Ciliary Neurotrophic Factor Prevents Acute Lipid-Induced Insulin Resistance by Attenuating Ceramide Accumulation and Phosphorylation of c-Jun N-Terminal Kinase in Peripheral Tissues

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Ciliary neurotrophic factor (CNTF) is a member of the gp130 receptor cytokine family recently identified as an antiobesity agent in rodents and humans by mechanisms that remain unclear. We investigated the impact of acute CNTF treatment on insulin action in the presence of lipid oversupply. To avoid confounding effects of long-term high-fat feeding or genetic manipulation on whole-body insulin sensitivity, we performed a 2-h Intralipid infusion (20% heparinized Intralipid) with or without recombinant CNTF pretreatment (Axokine 0.3 mg/kg), followed by a 2-h hyperinsulinemic-euglycemic clamp (12 mU/kgmin) in fasted, male Wistar rats. Acute Intralipid infusion increased plasma free fatty acid levels from 1.0 ± 0.1 to 2.5 ± 0.3 mEq/l, which subsequently caused reductions in skeletal muscle (insulin-stimulated glucose disposal rate) and liver (hepatic glucose production) insulin sensitivity by 30 and 45%, respectively. CNTF pretreatment completely prevented the lipid-mediated reduction in insulin-stimulated glucose disposal rate and the blunted suppression of hepatic glucose production by insulin. Although lipid infusion increased triacylglycerol and ceramide accumulation and phosphorylation of mixed lineage kinase 3 and c-Jun N-terminal kinase 1 in skeletal muscle, CNTF pretreatment prevented these lipid-induced effects. Alterations in hepatic and muscle insulin signal transduction as well as phosphorylation of c-Jun N-terminal kinase 1/2 paralleled alterations in insulin sensitivity. These data support the use of CNTF as a potential therapeutic means to combat lipid-induced insulin resistance. (Endocrinology 147: 2077–2085, 2006)

SKELETAL MUSCLE insulin resistance is a key factor in the pathogenesis of type 2 diabetes and is a metabolic disorder that precedes overt diabetes by several decades. Elevated plasma free fatty acid (FFA) levels are often observed in human and rodent models of type 2 diabetes or insulin resistance, and it has been shown that acute elevation of FFA levels, achieved via heparinized infusion of a triglyceride emulsion, leads to skeletal muscle and hepatic insulin resistance (1–3). An important consequence of chronically elevated circulating FFA levels is the accumulation of fatty acid metabolites within insulin-responsive tissues. Accumulation of these lipid intermediates, including ceramides and diacylglycerol, also correlate with insulin resistance (4, 5). The production of fatty acid metabolites within insulin-responsive tissues is known to activate a host of serine kinases including protein kinase Cθ, IKK-nuclear factor κB (NFκB), and c-Jun N-terminal kinase (JNK), which have been shown to impair insulin signal transduction and glucose disposal in vitro (3, 6).

Given that insulin sensitization is a key therapeutic strategy to combat type 2 diabetes, our laboratories have been actively investigating mechanisms to ameliorate insulin resistance by promoting fatty acid use in skeletal muscle. To this end, in the current investigation, we studied the impact of ciliary neurotrophic factor (CNTF) administration on acute lipid-induced insulin resistance. CNTF is a member of the gp130 cytokine family and was first identified as a trophic factor for motor neurons in the ciliary ganglion (7), and later as an antiobesity agent in rodents and humans (3, 6, 8–10). Because obesity is highly correlated with type 2 diabetes and because obese CNTF-treated mice exhibit an improved metabolic profile, (i.e. reduced basal levels of blood glucose, insulin, FFA, and triglycerides and improved glucose tolerance) (8, 9, 11, 12), we examined the mechanism(s) by which CNTF improves metabolism. Although other studies have described the effects of CNTF administration on improving insulin signaling in 3T3-L1 adipocytes (13) and steatotic hepatocytes isolated from diabetic mice (11), we investigated the impact of CNTF administration on skeletal muscle insulin action and fatty acid metabolism because skeletal muscle accounts for the majority (75–95%) of whole-body insulin-stimulated glucose disposal (14) and 25% of whole-body FFA uptake (15).

The CNTF receptor α heterodimerizes with the transmembrane gp130 receptor upon activation, and this heterodimer is highly expressed in skeletal muscle (16). Moreover, the gp130 receptor shares significant sequence homology with
the long form leptin receptor in that it contains a Src homology 2 domain capable of activating the Jak/Stat signaling pathway after ligand binding. It is well known that leptin increases fat oxidation and prevents insulin resistance (17); therefore, we hypothesized that activation of the CNTF receptor would induce similar metabolic actions to leptin, namely increased fatty acid oxidation (18) and reduced fatty acid deposition in skeletal muscle (19). Therefore, the aim of the present study was to elucidate the role of CNTF on insulin action in vivo and correlate these measures with analyses of skeletal muscle fat metabolite accumulation. To avoid confounding variables of long-term high-fat feeding or genetic manipulation, we imposed a lipid challenge via acute heparinized Intralipid infusions with or without exogenous CNTF treatment. After the lipid challenge, hyperinsulinemic-euglycemic clamp studies were performed to assess whole-body insulin sensitivity, and tissues subsequently were excised for analysis of insulin signal transduction and fatty acid metabolite accumulation. In this study, we report that although lipid overload caused marked accumulation of lipid intermediates, decrements in insulin signaling, and increased proinflammatory signaling in muscle and liver, CNTF pretreatment completely prevented these deleterious effects of lipid oversupply.

Materials and Methods

Animals

Male Wistar rats (Monash Animal Services, Clayton, Australia) aged 13–14 wk were housed two per cage at 22°C for subsequent experiments. Materials and Methods

Surgical procedures

Three days before the glucose clamp, all animals were chronically cannulated under single-dose anesthesia (100 mg/ml ketamine HCl, 20 mg/ml xylazine, 10 mg/ml acepromazine maleate; 1.5:3:1; 0.1 cc/100 g body weight administered im) in the jugular vein for infusion (glucose, [3H]glucose tracer, and insulin) [dual cannula; SILASTIC brand (Dow Corning Corp., Midland, MI), inside diameter 0.021 cm] and in the carotid artery (Clay Adams, PE-50) for arterial blood sampling. Cannulae were tunneled sc, exteriorized at the back of the neck, and encased in SILASTIC brand tubing (0.02 cm inside diameter) sutured to the skin. Animals were allowed 3 d of postoperative recovery and were fasted 12 h before the clamp experiment. To determine whether CNTF was effective at overcoming acute lipid-induced insulin resistance, rodents were randomly divided into one of three study groups: 1) control (Con) saline no CNTF, 2) Intralipid infusion no CNTF, and 3) CNTF treatment (0.3 mg/kg) + Intralipid infusion. Hyperinsulinemic-euglycemic clamp experiments

Animals were weighed and placed in a modified metabolic chamber at 140 min before the clamp. After 20 min, a baseline sample was drawn from the carotid line and plasma was stored at −20°C for subsequent analyses. A primed (5 μCi) constant infusion (0.16 μCi/min) of tritiated [3H]glucose (Amersham Biosciences, Buckinghamshire, UK) was then administered. After a 60-min tracer equilibration period and basal sampling at 15 min before and immediately before lipid infusion (to determine basal glucose turnover), either saline + tracer (0.16 μCi/min) or heparinized (40 U/ml) 20% lipid emulsion + tracer (Intralipid; Monash Medical Centre, Clayton, Australia) was infused at a rate of 0.0167 ml/min for 120 min. The total volume of Intralipid + heparin infused was 2.0 ml. Although others have typically infused lipid emulsions and heparin for 3–5 h to induce insulin resistance (3, 20–22), preliminary work from our laboratory demonstrated insulin resistance after 2 h of infusion and more reliable steady-state glucose infusion rates during the subsequent hyperinsulinemic-euglycemic clamp. Where appropriate, the CNTF analog Axokine, a gift from Regeneron Pharmaceuticals, was administered slowly as a bolus injection (0.3 mg/kg) into the carotid line and flushed into the animal with saline just before the constant infusions of Intralipid. Axokine is not identical to human CNTF as Gln63 is replaced by Arg63, which appears to make Axokine more specific for the CNTF receptor (23). Axokine at this dose does not induce cachexia, stress, inflammation, or nausea in rodents (8). During the saline or Intralipid infusion period preclamp, samples were drawn at 60 and 120 min for the measurement of FFA concentration. After this, the lipid or saline infusion was terminated and the euglycemic-hyperinsulinemic clamp was initiated. This infusion protocol was designed to increase intracellular lipid content in peripheral tissues, yet ensure low circulating fatty acid levels during the clamp procedure. Unlike previous studies that have implemented a triglyceride-heparin infusion throughout the hyperinsulinemic-euglycemic clamp, stopping the triglyceride-heparin infusion before the clamp allowed us to discriminate between events mediated within the muscle from events mediated by pharmacologically high circulating FFAs. Moreover, because glucose provides almost all of the substrate during hyperinsulinemic-euglycemic clamp, we did not expect a reduction in tissue lipids. After the saline or lipid infusion period, animals were each exposed to the same general glucose clamp protocol. Glucose (variable infusion; 50% dextrose; Abbott Labs, Chicago, IL) and tracer + insulin (16.7 μl/min) infusions were initiated simultaneously. Small blood samples (5 μl) were drawn at 10-min intervals and immediately analyzed for glucose (HemoCue, Angelholm, Sweden) to maintain the integrity of the glucose clamp throughout the duration of the experiment. Animals were clamped at a target glycemia 6 mm, and there were no differences in clamp glycemia between the groups (P = 0.72). Steady-state was achieved in most animals by 60 min and maintained until termination of the clamp at 120 min. Arterial samples (100 μl) for measurement of glucose turnover were drawn at 100 and 120 min. Specific activity of radiolabeled tracer in the blood was not different between the two time points (P = 0.35). Given that arterial blood glucose did not fluctuate more than 5% and that glucose infusion rate was held constant for a minimum of 30 min, we are confident that stringent steady-state conditions were achieved. After the blood sample for determination of glucose turnover was drawn, an additional 500-μl sample of blood was taken from the carotid line for the subsequent analysis of FFA and sterolinsulin content. Plasma samples were then mixed with a lethal dose of sodium pentobarbital (Nembutal, 100 mg/kg) administered iv. Tissue samples were rapidly excised and snap frozen for subsequent metabolic analyses.

Plasma metabolite and hormone analysis

Blood was analyzed for fasting concentrations of glucose, insulin, and FFA. Plasma was obtained by centrifugation of whole blood and stored at −80°C until analysis. Blood glucose levels were monitored with the MediSense2 Blood Glucose Testing System (MediSense Australia Pty, Ltd., Victoria, Australia). For determination of plasma metabolites and hormones, blood was withdrawn, immediately spun at 4°C (5 min at 5000 × g), and rapidly frozen to limit lipoprotein lipase activation and, thereby, prevent triglyceride degradation, which would result in overestimation of plasma FFA levels. Plasma insulin levels were determined using enzyme RIA kits (Linco, St. Charles, MO), and plasma FFA were measured using an enzymatic colorimetric assay kit (Roche, Basel, Switzerland).

Western blot analysis

Soleus muscle and liver were homogenized in ice-cold buffer consisting of 20 mM HEPES, 1 mM dithiothreitol, 1 mM Na3PO4.2 mM EDTA, 1% Triton X-100, 10% glycerol (vol/vol), 3 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 5 μl/ml phosphatase inhibitorcock-
tail 2 (Sigma, St. Louis, MO), 5 μl/mole probe inhibitor cocktail (Sigma) and rotated for 40 min at 4 C. Homogenates were centrifuged at 16,000 × g for 25 min and the supernatant was removed and rapidly frozen in liquid nitrogen. Protein concentration of the lysates was determined according to the bicinchoninic acid method (Pierce Kit; Progen Industries, Darra, Queensland, Australia). Tissue lysates (1 mg) were immunoprecipitated with anti-insulin receptor substrate (IRS)-1 or anti-IRS-2 antibody (Upstate, Lake Placid, NY) for 16 h and solubilized in Laemmli sample buffer and boiled for 5 min, resolved by SDS-PAGE on polyacrylamide gels, transferred to a nitrocellulose membrane, blocked with 5% milk, and immunoblotted with anti-IRS-1 tyrosine (Upstate) or anti-p85 (Cell Signaling Technology, Beverly, MA) primary antibody overnight. After incubation with horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, Castle Hill, New South Wales, Australia) and quantified by densitometry (Chemidoc XRS; Bio-Rad Laboratories, Regents Park, New South Wales, Australia). Membranes were stripped, washed, and reprobed for total protein content where appropriate. Primary antibodies for phospho-mixed lineage kinase 3 (MLK3) (Thr187/ Ser202), INK1, phospho-INK1 (Thr185/ Tyr191), NF-κB p65, phospho-NF-κB p65 (Ser536), phospho-IRS-1 (Ser636), and total IRS-1 were obtained from Cell Signaling Technology, and MLK3 total was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Protein contents were determined as described after solubilizing 80 μg protein for SDS-PAGE.

Lipid analysis

Triacylglycerol content was analyzed as previously described (24). Tissues were dissected free of visible connective tissue and blood. Lipid was extracted by a Folch extraction, the triacylglycerol was saponified in an ethanol/KOH solution at 60 C, and glycerol content was determined fluorometrically. Diacylglycerol and ceramide were extracted and quantified according to the methods of Preiss et al. (25). Lipids were extracted from freeze-dried, powdered soleus muscle using chloroform-methanol-PBS + 0.2% SDS (1:2:0.8). Diacylglycerol kinase and [32P]ATP (15 mCi/mmol cold ATP) were added to lysates preincubated with cardiolipin/octylglucoside, and the reaction was stopped after 2 h by the addition of chloroform-methanol (2:1). Samples were spotted onto thin-layer chromatography plates and developed. 32P-labeled phosphatidic acid and ceramide-1-phosphate were identified, dried, scraped from the thin layer chromatography plate, and counted in a liquid scintillation analyzer (Tri-Carb 2500TR; Packard, Canberra, Australia).

Cell culture

In later experiments, we determined the effects of CNTF administration on fat oxidation in skeletal muscle cell culture. L6 myoblasts were maintained at 37 C (95% O2/5% CO2) in α-modified Eagle’s medium containing 10% fetal bovine serum, 1% penicillin/streptomycin, and 5 mM glucose. Differentiation was induced by switching to medium containing 2% horse serum when the myoblasts were approximately 90% confluent. To examine the effects of CNTF on fatty acid oxidation, cells were serum starved for 4 h and, after washing twice with PBS, fresh media (2 ml) consisting of α-modified Eagle’s medium, 2% BSA, and 0.5 mM palmitate, 1 μCi/ml [9,10-3H]palmitate (Amersham Biosciences, Buckinghamshire, UK), was added to the cells. Cells were treated with 10 ng/ml CNTF, 2 mM 5-aminomidazole-4-carboxamide 1-β-d-ribofuranoside (AICAR), or 100 mM epinephrine. The reaction was stopped after 2 h. Fatty acid oxidation was determined by separation of3H2O from the substrate by adding a 1-mM media to chloroform-methanol (2:1), subsequent addition of 2 M KCl-2 M HCl, and removal of the upper phase for liquid scintillation counting.

Statistics

Data are expressed as the mean ± SEM. Statistical analysis was performed by one-way ANOVA with repeated measures, and specific differences were detected using a Student-Newman-Keuls post hoc test. Statistical significance was set at P < 0.05.

Results

Plasma metabolites and hyperinsulinemic-euglycemic clamp

In the basal fasted state, circulating levels of insulin, glucose, and FFA as well as glucose turnover rate (10.34 ± 0.42 mg/kg-min) were identical between the groups. After basal measurements were taken, CNTF or saline was acutely administered via the carotid line. Animals were then infused with a heparinized lipid emulsion or saline, and at the end of this 2-h period, FFA levels were elevated 3-fold above basal (P < 0.05; Fig. 1) for animals receiving the lipid emulsion, whereas FFA levels were not different from basal for animals infused with saline. After 2 h, these constant infusions were ceased and the subsequent effect of insulin on whole-body glucose disposal rate and suppression of hepatic glucose production (HGP) was measured during hyperinsulinemic-euglycemic clamps (12 mU/kg-min). Clamp insulin and glucose concentrations at 12 mU/kg-min averaged 840 ± 61 pm and 6.1 ± 0.2 mm, respectively, and by design, no differences for either parameter were detected between the groups (Table 1). Insulin infusion caused a decrease in plasma FFA levels (P < 0.05) from basal, and despite the 2-h lipid preinfusion, no differences in fatty acid concentration were detected at 60 or 120 min during the clamp procedure between the groups (Fig. 1). Despite the insulin-stimulated suppression of circulating FFA for animals preinfused with lipid, the glucose infusion rate required to maintain euglycemia was decreased by approximately 30% (P = 0.03) compared with Con saline-infused rats (Fig. 2A). Similar findings were observed during preliminary submaximal clamp studies (1.2 mU/kg-min) where only glucose infusion rate was assessed (data not shown). In animals receiving CNTF before lipid, the steady-state glucose infusion rate was identical to Con (saline infused) values. Assessment of tracer kinetics allowed us to distinguish whether these differences could be attributed to alterations in metabolism within liver, skeletal muscle, or both. Insulin caused a 93% suppression of HGP in...
untreated rats during clamps, whereas acute elevation in FFA concentration before the clamp caused a significant blunting ($P < 0.01$; Fig. 2B) in HGP suppression for high fat (HF). Despite HF, acute CNTF treatment restored the ability of insulin to suppress HGP to rates observed in Con (Fig. 2B). The insulin-stimulated glucose disposal rate (IS-GDR), reflecting predominantly skeletal muscle insulin sensitivity, was decreased by 25% ($P = 0.04$) after acute elevation of tissue lipid content (HF—see below). CNTF pretreatment caused a normalization ($P = 0.005$ vs. HF) of IS-GDR back to Con levels in HF animals. These data indicate that CNTF counteracts the deleterious effects of elevated circulating FFAs as it restored the ability of insulin to stimulate peripheral glucose disposal as well as suppress HGP despite prior lipid oversupply.

### Skeletal muscle lipids

The intracellular deposition of triacylglycerols and fatty acid metabolites have been implicated in lipid-induced skeletal muscle insulin resistance via alterations in insulin signaling (3, 26). Triacylglycerol concentration in skeletal muscle averaged 4.26 ± 0.29 mol/g wet mass and was increased by 155% ($P = 0.04$; Fig. 3A) after heparinized Intralipid infusion. CNTF pretreatment completely prevented this increase in intramyocellular triglyceride accumulation. Skeletal muscle ceramide content was elevated ($P = 0.06$) after acute heparinized Intralipid infusion, and CNTF pretreatment lowered these levels by 45% ($P = 0.03$; Fig. 3C). Unlike

### TABLE 1. Metabolic parameters during hyperinsulinemic-euglycemic clamp in male Wistar rats with (CNTF) or without (SAL) CNTF pretreatment and/or prior triglyceride and heparin infusion

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>HF SAL</th>
<th>HF CNTF</th>
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<tbody>
<tr>
<td>Body mass (g)</td>
<td>367 ± 15</td>
<td>401 ± 14</td>
<td>382 ± 13</td>
</tr>
<tr>
<td>Insulin (pmol)</td>
<td>835 ± 70</td>
<td>860 ± 26</td>
<td>828 ± 88</td>
</tr>
<tr>
<td>Glucose (mM)</td>
<td>6.0 ± 0.3</td>
<td>6.0 ± 0.4</td>
<td>6.2 ± 0.2</td>
</tr>
<tr>
<td>FFA (mM)</td>
<td>0.30 ± 0.13</td>
<td>0.16 ± 0.03</td>
<td>0.22 ± 0.04</td>
</tr>
<tr>
<td>Liver TG (µmol/g)</td>
<td>12.8 ± 1.8</td>
<td>8.4 ± 2.3</td>
<td>10.9 ± 0.8</td>
</tr>
</tbody>
</table>

Values are mean ± SEM, n = 6. TG, Triacylglycerol; CON, control.
the potent effects of CNTF on prevention of skeletal muscle triacylglycerol and ceramide accumulation, CNTF had no effect on the accumulation of diacylglycerol induced by lipid infusion (Fig. 3B). To determine whether the reduced skeletal muscle fatty acid accumulation seen in CNTF-treated animals is mediated by increased fat oxidation, we examined CNTF effects in skeletal muscle L6 myotubes. CNTF increased palmitate oxidation by 88%, which was comparable to other known activators of fatty acid oxidation including AICAR (2 mM, 39% increase) and epinephrine (100 nM, 55% increase).

Skeletal muscle insulin signaling

The increase in skeletal muscle triacylglycerol and ceramide in HF animals was associated with impaired insulin signaling. As previously shown, IRS serine phosphorylation leads to reduced tyrosine phosphorylation and reduced glucose transport (3, 27). Consistent with this, IRS-1 Ser307 phosphorylation tended to increase \((P = 0.06)\) in HF animals; however, CNTF treatment prevented this lipid-induced signaling effect \((P = 0.02; \text{Fig. } 4A)\). Although lipid preinfusion tended to cause a modest decrease \((P = 0.1, \text{not significant})\) in insulin-stimulated IRS-1 tyrosine phosphorylation (Fig. 4B) for HF vs. Con, similar to our findings for IS-GDR, CNTF completely prevented this effect. Insulin-stimulated association between IRS-1 and the p85 subunit of phosphatidylinositol 3 (PI3) kinase was also impaired \((P = 0.04)\) by prior lipid oversupply (HF) vs. Con, but was prevented by CNTF pretreatment (Fig. 4C). Insulin-stimulated phosphorylation of Akt at Ser473 was diminished by prior acute lipid infusion and CNTF pretreatment in HF animals completely restored insulin signal transduction from IRS-1 through Akt (Fig. 4D).

MLK3 is a member of the MAPK kinase family of kinases and is activated in mammalian cells by ceramide (28). MLK3 phosphorylation was elevated \((P = 0.03)\) in muscle after lipid infusion, and this finding is consistent with the increased ceramide levels observed in this group. CNTF treatment prevented the lipid-induced increase in ceramide content, and hence unlike HF alone, no subsequent elevation in MLK3 phosphorylation was observed for CNTF + HF (Fig. 5A). Additionally, because MLK3 activates JNK \textit{in vitro} (29), our findings are consistent with these observations in that JNK phosphorylation was elevated in HF rats alone \((P = 0.04, \text{Fig. } 5B)\), but not after CNTF treatment.

Hepatic lipids, insulin signaling, and serine kinase activation

Lipid infusion did not result in liver steatosis (Table 1), but activated proinflammatory pathways and inhibited insulin signaling. Insulin-stimulated IRS-1 association with the p85 subunit of PI3 kinase p85 was decreased \((P = 0.02)\) in HF Sal compared with Con, and although not significant \((P = 0.14, \text{not significant})\), IRS-1 Ser307 phosphorylation (A), IRS-1 tyrosine phosphorylation (B), IRS-1 PI3 kinase association (C), Akt Ser473 phosphorylation (D), representative blots after immunoprecipitation with IRS-1 and immunoblotting with anti-PI3 kinase p85 or anti-IRS-1 tyrosine or serine antibodies (E), and representative immunoblots after probing with anti-Akt Ser473 and anti-Akt antibodies. Values are means \(\pm SE, n = 6. \ast, \text{Different from Con; } \dagger, \text{different from HF CNTF, } P < 0.05.\)
there was a tendency for this marker of insulin signaling to be attenuated in HF CNTF (Fig. 6A). IRS-2 activation as assessed by IRS-2 association with p85 subunit of PI3 kinase was decreased ($P = 0.05$) in HF Sal compared with Con (Fig. 6B). CNTF restored PI3 kinase p85 association with IRS-2 after lipid infusion. Akt Ser$^{473}$ phosphorylation was decreased in both HF groups compared with Con ($P < 0.05$, Fig. 6C). The phosphorylation of Ser$^{536}$ on the p65 subunit of NF-κB, a critical event in the regulation of NF-κB signaling, was elevated in HF Sal compared with Con ($P = 0.01$) (Fig. 6D). HF + CNTF partially blocked the phosphorylation of Ser$^{536}$. We observed no increase in Ser$^{32}$ phosphorylation of IκBα, or IκBα degradation, both critical events in the activation of the NF-κB signaling complex. However, these events occur before the phosphorylation of Ser$^{536}$ on the p65 subunit of NF-κB, and are tightly regulated so as to avoid the generation of highly proinflammatory conditions. Given that we collected tissue 2 h after the cessation of lipid infusion, it is likely that we missed these early activation markers of the NF-κB signaling complex. The phosphorylation of both p46 JNK1/2 and p54 JNK1/2 were elevated after HF Sal compared with Con ($P < 0.05$; Fig. 6E). HF CNTF completely prevented JNK1/2 phosphorylation.

**Discussion**

The beneficial effects of CNTF on insulin action have been previously attributed to occur secondary to effects on lipogenic gene expression and decreased lipid accumulation in nonadipose tissues (11). Our findings show that acute CNTF treatment prevents decrements in whole-body insulin sensitivity induced by acute lipid infusion. This was accompanied by increased insulin-stimulated tyrosine phosphorylation of IRS-1, p85 association with IRS-1, and activation of Akt in skeletal muscle samples obtained from animals undergoing a hyperinsulinemic-euglycemic clamp. CNTF treatment also prevented the lipid-induced increase in skeletal muscle triacylglycerol and ceramide content, and subsequent lipid-induced MLK3 and JNK activation. We show that CNTF protects insulin action in the face of a lipid challenge via mechanisms other than altered lipogenic gene expression. These mechanisms are likely associated with the prevention of cellular accumulation of ceramides and phosphorylation of JNK in peripheral tissues, thereby preventing impaired insulin signal transduction.

A strong correlation between skeletal muscle lipid accumulation and the severity of insulin resistance suggests that the accumulation of lipid-derived metabolites may antagonize directly insulin signal transduction (4, 5). In the present study, lipid infusion increased skeletal muscle triacylglycerol, diacylglycerol, and ceramide content in conjunction with reduced insulin sensitivity. CNTF treatment completely prevented the increase in IMTG and ceramide, but not diacylglycerol, suggesting CNTF may mediate its insulin-sensitizing effects by reducing select skeletal muscle fatty acid metabolite levels, perhaps via increased fatty acid oxidation. Ceramide is a fatty acyl CoA metabolite that is elevated in the skeletal muscle from insulin-resistant humans (30) and rodents (31, 32), and is correlated negatively to insulin sensitivity as assessed by the hyperinsulinemic-euglycemic clamp technique (33). Studies using short-chain ceramide analogs or saturated fatty acids to increase ceramide levels in skeletal muscle cells show that ceramide inhibits Akt phosphorylation/activation (26, 34) by dual mechanisms: first by preventing translocation of Akt and second via dephosphorylation of Akt by protein phosphatase 2A (35, 36). The reduction in ceramide content and increased insulin-stimulated Akt Ser$^{473}$ phosphorylation after CNTF administration in lipid-treated rats is consistent with these *in vitro* observations and may explain, at least in part, the insulin sensitizing effects of CNTF.

The decrease in ceramide content may also explain the improved insulin-stimulated IRS-1 tyrosine phosphorylation seen in CNTF-treated rats after the lipid challenge (37), although this remains controversial (26). Fatty acids promote serine kinase activation resulting in increased serine phosphorylation of IRS-1. Phosphorylation of IRS-1 on certain Ser residues impairs insulin-stimulated tyrosine phosphorylation and downstream PI3 kinase activation, and thus insulin-stimulated glucose disposal is reduced (38). Although many kinases phosphorylate IRS-1, evidence suggests JNK, a member of the MAPK family, is an important negative mediator of fatty acid-induced insulin resistance. *In vitro*, JNK activation causes phosphorylation at IRS-1 on residue Ser$^{307}$ and
reduces tyrosine phosphorylation, effects which are reversed by inhibition of JNK in cultured cells (27, 39), whereas similarly in vivo, ob/ob and high fat-fed mice have elevated JNK activity in peripheral tissues (14). Additional support for JNK1 in the pathogenesis of lipid-induced insulin resistance is provided by the JNK1 null mice which are protected from the deleterious effects of high fat feeding on whole-body insulin sensitivity (40). Although the molecular pathway linking JNK to reduced insulin action is well supported, the in vivo effects of ceramide in this pathway remain unknown. Previous studies using dsRNAi-mediated gene silencing and cell-permeable agonists show that ceramide activates MLK3 and thus JNK (28). In the present in vivo experiments, elevated ceramide content (1.5-fold) was associated with increased MLK3, JNK, and IRS-1 phosphorylation on residue Ser307 in skeletal muscle, and decreased whole-body insulin-stimulated glucose disposal. Moreover, these signaling and biological effects were prevented with CNTF pretreatment, which also coincided with prevention of ceramide accumulation.

It is possible that other serine kinases phosphorylate IRS-1 at Ser307 during lipid infusion. IKKβ can directly bind to IRS-1 Ser307 (6); however, we measured no change in skeletal muscle NFKβ signaling, which is consistent with human studies using acute lipid infusion (1). Fatty acids may also cause insulin resistance through diacylglycerol activation of a serine kinase cascade involving protein kinase Cθ (2, 3). Although diacylglycerol and IRS Ser307 were increased after lipid infusion, CNTF had no effect to diminish diacylglycerol after Intralipid infusion despite improved skeletal muscle insulin signaling and whole-body glucose disposal. The disconnect between skeletal muscle diacylglycerol concentration and peripheral insulin action, particularly CNTF-induced insulin sensitization in this case, indicate that diacylglycerol and protein kinase C activation may not be essential to the etiology of lipid-induced insulin resistance (1, 3).

Lipid-induced hepatic insulin resistance in the present study was associated with reduced IRS-1 and IRS-2 association with the p85 subunit of PI3 kinase, which are important components of hepatic insulin signal transduction. The finding that CNTF administration partially reversed these deleterious events in lipid-infused rats is consistent with findings from Sleeman et al. (11), who demonstrated that 10 d of Axokine treatment improved insulin-stimulated signaling in db/db mice exhibiting severe hepatic steatosis. However, we show that CNTF can improve insulin action acutely in liver, and insulin sensitization in our model does not occur due to reductions in liver steatosis and/or alterations in the expression of key hepatic genes. There is now an abundance of...
evidence linking inflammation, serine/threonine kinase activation, and insulin resistance (41), and accordingly, we demonstrated increased NFkB and JNK activation and hepatic insulin resistance after lipid infusion. A significant finding of the present study was the ability of CNTF to completely suppress lipid-induced JNK activation and partially prevent NFkB signaling. Thus, our data ascribes an acute direct role for CNTF on hepatic insulin action.

In summary, these data provide new insights into the mechanisms of CNTF-induced insulin sensitization in skeletal muscle and liver. In this study, we provide compelling evidence that CNTF prevents the accumulation of triacylglycerol and ceramides in skeletal muscle and this yields protection against acute lipid-induced insulin resistance. The maintenance of normal insulin signaling despite lipid oversupply is associated with 1) elimination of ceramide-induced Akt inhibition and or 2) enhanced IRS-1 tyrosine phosphorylation secondary to attenuated MLK3/JNK activation and IRS-1 Ser307 phosphorylation. Acute CNTF administration also enhances hepatic insulin action acutely by inhibition of inflammatory serine kinases. These data confirm the efficacy of CNTF receptor ligands as potential therapeutic targets for lipid-induced insulin resistance.

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Erratum

The article “Evaluation of an Estrogen Receptor-β Agonist in Animal Models of Human Disease” by Heather A. Harris, Leo M. Albert, Yelena Leathurby, Michael S. Malamas, Richard E. Mewshaw, Chris P. Miller, Yogendra P. Kharode, James Marzolf, Barry S. Komm, Richard C. Winneker, Donald E. Frail, Ruth A. Henderson, Yuan Zhu, and James C. Keith, Jr. (Endocrinology 144:4241–4249, 2003) described a rat mammarytrophic model in which ERB-041 was reported to regulate gene expression. The authors have determined that the gene marker measured in this model was, in fact, not casein kinase II and at present have not identified the gene product measured. The authors regret the error.