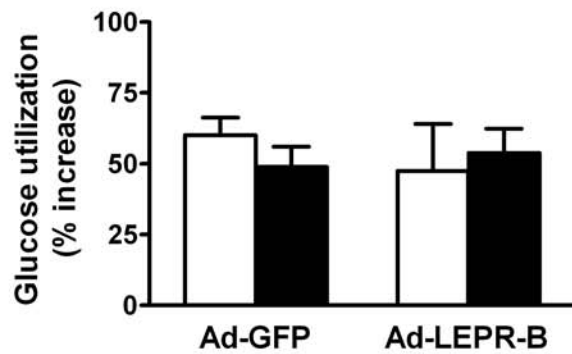
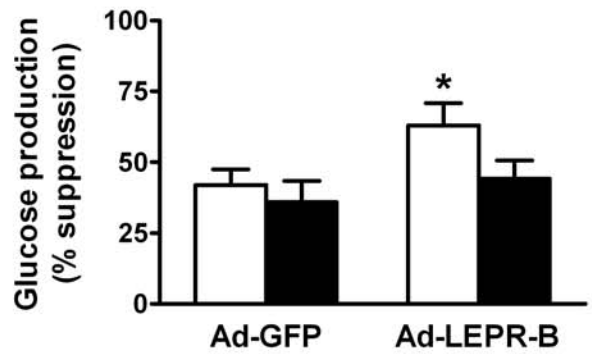
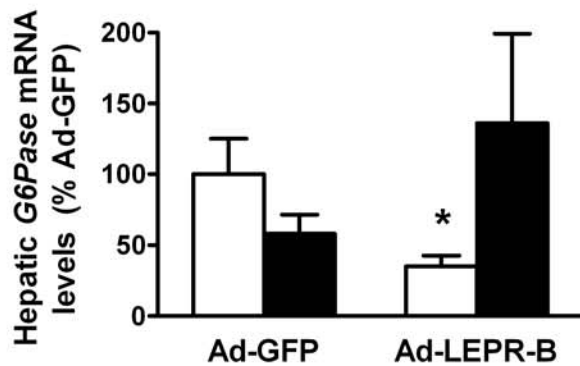
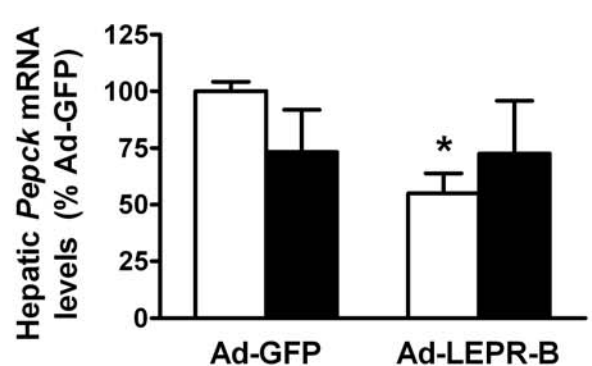
FIG. 2**A****B****C****D****E****F****G****H**

FIG. 3

