Central infusion of leptin does not increase AMPK signaling in skeletal muscle of sheep


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Abstract

Type 2 diabetes and obesity are characterized by impaired glucose (68) and fat (46) metabolism in peripheral tissues, especially skeletal muscle. It has been proposed that inactivity of the energy-sensing enzyme AMPK, may play an important role in the development of these disorders (16, 17, 67). Acute pharmacological activation of AMPK by 5-aminimidazole-4-carboxamide-ribonucleoside (AICAR) increases glucose uptake (20, 32, 40) and fat oxidation (40, 43) in skeletal muscle.

Administration of AICAR on a daily basis leads to favorable alterations in cellular bioenergetics (66) that can prevent diabetes in obese Zucker rats (50).

Physiologically, AMPK is activated during exercise by an energy deficit in the cell via an increase in the AMP:ATP ratio (19). However, AMPK can also be activated in skeletal muscle by a number of other signals, including upstream AMPK kinase(s) (19), metformin (44), and in rodent skeletal muscle, by the adipocyte-derived hormone, leptin (43). Blood leptin levels are generally proportional to adipose tissue mass (15, 35), and whether it is administered centrally or peripherally, leptin reduces food intake (41, 57) and increases energy expenditure (27, 48). However, the mechanisms by which central leptin increases energy expenditure are not clear.

We have shown in sheep that acute central (intracerebroventricular) leptin infusion not only reduces food intake but increases energy expenditure, potentially via increasing postprandial heat production in skeletal muscle and fat tissues (23). This effect of central leptin on the temperature of gluteal fat, visceral fat, and skeletal muscle occurred an hour before increases in core temperature were observed, indicative of a true thermogenic effect. Minokoshi et al. (43) have shown that central leptin can acutely activate skeletal muscle AMPK for at least 6 h in mice. These central effects of leptin are thought to involve control of the sympathetic nervous system, via α-adrenergic signaling (43). Thus, the well-recognized property of leptin to cross the blood-brain barrier (1) and activate the SNS (14, 52) is thought to lead to increased skeletal muscle AMPK activity (43), although this effect has only been shown in lower-order mammals, such as mice. Therefore, the aim of the current study was to examine whether acute central leptin administration activates AMPK in skeletal muscle of sheep.

Although leptin has these acute effects on muscle and fat temperature, core temperature, and energy expenditure, it is, however, an adipostat hormone, providing long-term signaling of energy status to the brain. Thus, data provided in these studies (23, 43), involving acute leptin administration, may not pertain to physiological conditions of chronic alteration in leptin status. Indeed, while obesity is characterized by high levels of circulating leptin (34), skeletal muscle AMPK activity is not elevated in either obese humans or rodents fed a high-fat diet (34, 36, 40, 62). Circulating leptin levels are chronically elevated in obese individuals with type 2 diabetes, (34) but “leptin resistance” occurs such that the effects of leptin on food intake, energy expenditure, and skeletal muscle AMPK activation are diminished (59, 60). Therefore, a second aim was to examine the effects of chronic central leptin administration...
(7–8 days) on AMPK activation in skeletal muscle and fat, as well as on metabolism in sheep. We used the large mammal model of sheep since, unlike rodents, they are diurnal, as are humans, and sheep display similar hormonal responses to humans over a range of metabolic challenges. For example, under fasted conditions, both sheep (13) and humans (26) exhibit an increase of growth hormone secretion, whereas rodents (63) exhibit a decrease. On the basis of the studies in rodents, we hypothesized that acute central leptin infusion would activate AMPK but that this effect would be attenuated after chronic leptin infusion, indicative of leptin resistance.

MATERIALS AND METHODS

Animals

Corriedale ewes aged 3–4 years were obtained from and housed at the Monash University large animal facility. Ethics approval was obtained from the Department of Primary Industries Animal Ethics Committee, and all animal experimentation was conducted in accord with accepted standards of humane animal care. Under general anesthesia, induced by 20 mg/kg iv pentobarbital sodium and maintained with 1–2% isoflurane and oxygen, all animals were ovarioctomized to avoid cyclic variations in plasma levels of ovarian hormones (10, 28). At least 1 mo later, animals underwent a second surgery, also under general anesthesia (see above), for placement of an infusion cannula into the lateral ventricle (LV) of the brain (49).

The ewes were then allowed to recover for 4–6 wk in the field, and 1 wk prior to the experimental period, they were introduced to individual pens (alongside other sheep), in the experimental facility to get them familiarized with the environmental conditions and surroundings. The animals were subjected to normal environmental variation in temperature and photoperiod throughout the month of September in single pens, the animals were fed an ad libitum diet.

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Ser221 phosphorylation, lysates of muscle, liver, heart, kidney (130 μg) and PBS with the standard. Lysates were solubilized in Laemmli sample buffer and incubated at 100°C for 5 min.

For determination of AMPKα Thr172 phosphorylation and ACCβ Ser212 phosphorylation, lysates of muscle, liver, heart, kidney (130 μg), and PBS with the standard. Lysates were solubilized in Laemmli sample buffer and incubated at 100°C for 5 min.

Plasma analysis. Plasma epinephrine and norepinephrine concentrations were measured using an enzyme immunoassay kit (Labor Diagnostika Nord, Nordhorn, Germany). Plasma insulin concentration was determined using a porcine insulin radioimmunoassay kit (Linco Research, St. Charles, MO) previously shown to be compatible for ovine insulin measurements (24). Plasma free fatty acid concentration was determined using an enzymatic calorimetric procedure (Wako, Osaka, Japan) (29). Plasma glycerol concentration was determined using a fluorometric technique (7) on a 96-well plate. Plasma concentrations of growth hormone and cortisol were measured by radioimmunoassay, as previously described (25, 64), and pulse analysis of growth hormone concentrations was undertaken using PULSAR analysis (25).

Whole body glucose kinetics. Plasma glucose concentration was determined using the glucose oxidase method (ABL 615 Radiometer Medical A/S, Copenhagen, Denmark). Tritiated glucose within each sample was measured, as described previously (39). Briefly, the samples were deproteinized and spun; the supernatant was incubated in an oven overnight to evaporate any tritiated water. Milli-Q water was then added, and the samples were counted using a liquid scintillation counter (Packard Instrument, Meriden, CT). Glucose kinetics were estimated using a modified one-pool nonsteady-state model (51, 58a). We assumed 0.65 as the rapidly mixing portion of the glucose pool (5) and estimated the apparent glucose space as 25% of the glucose Rd by the “treatment” (leptin, control, or pair-fed) was defined as the between-subject factor, while “time” was defined as the within-subject factors (i.e., repeated measures). Significance level was set at P < 0.05. If the ANOVA detected a statistically significant difference, specific differences were located using the Fisher’s least significant difference post hoc test. As discussed below in Pair-Fed Group, there were initial significant differences between the pair-fed (PF) group and the other two groups prior to the commencement of infusions, and since there was a lack of change in any variable in the PF group in response to the aCSF infusion, subsequent statistical comparisons were made between the leptin-treated animals and the control aCSF infusion (normally fed) animals only.

RESULTS

Central leptin infusion: food intake and body weight. Food intake across the 8 days of the experiment was lower (P < 0.05) in the leptin-treated (6.0 ± 3.0 kg) and pair-fed (7.3 ± 1.4 kg) groups than in the controls (13.9 ± 2.4 kg) with no difference between the leptin-treated and pair-fed groups. Consistent with the reduced food intake, body weight decreased (P < 0.05) in the leptin-treated (65.6 ± 1.4 to 59.8 ± 2.8 kg) and pair-fed (69.9 ± 4.1 to 65.5 ± 3.2 kg) groups by a similar amount, which was significantly (P < 0.05) different to the effect of vehicle control infusion (67.4 ± 2.2 to 69.5 ± 2.1 kg).

Pair-Fed Group

For unknown reasons, prior to commencement of the infusions, the PF group displayed elevated plasma insulin concentrations (P < 0.05), elevated whole body glucose turnover (P < 0.05), and a tendency toward elevated plasma glucose concentrations compared with the other groups. Importantly, however, the levels of these variables did not change significantly throughout the infusion and pair-feeding period. Basal plasma catecholamine, glycerol, and free fatty acid (FFA) concentrations; skeletal muscle glycogen content and AMPK; and ACC phosphorylation in all tissues measured in the pair-fed group were not significantly different to the other groups.
prior to commencement of the infusions and did not change throughout the experimental period. As mentioned in statistical analysis, because of the initial differences between the PF group and the other groups prior to the commencement of infusions and the lack of change in any variable in the PF group in response to the aCSF infusion, subsequent statistical comparisons were made between the leptin-treated animals and the control aCSF infusion (normally fed) animals, only.

Central Leptin Infusion: Plasma Catecholamines, Cortisol, and Growth Hormone Concentrations

Central leptin infusion caused an acute increase ($P < 0.05$) in plasma epinephrine over the first 6 h of infusion, and at 6 h, plasma epinephrine was higher in the leptin group than the CON group (Fig. 2A). After 7 days of leptin infusion, plasma epinephrine concentrations remained higher ($P < 0.05$) than the preinfusion level in the leptin-treated animals, and values were higher ($P < 0.05$) than in the CON group (Fig. 2A). Plasma norepinephrine levels were not significantly affected by leptin infusion (Fig. 2B). Plasma cortisol and growth hormone (GH) concentrations, and mean GH interpulse intervals and pulse amplitude were not different in control and leptin-treated sheep during the 6 h immediately prior to, and the 6 h after, the commencement of the leptin infusion (data not shown).

Central Leptin Infusion: Plasma Glycerol, FFA, Glucose, and Insulin

There was no significant effect of acute and chronic leptin infusion on plasma glycerol, NEFA, glucose, or insulin concentrations (data not shown).

Central Leptin Infusion: Whole Body Glucose Kinetics

Central leptin infusion had no acute effect on glucose Ra, but after 7 days of leptin infusion, glucose Ra was lower ($P < 0.05$) in the leptin-treated animals (Fig. 3A). Glucose Rd decreased with acute leptin infusion and was lower ($P < 0.05$) after 5 and 6 h in the leptin group compared with the vehicle-infused CON group (Fig. 3B). In addition, glucose Rd was lower ($P < 0.05$) in the leptin group than CON after 7 days of leptin infusion (Fig. 3B). Glucose clearance rate was also lowered ($P < 0.05$) by both acute and chronic leptin infusion (Fig. 3C).

Central Leptin Infusion: Muscle and Liver Glycogen Content

Basal muscle glycogen content did not differ between groups ($55 \pm 4$ and $53 \pm 11$ mmol/kg wet weight in CON and leptin, respectively). There was no effect of leptin infusion on muscle glycogen content after 6 h or after chronic infusion (7
days; data not shown). Liver glycogen content did not differ between the groups at 8 days (89 ± 4 and 88 ± 10 mmol/kg wet weight in CON and leptin, respectively).

**Central Leptin Infusion: Skeletal Muscle AMPKα Phosphorylation and ACCβ Phosphorylation**

There was no effect of 6 h or 7 days of central infusion of leptin on total protein abundance of either AMPKα or ACCβ (data not shown). In addition, central leptin infusion had no acute or chronic effect on skeletal muscle AMPK phosphorylation of AMPKα Thr[172] (data not shown) or when normalized to total AMPKα protein (Fig. 4A). Similarly, ACCβ Ser[221] phosphorylation and ACCβ protein content in subcutaneous fat were unaffected by acute and chronic leptin infusion (data not shown).

**Central Leptin Infusion: Post Mortem Liver, Heart, and Kidney AMPKα Phosphorylation and ACCβ Phosphorylation**

Chronic leptin infusion had no significant effects on AMPKα Thr[172] phosphorylation or total AMPKα abundance in the liver, heart, and kidney (data not shown).

**Intra-arterial AICAR Infusion: Peripheral AICAR Infusion and AMPKα Phosphorylation**

Femoral artery infusion of AICAR resulted in an increase (P < 0.05) in skeletal muscle AMPKα phosphorylation at both 1 h and 6 h of infusion compared with the same time points during vehicle infusion (Fig. 5).

**DISCUSSION**

The major finding of this study was that acute and chronic central administration of leptin in sheep reduced food intake and whole body glucose turnover and increased plasma catecholamine concentrations but had no effect on AMPK phosphorylation in skeletal muscle, fat, or any other peripheral tissue examined. Importantly, femoral artery infusion of

![Fig. 4. Mean AMPKα Thr[172] phosphorylation normalized to total AMPKα before, after 6 h, and following 7 days of central leptin (Leptin) or aCSF (CON) infusion in skeletal muscle (A) and in subcutaneous fat (B). Data are expressed as means ± SE; n = 4 per group.](image)

![Fig. 5. Mean AMPKα Thr[172] phosphorylation normalized to total AMPKα in skeletal muscle after 1 h or 6 h of femoral artery AICAR or saline infusion. Data are expressed as means ± SE; n = 4 per group. P < 0.05 vs. saline.](image)
AICAR activated skeletal muscle AMPK in sheep. These results suggest differences may exist between rodent and ovine models with respect to the regulation of AMPK in the skeletal muscle and other peripheral tissues.

To determine whether the physiological effects of leptin infusion in the brain were translated to the periphery through activation of the SNS, plasma epinephrine and norepinephrine were measured as an indicator of sympathetic nerve activity (SNA), due to spillover from the sympathetic nerve terminals. Previous studies in rodents have shown that a single intracerebroventricular injection of leptin leads to an increase in renal SNA (53), and acute peripheral leptin increased SNA in brown adipose tissue, hindlimb, adrenal gland, and kidney (21). In the current study, central leptin infusion increased plasma epinephrine concentrations within 6 h, and the effect was sustained for at least 7 days. These results suggest that any peripheral effects of centrally administered leptin are transmitted via the SNS. Similarly, intracerebroventricular infusion of leptin increases the postprandial thermogenic response in fat and in muscle of sheep (23), most likely transmitted via the SNS. The increase in plasma epinephrine without associated changes in cortisol and growth hormone secretion, argues strongly against a non-specific stress-induced change in hormone concentrations.

This is the first study to report a reduction in whole body glucose turnover during central leptin infusion with rodent studies reporting that acute central leptin injection increases glucose uptake in skeletal muscle, brown adipose tissue, heart, and whole body glucose turnover (18, 30, 42, 65). The previous rodent studies and the current sheep study found that the effects on glucose turnover of leptin occurred independently of changes in plasma glucose and insulin concentration. In rodents, leptin activates AMPK, and it has been shown, using the AMPK activator AICAR, that AMPK increases glucose uptake (3, 20). Since AMPK is not activated by central infusion of leptin in sheep, it is perhaps not surprising that leptin does not increase glucose uptake in sheep. Elevations in plasma epinephrine inhibit glucose uptake in skeletal muscle (4), and elevated plasma FFAs inhibit glucose transport and disposal in skeletal muscle (31, 54). Therefore, the increased plasma epinephrine and trend toward elevated FFAs following leptin treatment may contribute to the reduced rate of glucose disposal observed in the present study.

We also found that leptin infusion decreased the glucose Ra. As sheep are ruminants, we did not attempt to fast them prior to the commencement of the leptin infusion protocol, because emptying of the stomachs would take a number of days. Ruminants do not absorb carbohydrates from the gut and rely heavily on gluconeogenesis in the liver for their glucose supply (22). Therefore, we assume that glucose is predominantly appearing from the liver rather than the gut. The fatty acid, propionate, is the major gluconeogenic substrate of the ruminant in the fed state (11). Therefore, it is possible that the reduced food intake observed with leptin, particularly after 7 days, caused a reduction in the rate of glucose appearance by decreasing gluconeogenesis in the liver.

Whereas central leptin administration in mice increases skeletal muscle AMPK signaling, with concomitant elevation of SNA (43), in our current study, skeletal muscle AMPK signaling was not increased, despite increases in plasma catecholamines. Importantly, we found that local AICAR infusion was able to increase hindlimb skeletal muscle AMPK phosphorylation in sheep, as it does in rodents (3). Therefore, although sheep skeletal muscle AMPK can be activated acutely by local AICAR infusion, sheep skeletal muscle AMPK is not activated in response to acute or chronic central leptin infusion. These results contrast to those previously demonstrated in mice (43) and, therefore, suggest a species difference. As discussed in the introduction, sheep are physiologically closer to humans than rodents in a number of respects (13, 26, 63).

We have previously shown that muscle temperature is increased with acute central leptin infusion in sheep (23). Given that we now show that central leptin infusion does not cause skeletal muscle AMPK activation, it would appear that the thermogenic effect of central leptin infusion is due to factors other than AMPK activation.

Interestingly, infusion of AICAR into the femoral artery of humans at an identical rate as we used in the current sheep study (10 mg/h) increased skeletal muscle extracellular signal-regulated kinase 1/2 phosphorylation but not AMPK phosphorylation (9). Therefore, although skeletal muscle AMPK in humans is activated by exercise, it is not activated by a low-dose infusion of AICAR, which activates skeletal muscle AMPK in sheep. This suggests that sheep skeletal muscle may be more sensitive to AICAR than human skeletal muscle. Importantly, in the current study, central leptin infusion in sheep had effects on food intake, catecholamine levels, and glucose turnover but did not affect skeletal muscle AMPK phosphorylation despite the fact that the skeletal muscle AMPK in sheep was activated by peripheral infusion of AICAR. This shows a clear species difference with the mouse data in the study by Minokoshi et al. (43), in which both central and peripheral AICAR administration activated skeletal muscle AMPK.

Given that projections of the SNS are distributed throughout the body, it is possible that central leptin administration could activate AMPK and modulate metabolism in other tissues in addition to skeletal muscle. In rodents, AMPK activation in adipose tissue decreases lipogenesis (37) and increases fat oxidation (55). Sustained hyperleptinemia induced in normal rats by adenovirus transfer of the leptin cDNA results in virtually complete disappearance of body fat within 7 days (6, 58). Chronic adenovirus-induced hyperleptinemia reduces fat mass and has been shown to cause AMPK activation in some (45), but not all (33), studies. Here, we report that 6 h and 7 days of central leptin infusion does not change AMPK activation in subcutaneous fat, consistent with our findings in skeletal muscle. As mentioned above, the tendency toward elevated plasma FFA concentration following leptin treatment could reflect epinephrine-stimulated adipocyte lipolysis (12, 61). However, there was no significant increase in plasma glycerol concentration with leptin infusion. To confirm whether leptin treatment leads to increased adipocyte lipolysis, follow-up studies will need to use labeled glycerol.

Although acute central leptin has been shown in mice to increase glucose uptake in the heart (30, 42), the mechanism by which this occurred was not investigated. In the heart, previous rodent studies suggest that acute elevation of AMPK activity increased glucose uptake (8, 56) and fat oxidation (8) similar to skeletal muscle. Seven days of continuous central leptin infusion had no effect on AMPK activity in the sheep heart, consistent with our finding in skeletal muscle. Chronic adenovirus-induced hyperleptinemia has been shown to reduce fat...
storage in the liver, but it has no effect on liver AMPK activity (33). Similarly, we found no effect of chronic central leptin infusion on liver AMPK phosphorylation.

In conclusion, consistent with the well-known actions of leptin on appetite and the SNS, the current study demonstrated that acute and chronic central administration of leptin in sheep reduced food intake in conjunction with increased plasma epinephrine. These physiological effects occurred without changes in skeletal muscle, fat, or other peripheral tissue AMPK activation, and given that reduced whole body glucose turnover was also observed, may indicate species differences between the rodent and ovine models. Further studies will be required to fully elucidate the role leptin plays as a long-term signal and how the actions of central leptin might play a role in obesity and leptin resistance.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES


Leptin and Skeletal Muscle AMPK in Sheep


