Diet-induced Obesity Alters AMP-Kinase Activity in Hypothalamus and Skeletal Muscle

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AMPK is a key regulator of cellular energy balance and of leptin’s effects on food intake and fatty acid oxidation. Obesity is usually associated with resistance to leptin’s effects on food intake and body weight. To determine whether diet-induced obesity (DIO) impairs the AMPK response to leptin in muscle and/or hypothalamus, we fed FVB mice a high fat (55%) diet for 10-12 weeks. Leptin acutely decreased food intake by ~30% in chow-fed mice. DIO mice tended to eat less and leptin had no effect on food intake. Leptin decreased respiratory exchange ratio (RER) in chow-fed mice indicating increased fatty acid oxidation. RER was low basally in high fat fed mice and leptin had no further effect. Leptin (3mg/kg ip) increased α2-AMPK activity 2-fold in muscle in chow-fed mice but not in DIO mice. Leptin decreased acetyl-coA carboxylase (ACC) activity 40% in muscle from chow-fed mice. In muscle from DIO mice, ACC activity was basally low and leptin had no further effect. In paraventricular, arcuate and medial hypothalamus of chow-fed mice, leptin inhibited α2-AMPK activity but not in DIO mice. In addition, leptin increased STAT3 phosphorylation 2-fold in arcuate of chow-fed mice but this effect was attenuated due to elevated basal STAT3 phosphorylation in DIO mice. Thus, DIO in FVB mice alters α2-AMPK in muscle and hypothalamus and STAT3 in hypothalamus and impairs further effects of leptin on these signaling pathways. Defective responses of AMPK to leptin may contribute to resistance to leptin action on food intake and energy expenditure in obese states.
INTRODUCTION
Obesity has reached epidemic proportions worldwide and currently affects 1 in 3 Americans (1,2). Most obese people are resistant to the actions of insulin. Obesity is a major risk factor for developing type 2 diabetes and cardiovascular disease and for some forms of cancer (1,2). Leptin (Ob), a hormone secreted by the adipocyte in proportion to fat stores, plays a major role in regulating energy homeostasis by decreasing food intake and increasing energy expenditure. While these effects are primarily through actions in the hypothalamus (3), peripheral actions of leptin have also been described (4,5).

Rodents with diet-induced obesity (DIO) and most obese humans are resistant to the effects of leptin (6,7). Leptin resistance is defined as decreased sensitivity to the anorexigenic or weight-loss effects of leptin. A hallmark of leptin resistant states is hyperleptinemia. The mechanism for leptin resistant states is of great interest and understanding it could lead to new approaches to prevent or treat obesity and the accompanying risk for type 2 diabetes.

Leptin binds to its cell surface receptor, ObRb, which is a member of the class 2 cytokine family that classically signals through the JAK/STAT pathway. Leptin activates JAK2 and STAT3 as well as the mitogen-activated protein (MAP) kinase (Erk 42/44) and the phosphoinositide 3-kinase (PI3K) pathways. Intact STAT3 signaling is necessary for leptin’s effects on food intake. Mice that lack either the binding site for STAT3 on the leptin receptor (8) or neuronal STAT3 protein (9) are obese and hyperphagic. Leptin also modulates the activity of the AMP-activated protein kinase (AMPK) and inhibition of AMPK in discrete hypothalamic regions is also critical for leptin’s anorexigenic effects (10). In addition, activation of PI3K may be important for leptin’s effects on food intake through changes in membrane potential in target neurons (11,12).

Several studies have demonstrated that rodents with DIO are biologically and biochemically resistant to leptin. They do not decrease their food intake or body weight in response to peripheral leptin administration (6,13). In addition, leptin injection peripherally fails to induce phosphorylation of STAT3 in the hypothalamus. One proposed mechanism for leptin resistance in DIO is decreased leptin transport across the blood brain barrier (14,15). However, this appears to account for only part of the defect in leptin action. While intracerebroventricular (icv) leptin administration induces STAT3 phosphorylation in DIO, this phosphorylation is dramatically reduced when compared to chow-fed mice (13,16). Taken together, the studies of peripheral and central leptin administration in rodents on a high fat diet suggest that resistance to leptin signaling at the level of STAT3 is due in part to decreased leptin transport into the brain and in part to defective signaling in the hypothalamus. Both suppressor of cytokine signaling 3 (SOCS-3) and protein tyrosine phosphatase-1B (PTP-1B) negatively regulate the leptin signaling pathway (17-19). Neuronal-specific deletion or whole-body haploinsufficiency of SOCS-3 increases leptin sensitivity and confers resistance to diet-induced obesity (20,21). PTP-1B -/- mice exhibit similar characteristics (18,19,22,23).

Leptin has metabolic functions independent of its role as a satiety factor. For instance, leptin partitions fatty acids away from storage towards oxidation in skeletal muscle (24). However, when mice are fed a high fat diet, there is no effect of leptin on fatty acid oxidation in muscle ex vivo (25) indicating leptin resistance. Similarly, when human skeletal muscle is incubated ex vivo with leptin, fatty acid oxidation is increased in muscle from lean subjects only, not in muscle.
from obese subjects (26). Until recently, the signaling pathways underlying leptin's effects on fatty acid oxidation were not known. We demonstrated that leptin increases fatty acid oxidation in skeletal muscle by activating AMPK (5). AMPK is a key regulatory enzyme in cellular energy homeostasis (27,28). It is a heterotrimERIC protein consisting of catalytic α- and regulatory β- and γ-subunits, that is activated allosterically by increases in the AMP: ATP ratio as well as by phosphorylation on Thr172 by upstream kinases. Recently, two upstream AMPK kinases have been identified, LKB1 (29,30) and Calmodulin kinase kinase (31-33).

Once activated, AMPK switches on energy-producing pathways at the expense of energy-depleting processes. AMPK regulates fatty acid metabolism by stimulating fatty acid oxidation through phosphorylation of acetyl CoA carboxylase (ACC) thereby decreasing malonyl CoA levels, which disinhibits carnitine palmitoyl transferase-1 and increases fatty acid entry into mitochondria. In addition, AMPK phosphorylates target proteins involved in a number of metabolic pathways, including lipolysis (adipocytes), lipid metabolism (liver and muscle), glucose transport (muscle and adipocytes), and glycogen metabolism (muscle and liver) (34).

We demonstrated a direct, transient effect of leptin on AMPK activation in oxidative muscle and a more sustained effect that is mediated through the hypothalamus and sympathetic nervous system (5). Both effects involved acute phosphorylation and activation of the α2 catalytic subunit of AMPK without changes in the level of the α2 subunit protein and without changes in activation of the α1 catalytic subunit. Steinberg et al. showed that chronic leptin treatment increases AMPK activity due to an increase in the α catalytic subunit protein levels in rat muscle (35). In addition, transgenic mice overexpressing leptin in liver have increased phosphorylation of AMPK and decreased triglyceride content in soleus muscle (36). Moreover, a crucial role for AMPK in the hypothalamus in the regulation of food intake by leptin and other hormones has been demonstrated (10,34,37,38). Thus, AMPK mediates multiple critical effects of leptin on energy homeostasis.

In the present study, we sought to determine whether impaired response of the AMPK pathway to leptin could contribute to the molecular pathogenesis of leptin resistance in mice on a high fat diet. We demonstrate that by 12 weeks of high fat feeding, DIO mice are resistant to the effects of leptin administration on AMPK activity in both muscle and hypothalamus. This may be due, at least in part, to constitutive alterations in the AMPK signaling pathway in the absence of leptin administration. Basal activity of AMPK tends to be increased and basal ACC activity is decreased in muscle. In paraventricular nucleus (PVN) from DIO mice, AMPK activity is constitutively decreased and in PVN, arcuate and medial hypothalamus, leptin fails to suppress AMPK activity. These data suggest that the AMPK pathway is dysregulated in muscle and hypothalamus in obese states resulting from high fat feeding and that lack of dynamic responsiveness of this pathway may play a role in the pathophysiology of leptin resistance in diet-induced obesity.
METHODS

Mice and diets
Male FVB mice were obtained from Taconic at approximately three weeks of age. After an acclimation period of one week, mice were randomly assigned into two groups: Chow or High Fat (DIO). Chow mice were fed Purina chow diet 5008 (4.5% calories from fat) while DIO mice ate a diet high in fat (55% calories from fat, Harlan Teklad 93075) for 5-12 weeks. Mice were housed one per cage in a temperature-controlled room and were maintained on a 14/10-hour light-dark cycle. Mice had ad libitum access to both food and water.

Treatment and tissue harvesting
Mice were handled for 3-5 days prior to experiments to reduce stress during the experiment. After an overnight fast, mice were injected with saline (con) or leptin (3mg/kg; A. F. Parlow, National Hormone & Peptide Program, Torrance, CA) intraperitoneally (ip). Five hours later, mice were anesthetized with ketamine/xylazine and killed by decapitation. Hypothalamic nuclei and peripheral tissues were rapidly dissected and frozen in liquid nitrogen. Each hypothalamic region was dissected from 1-mm-thick sagittal sections of fresh brain. PVN, ARC, VMH and DMH were dissected from the first sections from the midline of the brain (10). All assays were performed on hypothalamic regions from individual mice.

Metabolic Parameters
Body weights were measured weekly at the same time. Random fed mice were bled prior to starting the diets and again a week before sacrifice. Plasma samples were centrifuged and serum was stored at –20°C until it was assayed. Plasma glucose was measured using the One-touch Ultra glucometer. Plasma insulin and leptin levels were determined by their respective ELISA kits (Crystal Chem, Ill). For the glucose tolerance test (GTT), mice were fasted for 16 hours and 2mg/kg glucose was injected ip. Blood glucose was measured at 0, 15, 30, 60 and 120 minutes after injection. For the insulin tolerance test, food was removed at 8am. Four hours later, mice were injected with 1U/kg human insulin (Lilly) ip. Blood was withdrawn from the tail vein at 0, 15, 30, 45, 60 and 90 minutes.

Food Intake
After ten weeks of DIO or chow diet, six mice from each group received an i.p. injection of leptin (3mg/kg body weight) or saline at the start of the dark cycle and again 12 hours later. Body weight and food intake were measured 12 and 24 hours after the first injection.

Indirect Calorimetry
The metabolic rate of mice was measured by indirect calorimetry in eight open-circuit oxymax chambers that are a component of the Comprehensive Lab Animal Monitoring System (CLAMS) (Columbus Instruments, Columbus, OH). Mice were housed singly and maintained at 24°C under a 12-hour light dark cycle (dark period 20:00-8:00). Food and water were available ad libitum. All mice were acclimated to monitoring cages for 24 hours prior to beginning the physiological recordings. Mice were injected with either saline or leptin (3mg/kg) at 4 pm on day 1 and again at 8am and 4 pm on day 2. To calculate oxygen consumption (VO₂), carbon dioxide production (VCO₂), and RER (ratio of VCO₂ to VO₂), gas concentrations were measured at the inlet and outlet of the sealed chambers.
**Western blot analysis**

Tissue lysates were prepared as described previously (10). Phosphorylation of STAT3 in hypothalamic regions was determined with 7.5% SDS acrylamide gels using an antibody against the phospho-tyrosine705 (Cell Signaling) of STAT3. Phosphorylation of the α subunit of AMPK in soleus lysates was determined with 10% SDS acrylamide gels by using antibodies that recognize phosphoThr 172 of the α-subunit of human AMPK (Cell Signaling). Blots were re-probed with antibodies to α2-AMPK (generous gift from Dr. D. Carling) or ACC (streptavidin–horseradish peroxidase from Amersham Pharmacia). Chemiluminescence (Western Lightning, Perkin Elmer) was quantified by laser densitometry within the linear range (Molecular Dynamics) or GeneSnap.

**Activity Assays**

AMPK activity was measured in soleus muscle or hypothalamic regions by immunoprecipitation of α2-AMPK from muscle lysates (100 μg of protein) or brain regions (40-50 μg) with specific antibodies against the α2 catalytic subunits bound to protein-G/sepharose beads. Kinase activity was measured using synthetic 'SAMS' peptide and [γ-32P] ATP as described previously (10). The activity of ACC in red (slow twitch) muscle lysates was measured by 14CO2 fixation to acid-stable products in the presence of citrate (2 mM), an allosteric activator of ACC.

**Statistical Analyses**

All data are expressed as means ± S.E.M. Significance is set at p < 0.05. For GTT, ITT, and RER statistical analyses were performed using repeated measures ANOVA with Bonferroni post-test. Comparisons of mean plasma insulin, plasma leptin, food intake, AMPK activity, phosphorylated AMPK, ACC activity, and phosphorylated STAT3 in DIO vs. chow were made by one-way ANOVA with Bonferroni’s post-test. Comparisons of total protein levels (AMPK, ACC, STAT3) between two groups (Chow and DIO) were made using Student’s t-test.
RESULTS

Male FVB mice were randomized to either chow or DIO so initial body weights were similar in both groups. By two weeks on the high fat diet, the DIO mice were heavier than their chow-fed counterparts and the weights continued to diverge throughout 11 weeks on the high fat diet (p<0.05) (Figure 1A). After one week on the high fat diet, serum insulin levels were normal but after 10 weeks insulin levels were ~two-fold higher in DIO mice than in chow-fed mice (Figure 1B). After 1 week on the diet, plasma leptin levels in the fed state were not different in DIO mice compared to chow-fed mice. Serum leptin levels increased ~3-fold in chow-fed mice between 1 and 11 weeks of the study while serum leptin levels in DIO mice increased ~7-fold during this same period. After 11 weeks on the diet, leptin levels in the fed state were three-fold higher in DIO mice compared to chow-fed mice (Figure 1C). An overnight fast decreased serum leptin levels by 51% in chow-fed mice and 78% in DIO mice. Thus, in the fasted state, serum leptin was not elevated in DIO mice compared to fasted chow fed mice. Overnight fasted DIO mice had elevated blood glucose (chow 101 ± 13 vs. DIO 145 ± 5 mg/dl, p<0.05). Glucose tolerance tests revealed overt diabetes in DIO mice (Figure 1D) and these mice were unresponsive to exogenous insulin during an insulin tolerance test (Figure 1E), indicating marked insulin resistance.

One of the primary biological indicators of leptin resistance is the inability of leptin to decrease food intake. After ten weeks on the diets, mice were injected with leptin intraperitoneally (ip) and 24-hour food intake was measured. Two injections of leptin (3mg/kg, each) over 24 hours decreased food intake in the chow-fed mice by more than 30%. Saline-injected DIO mice tended to eat less than saline-injected chow-fed mice and leptin did not reduce food intake in DIO mice (Figure 2A). We also measured food intake over 48 hours and saw no effect of leptin in DIO mice (not shown). These data suggest that the DIO mice are resistant to the effects of leptin administration on food intake.

In addition to its ability to decrease food intake, leptin also increases energy expenditure and fatty acid oxidation. To determine whether DIO mice are resistant to leptin’s effects on fat utilization, mice received three ip injections of leptin (3mg/kg, each) over a 24-hour period while in the indirect calorimeter. The respiratory exchange ratio (RER) is a ratio of carbohydrate oxidation to lipid oxidation. An RER of 1.0 indicates high utilization of carbohydrate for energy while an RER of 0.7 indicates increased fatty acid oxidation (39). Figures 2B and 2C show the RER after the third ip injection of leptin. Prior to the third injection, RER was lower in the leptin-injected chow group compared to the saline-injected chow-fed mice due to the previous two leptin injections. The third injection of leptin led to a sustained decrease in RER in mice on the chow diet compared to saline-injected chow-fed mice (p<0.01). Even though RER rose in both chow-fed groups as they ate more starting around 18:00, RER remained lower in the leptin-injected chow-fed mice throughout most of the dark cycle. DIO mice have a lower baseline RER because they utilize fat for energy. Leptin had no effect on RER in DIO mice. Figure 2C is a quantitation of the RER from 18:00-6:00 hours after three i.p. leptin injections.

Whether the inability of leptin to further decrease RER in DIO mice is due to leptin resistance or because DIO mice already are at the lower biological limit for RER from the high fat content of the diet is unknown. The lowest RER we are aware of is 0.7 and is seen in mice on a ketogenic diet with an extremely low carbohydrate content (E. Maratos-Flier, personal communication). Lower RER in DIO mice reflects increased fatty acid utilization and we demonstrated that leptin increases fatty acid oxidation through
activation of AMPK in muscle (5). Thus, changes in AMPK and ACC activities in muscle in DIO mice might explain, at least in part, their lower baseline RER.

We previously demonstrated that leptin activates AMPK in muscle both directly and through the hypothalamic-sympathetic nervous system (SNS) (5). Because the physiological contribution of direct leptin action on peripheral tissues to whole body energy homeostasis is controversial, we focused on the effects of leptin on AMPK that are mediated by the SNS pathway. Leptin's effects on AMPK in the hypothalamus are more pronounced after an overnight fast (10). In fasted chow-fed mice, leptin increased AMPK phosphorylation in soleus muscle ~2-fold at five hours after ip injection (Figures 3A and 3B). In fasted DIO mice, there was a tendency for increased basal AMPK phosphorylation but this was not statistically significant. Leptin had no effect on AMPK phosphorylation in DIO mice. Neither leptin injection nor diet affected total α2 AMPK protein levels (Figure 3C). AMPK activity parallels AMPK phosphorylation. Leptin increased AMPK activity by 75% in soleus muscle from chow mice (Figure 3D). There was no effect of leptin on AMPK activity in soleus from DIO mice. Similar results were seen in mice fed a high fat diet for either 12 or 22 weeks. Treatment with 5-aminimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) stimulated AMPK phosphorylation to the same degree in soleus muscle from both chow-fed and DIO mice (not shown), indicating that the AMPK pathway is intact in DIO mice.

AMPK increases fatty acid oxidation by phosphorylating ACC, which decreases ACC activity. As expected, leptin decreased ACC activity in muscle from chow mice (Figure 4A). However, in DIO mice, there was a significant decrease in basal ACC activity in muscle to the level of leptin’s effect in chow mice and leptin had no further effect to decrease ACC activity in muscle from DIO mice. Total ACC protein levels were 17% higher in DIO mice (Figure 4B) and leptin treatment had no effect on total ACC levels (not shown). We can measure total or phosphorylated ACC but we cannot measure the dephosphorylated form. The increased total ACC protein levels observed (Figure 4B) in the setting of decreased ACC activity (Figure 4A) could be due to an increase of the inactive form of ACC.

While leptin increases AMPK activity in skeletal muscle (5), it inhibits it in hypothalamus (10,37). This decrease in AMPK activity in the hypothalamus is required for leptin’s anorexigenic actions (10). In chow-fed mice, leptin decreased AMPK activity in the ARC, paraventricular nucleus and medial hypothalamus but leptin failed to attenuate AMPK activity in these hypothalamic regions of DIO mice (Figure 5). Basal AMPK activity in the PVN of DIO mice was already suppressed to the level of leptin’s effect in chow-fed mice and there was no further effect of leptin. The lower basal AMPK activity in PVN of DIO mice may be due to effects of hyperinsulinemia and/or hyperglycemia which also suppress AMPK activity in multiple hypothalamic nuclei (10). There was a tendency, although not significant, for basal AMPK also to be lower in the other hypothalamic regions. In the medial hypothalamus, containing dorsomedial and ventromedial hypothalamus, leptin increased AMPK in DIO. This increase is likely to contribute to resistance to leptin’s biologic effects.

STAT3 phosphorylation and activation are required for leptin’s effects on food intake and energy homeostasis (8), and Munzberg et al. recently demonstrated defects in leptin-induced STAT3 phosphorylation in the arcuate hypothalamus of DIO mice (40). In our study, STAT3 phosphorylation increased 2-fold in arcuate of chow-fed mice five hours after i.p. leptin (Figure 6). Similar to the “leptin-like”
effect on basal AMPK activity in PVH and tendency toward this effect in ARH and MH of DIO mice, there is a 73% increase in basal STAT3 phosphorylation in arcuate of DIO mice. This appeared to be due to increased activation of STAT3 since there was no difference in total STAT3 protein levels between the groups (Figure 6). Leptin tended to increase STAT3 phosphorylation ~40% in the arcuate of DIO mice although this was not statistically significant.

Leptin administration had no effect on AMPK activity in muscle and hypothalamus or STAT3 phosphorylation in ARC from DIO mice. There was a leptin-like effect on the AMPK-ACC signaling pathway in muscle and on AMPK activity and STAT3 phosphorylation in hypothalamus in the basal state in DIO mice on a high fat diet for 12 weeks. To determine whether these effects could result from the elevated leptin levels in these mice, we studied mice that were fed a high fat diet for only 5 weeks. After 5 weeks on the diet, DIO mice were heavier than the chow-fed mice (26.6 ± 0.2 g, chow vs. 29.5 ± 0.4 g, DIO p<0.01). As seen in Figure 7A, 5 weeks of high fat feeding increased leptin levels ~7-fold, similar to that observed in DIO mice on a high fat diet for 12 weeks. Despite this dramatic increase in serum leptin levels, basal STAT3 phosphorylation in ARC from DIO mice fed a high fat diet for 5 weeks was comparable to that in chow-fed mice. Leptin-stimulated phosphorylation of STAT3 was also similar in chow-fed and DIO mice (data not shown). In addition, basal AMPK activities in the PVN, ARC and medial hypothalamus (Figures 7B and 7C) from DIO mice fed a high fat diet for 5 weeks were not different from chow-fed mice. In muscle, there was also no difference in basal AMPK activity between the two diet groups after 5 weeks on the diets (5.9 ± 0.3 nmol/mg/min, chow vs. 6.8 ± 0.3 nmol/mg/min, DIO).
DISCUSSION

Defects in the genes encoding leptin or its receptor lead to hyperphagia and severe obesity in animals and humans (3). Treatment of leptin-deficient rodents or humans with exogenous leptin ameliorates the obesity (7,41,42). However, the vast majority of obese humans are not leptin deficient. In fact, obese humans are hyperleptinemic yet this elevated leptin fails to suppress food intake or cause weight loss, indicating resistance to the major effects of leptin on energy balance. Feeding rodents a high fat diet induces an obese phenotype with many similarities to human obesity including resistance to leptin’s actions on food intake and body weight. Leptin influences body weight not only by suppressing food intake but also by increasing energy expenditure reflected in metabolic rate and oxygen consumption. One of the major actions of leptin on fuel utilization is to increase fatty acid oxidation and decrease fat storage in muscle via activation of AMPK. However, in both obese humans and rodents, treatment with leptin fails to stimulate fatty acid oxidation in muscle (25,26). The major objective of the present study was to determine whether this biological leptin resistance present in many forms of obesity is caused by impairment in the actions of leptin to increase AMPK activity in muscle or decrease it in the hypothalamus.

We found that the anorexigenic effect of leptin that was present in chow-fed mice was absent in DIO mice, but DIO mice tended to eat less at baseline. In addition, leptin decreased RER in chow-fed mice but had no effect on RER in DIO mice, although baseline RER was decreased in DIO mice and this could preclude a further reduction by leptin. The lower RER in DIO mice is consistent with increased AMPK activity (Figure 3) and decreased ACC activity (Figure 4) in muscle. We investigated whether these defects could be due to dysregulation of AMPK activity. The normal stimulation of AMPK phosphorylation and activity in soleus muscle of chow-fed mice by leptin administration was completely abrogated in DIO mice. However, we saw normal stimulation of AMPK activity by AICAR in muscle of DIO mice indicating that the AMPK pathway is intact. This observation agrees with data from human studies in which AICAR activates AMPK in skeletal muscle from lean and obese humans to the same extent (43). Phosphorylation of ACC, a downstream target of AMPK, inhibits its activity. Basal ACC activity was lower in muscle of the DIO mice than in chow-fed mice and there was no further response to leptin. The lower ACC activity could reflect hyperleptinemia in DIO mice, although serum leptin levels were not higher in DIO mice than in chow-fed mice in the fasting state (Figure 1C) in which the studies were performed. Furthermore, elevation of leptin alone is unlikely to cause the alterations in signaling in the basal state since leptin levels were similarly elevated in mice on the high fat diet for 5 weeks, but basal AMPK activity and STAT3 phosphorylation were normal in these mice (Figure 7). Other signaling pathways also regulate ACC in muscle including insulin, which normally stimulates ACC activity in muscle (44,45). Reduced ACC activity could reflect insulin resistance in muscle of DIO mice. The amount of total ACC protein was increased in DIO. This may be primarily the inactive (phosphorylated) form since ACC activity is decreased.

The tendency toward higher basal AMPK phosphorylation and activity and the lower ACC activity seen in our DIO mice differs from data reported in obese humans who showed no changes in the basal activity of AMPK or in basal ACCβ phosphorylation in skeletal muscle compared to lean controls (43). Others have shown that AMPK protein and activity in muscle are unaltered in obese and/or lean type 2 diabetics compared to nondiabetic controls (46,47). One possible explanation for this discrepancy is the
differences in muscle fiber type between humans and mice since AMPK isoform expression and activity differs in glycolytic versus oxidative muscle fibers (48).

Changes in hypothalamic AMPK activity regulate food intake (10,37,38); orexigenic factors (e.g. ghrelin, (49)) activate hypothalamic AMPK whereas leptin and other anorexigenic agents suppress AMPK activity in the hypothalamus. Furthermore, inhibition of AMPK is necessary for leptin's anorexigenic effects (10). In our study, basal AMPK activity was lower in PVN in the DIO mice and leptin had no further effect. Similar to the effects on AMPK in muscle, this may result from the chronic 7-fold elevation of leptin in the fed state. However, we do not see the same reduction of basal hypothalamic AMPK activity in DIO mice after five weeks of high fat feeding, despite similarly increased fed serum leptin levels. Taken together, these data suggest that hyperleptinemia alone is not responsible for the decreased hypothalamic AMPK activity in the basal state. Hyperglycemia and hyperinsulinemia could also contribute to lower basal AMPK activity, since they have both been shown to suppress hypothalamic AMPK activity (10,50). In support of this, we have also found lower AMPK activity in hypothalamic nuclei of db/db mice that are hyperglycemic and hyperinsulinemic (Y. Minokoshi and B.B. Kahn, unpublished data). The fact that constitutively lower AMPK activity does not effectively suppress food intake in these models indicates that a dynamic change in AMPK activity, rather than an absolute level, may be necessary to alter food intake. Alternatively, AMPK acts in conjunction with other signaling pathways that are also affected by DIO. SOCS-3 is increased in DIO and total body SOCS-3 haploinsufficiency or neuron-specific absence of SOCS-3 protects mice from DIO and leptin resistance (20,21). Similarly, deletion of PTP-1B, also enhances leptin sensitivity and prevents obesity in mice on a high fat diet (18,19,22,23). Defects in one of these pathways or in a pathway downstream of AMPK may be critical for resistance to leptin's effects on food intake and body weight.

Signaling through STAT3 in the hypothalamus is required for leptin's effects on energy homeostasis (8). In the present study, DIO mice showed increased basal STAT3 phosphorylation in the arcuate and a blunted response to leptin treatment consistent with the AMPK/ACC data in these mice. Therefore several leptin signaling pathways appear to be affected in the basal state with blunted response to exogenous leptin. Previous reports have shown that DIO mice on the C57/BL6 background have no STAT3 phosphorylation and/or DNA-binding activity in hypothalamus in response to peripheral leptin (16,40). However, in DIO rats, basal STAT3 phosphorylation is higher, similar to our data (51). Moreover, in aged obese rats with a six-fold elevation of serum leptin levels, basal STAT3 activation is also increased (52). As genetic background can influence susceptibility to DIO (13), strain differences or species differences may influence the effects of obesity on basal STAT3 phosphorylation (13,51,52).

The nature of the defective leptin signaling in hypothalamus remains unknown. It could be due to defective leptin transport across the blood brain barrier, which has been suggested as a mechanism for resistance to leptin's effect on food intake in DIO. However, when leptin is administered centrally, DIO rats and mice still have an impaired response both biologically, i.e. a reduced leptin effect on food intake, and in terms of signaling, i.e. impaired activation of STAT3 (6,16,40,53-55). In studies from obese humans, there is a threshold for leptin concentration and action above which the blood-brain transport of leptin is saturated and diminished sensitivity to leptin's effects on food intake and body weight occurs (56,57).
In conclusion, high fat feeding alters the AMPK signaling pathway in both muscle and hypothalamus and blunts the response to leptin administration. These changes do not appear to be due to hyperleptinemia, per se. These data suggest that responses of the AMPK signaling pathway are dysregulated in longstanding DIO, which may be due to constitutive leptin-like signaling effects. This lack of dynamic responsiveness of AMPK to leptin could provide a molecular mechanism underlying the biological resistance to leptin in DIO. Most likely, other pathways such as SOCS3 and PTP-1B are also involved. Nevertheless, further altering AMPK activity in DIO could have therapeutic effects since AMPK activators have been shown to cause weight loss in some obese rodent models (58), presumably through peripheral mechanisms. Thus, activating AMPK in muscle and other peripheral tissues could be a viable strategy for treating obesity.

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ABBREVIATIONS

The abbreviations used are: ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; AMP, adenosine monophosphate; AMPK, AMP-activated protein kinase; ARC, arcuate nucleus; ATP, adenosine tri-phosphate; CaMKK, Calmodulin kinase kinase; CLAMS, Comprehensive Lab Animal Monitoring System; DIO, diet induced obesity; GTT, glucose tolerance test; ITT, insulin tolerance test; JAK, janus kinase; MAPK, mitogen-activated protein kinase; MH, medial hypothalamus; ObRb, leptin receptor b; PI3K, phosphoinositide 3-kinase; PTP-1B, protein tyrosine phosphatase-1B; PVN, paraventricular nucleus; RER, respiratory exchange ratio; SOCS-3, suppressor of cytokine signalling 3; STAT3, signal transducer and activator of transcription 3.
FIGURE LEGENDS

Figure 1. Effect of DIO (diet-induced obesity) on body weight, serum insulin and leptin levels and glucose tolerance and insulin sensitivity.

A, Growth curves of male FVB mice maintained on a standard chow (chow) or a high fat diet (DIO) for 5-12 weeks. Mice were weighed weekly. B, Mice were bled between 8-10 am for measurement of serum insulin. C, Fed or over-night fasted mice were bled between 8-10 am for measurement of and serum leptin. D, GTT, after an overnight fast, mice were injected with D-glucose (2 mg/g of body weight) intraperitoneally. E, ITT, food was removed at 8 am and four hours later mice were injected with insulin (1.0 unit/kg of body weight). Values are means ± SEM; n=20 per group. Panels A, D, E *P<0.05 vs. chow. Panels B and C *P<0.05 vs. all other groups.

Figure 2. Attenuation of leptin's effects on food intake and respiratory exchange ratio (RER) in DIO mice.

A, After ten weeks of high fat or chow diet, six mice from each group received two i.p. injections of leptin (3ug/g body weight, each) or saline and food intake of individually housed mice was measured as described in Materials and Methods. Data are presented as kilocalories consumed/mouse per twenty-four hours. B, 24-hour RER during 24-hour treatment with leptin (3mg/kg) in DIO or chow-fed mice. After acclimating for 24 hours in the calorimeter, mice received two injections of saline and then three leptin injections. The 24-hour period following the third and final leptin injection is shown. RER was measured using indirect calorimetry and was calculated as the ratio of VCO₂ to the VO₂. The arrow indicates the leptin injection and the shaded area indicates the dark cycle (lights off). C. The 12-h (6pm-6am) RER area under the curve (AUC) was quantitated for each mouse. All data are presented as mean ± SEM. Statistical analyses were performed with two-way ANOVA using repeated measures with Bonferroni post-tests. *P<0.05 vs. chow saline, n=7-9 mice per group.

Figure 3. Decreased leptin-mediated AMPK phosphorylation and activity in skeletal muscle from DIO mice on a high fat diet for 12 weeks.

A and B, AMP-activated protein kinase-Thr172 phosphorylation in soleus 5 hours after ip injection of saline (white bars) or leptin, 3mg/kg (black bars). A, representative Western blot of Phospho-AMPK and total α2-AMPK (n=6-8 mice/treatment). This is representative of three independent experiments. B, quantitation of blots shown in A. Bars represent phosphoAMPK normalized to total α2-AMPK in soleus. C, total α2-AMPK protein levels from chow (white bars) or DIO (black bars) (n=6-8 mice/treatment). D, α2-AMPK activity in soleus muscle 5 hours after leptin, 3mg/kg (black bars) or saline (white bars); n = 9-15 mice per treatment. *P<0.05 vs. chow saline. †P<0.055 vs. chow saline.
Figure 4. **Effect of leptin on Acetyl CoA Carboxylase (ACC) activity in muscle from chow-fed or DIO mice on a high fat diet for 12 weeks.**

A, ACC activity in muscle from chow or DIO mice was measured 5 hours after ip injection of saline (white bars) or leptin, 3mg/kg (black bars). *P<0.05 vs. chow saline. B, Total ACC protein levels from chow (white bars) or DIO (black bars) mice. n=5-7 mice per treatment. *P<0.05 vs. chow. These data are representative of two separate experiments.

Figure 5. **Effect of high fat diet (12 weeks) on α2-AMPK activity in micro-dissected hypothalamic extracts.**

Chow or DIO mice were sacrificed 5 hours after ip injection of saline (white bars) or leptin, 3mg/kg (black bars); n=9-15 per treatment. *P<0.05 vs. chow saline. †P<0.05 vs. DIO saline. PVN = paraventricular nucleus; ARC = arcuate nucleus; MH = medial hypothalamus

Figure 6. **Decreased effect of leptin on STAT3 phosphorylation in arcuate nucleus from DIO mice on a high fat diet for 12 weeks.** Chow or DIO mice were sacrificed 5 hours after ip injection of saline (white bars) or leptin, 3mg/kg (black bars). The lysates were prepared as described under "Material and Methods" and subjected to SDS-PAGE and immunoblotted for phospho-STAT3 and re-probed for total STAT3. Bars show quantitation of phosphorylated STAT3 normalized to total STAT3. This is representative of two separate experiments on a total of 10-14 mice/treatment. The blots were quantified using GeneSNap software. *P<0.05 vs. chow saline.

Figure 7. **Short-term (5 weeks) high fat feeding does not affect basal AMPK activity or STAT3 phosphorylation.**

A, Mice were fed chow (white bar) or a high fat diet (DIO, black bar) for 5 weeks. Mice were bled between 8-10 am to determine serum leptin levels. ( * P <0.05 vs. all other groups; # P< 0.05 vs. all other groups). B, STAT3 phosphorylation in arcuate. Chow-fed (white bar) or DIO (black bar) mice were sacrificed 5 hours after ip injection of saline. Lysates were prepared as described under "Material and Methods" and subjected to SDS-PAGE and immunoblotted for phospho-STAT3. Blots were re-probed for total STAT3. Bars show quantitation of phosphorylated STAT3 normalized to total STAT3. C, AMPK activity was measured in hypothalamic nuclei (see "Materials and Methods"). Chow (white bar) or DIO (black bar) mice were sacrificed 5 hours after ip injection of saline; n=9-15 per treatment. PVN = paraventricular nucleus; ARC = arcuate nucleus; MH = medial hypothalamus.
Figure 3

A

AMPK Phosphorylation in Muscle

<table>
<thead>
<tr>
<th>Chow</th>
<th>DIO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospho AMPK</td>
<td>-</td>
</tr>
<tr>
<td>Total α2 AMPK</td>
<td>-</td>
</tr>
<tr>
<td>Leptin:</td>
<td>-</td>
</tr>
</tbody>
</table>

B

P-AMPK/total AMPK (% of Chow saline)

<table>
<thead>
<tr>
<th>Chow</th>
<th>DIO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>-</td>
</tr>
<tr>
<td>Leptin</td>
<td>-</td>
</tr>
</tbody>
</table>

C

Total α2-AMPK Protein in Muscle

<table>
<thead>
<tr>
<th>Chow</th>
<th>DIO</th>
</tr>
</thead>
<tbody>
<tr>
<td>α2-AMPK Protein (arbitrary units)</td>
<td>0.5</td>
</tr>
</tbody>
</table>

D

α2-AMPK Activity in Muscle

<table>
<thead>
<tr>
<th>Chow</th>
<th>DIO</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of Chow saline</td>
<td>*</td>
</tr>
</tbody>
</table>

[Graphs and data showing AMPK phosphorylation and activity in muscle under different conditions.]
Figure 4

(A) ACC Activity in Muscle

(B) Total ACC protein in Muscle
Figure 5

α2-AMPK Activity in Hypothalamic Nuclei

% of Chow saline

- Chow
- DIO
- PVN
- ARC
- MH

saline
leptin

* p < 0.05
† p < 0.01
Figure 6

STAT3 Phosphorylation in ARC

Total STAT3 Protein in ARC
Figure 7

A  
Serum Leptin Levels

B  
STAT3 Phosphorylation in ARC

C  
α2-AMPK Activity in Hypothalamic Nuclei

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Tonya L. Martin, Thierry Alquier, Kenji Asakura, Noburu Furukawa, Frederic Preitner and Barbara B. Kahn

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