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Altered levels of adiponectin and adiponectin receptors may underlie the effect of Ciliary Neurotrophic Factor (CNTF) to enhance insulin sensitivity in diet-induced obese mice

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INTRODUCTION

Ciliary neurotrophic factor (CNTF), a neuronal growth factor originally studied in amyotrophic lateral sclerosis, induces weight loss and improves insulin resistances in humans and rodents (1). It has been recently shown that CNTF induces hypothalamic neurogenesis and that it signals through the CNTFR_α-IL-6R-gp130 β receptor complex to increase fatty-acid oxidation and to reduce insulin resistance in skeletal muscle by activating AMP-activated protein kinase (AMPK) in peripheral tissues (2–4).

Adiponectin, a hormone secreted by adipose tissue, controls glucose and lipid metabolism, prevents hepatic steatosis, and plays an important role in the regulation of insulin resistance and energy homeostasis by binding and activating two cell membrane receptors. Adiponectin receptor 1 (AdipoR1) is most abundantly expressed in muscle, and adiponectin receptor 2 (AdipoR2), most predominantly in liver (5). The adiponectin receptors can ligand-dependently and dose-dependently activate AMPK, p38 mitogen-activated protein kinase, and PPAR α , thus stimulating fatty-acid oxidation and glucose uptake in hepatocytes and myocytes, limiting deposition of fat, and maintaining insulin responsiveness in liver and muscle. In addition, the weight-reducing effects of CNTF appear to result from direct effects on skeletal muscle and may not require central signaling, in contrast to the effects of leptin (3,4).

Although accumulating evidence suggests that adiponectin and its receptors play an important role in obesity and insulin resistance, no previous study has investigated whether administration of CNTF_{AX15} may improve insulin resistance by altering expression of adiponectin and its receptors above and beyond what would be expected based on reduction of caloric intake or body weight alone. We hypothesize that the effect of CNTF_{AX15} on insulin resistance could be mediated, in part, through alterations of secretion- and expression patterns of adiponectin and its receptors. Therefore we studied the effect of treatment with CNTF_{AX15} vs. placebo treatment and hypocaloric pair feeding for 7 days on circulating adiponectin and leptin levels as well as expression patterns of adiponectin and its receptors in white adipose tissue, liver and muscle in insulin- and leptin-resistant, hyperinsulinemic diet-induced obese (DIO) C57Bl/6J mice, the mouse model most closely associated with human obesity (1).

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MATERIAL AND METHODS

Animals

3–5 week old C57Bl/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME) and used as previously described (1). In brief, all mice were given a 7-day acclimation period with access to normal chow (Purina Rodent Chow #5008) and water *ad libitum* and were maintained at 25°C with a 12-hour light/dark cycle for the duration of the study. After the acclimation period, mice were placed on a Western diet (Teklad, Madison, WI. TD 88137: 42.2% of calories from fat, 42.8% from carbohydrates, 15.0% from protein; caloric content: 4.53 kcal/g) for 12 weeks in order to obtain diet-induced obese mice (DIO-C57Bl/6J). All animals were handled in accordance with the National Institutes of Health guidelines (1).

Experimental procedures

Mice were divided into groups of equal mean body weight prior to the experiment ($n = 10$). DIO-C57Bl/6J mice were treated with CNTF_{AX15} (Axokine®, a second generation CNTF analogue manufactured and provided by Regeneron Pharmaceuticals Inc., Tarrytown, NY) at 0.1 and 0.3 µg/g/d s.c. for 7 days. Body weights and food intake were measured daily between 0800 and 1000 AM. Mice were sacrificed 4 days after the last injection. Food and caloric intake data represent the amount of food eaten over the course of the 11-day experiment. Phosphate-buffered saline (PBS)-treated mice had access to food and water *ad libitum* and mice pair fed (PF) to the 0.3 µg/g/d –treated mice were included as control groups. Each group of mice was sacrificed between 0830 and 1130 AM and serum was collected. White adipose tissue (WAT), liver and muscle were shock-frozen and stored at –80°C (1).

Measurement of circulating hormone levels

Sera were collected and assayed in duplicate for adiponectin and leptin by RIA (Linco Research Institute Inc., St. Louis, MO).

Quantitative PCR analysis of Adiponectin and AdipoR1/R2 mRNA expression

Total RNA isolation and cDNA synthesis from muscle, liver and WAT tissues was performed as previously described (1). Adiponectin mRNA expression in WAT, as well as AdipoR1/R2 mRNA expression in muscle and/or liver was assayed and quantified using real-time quantitative PCR (RT-PCR) with mouse specific “gene expression assays” (Applied Biosystems Inc.; LaJolla, CA). RT-PCR reactions were performed in triplicate in an automated Stratagene Mx3000 QPCR System (Stratagene; LaJolla, CA) using Taqman Universal PCR Master Mix (Applied Biosystems Inc.; LaJolla, CA). The reaction conditions for all templates were: 10 min at 95°C, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min.

Amplification was performed using a FAM/TAMRA labeled gene-specific probe in a 20 µl reaction mixture. Relative quantities of Adiponectin and AdipoR1/R2 mRNA were normalized to respective mouse cyclophilin levels.

Statistical analyses

Descriptive characteristics are expressed as mean values $\pm$ standard error ($\pm$ SE). Data for mRNA-expression are presented as percent change from respective control groups. Statistical analysis was performed using Statview™ (Abacus/CA, USA). Statistical significance was assessed by standard Student t-tests, or paired two-tailed t-test and ANOVA with post hoc tests (LSD) as appropriate. Values were considered to be significant at the two-tailed $p \leq 0.05$ value.

RESULTS

Effect of CNTF_{AX15} or hypocaloric feeding on body weight, food intake, and serum hormones

Body weight and food intake—7 days of CNTF_{AX15} -treatment (at both the 0.1 and 0.3 µg/g/d dose) resulted in significant weight loss (Table 1), and 7 days of CNTF_{AX15} treatment (at the 0.3 µg/g/d dose) resulted in significant weight loss above and beyond pair feeding alone ($p < 0.01$; Table 1). Both cumulative food and caloric intake were significantly decreased following 7 days of CNTF_{AX15} treatment (Table 1).

Serum Hormone Levels—A dose dependent effect of CNTF_{AX15} to decrease serum leptin levels was observed, but this decrease reached statistical significance compared to the pair-fed group only at the 0.3 µg/g/d dose (Table 2). CNTF_{AX15}-treatment at the 0.3 µg/g/d dose resulted in a significant increase in serum adiponectin, above and beyond what was observed with pair feeding alone ($p < 0.05$ for both, vs. PBS-treated and PF controls; Table 2).

Effect of CNTF_{AX15} on Adiponectin-, AdipoR1- and AdipoR2 mRNA expression

No significant alterations in adiponectin-mRNA expression in WAT following 7 days of CNTF_{AX15} -treatment for 7 days or pair feeding could be observed in DIO-C57Bl/6J mice (Table 2). AdipoR1-mRNA expression was increased following 7 days of CNTF_{AX15} treatment in liver and muscle in a dose-dependent manner. However, statistical significance could only be reached in the group treated with CNTF_{AX15} at the 0.3 µg/g/d dose. A similar effect could be seen in the pair-fed group, suggesting that the increase in AdipoR1-mRNA expression was not a CNTF_{AX15} specific effect. There was no significant change in mRNA-expression of AdipoR2 in liver by either CNTF_{AX15} or hypocaloric feeding during this 7-day experiment.

DISCUSSION AND CONCLUSIONS

Treatment with CNTF_{AX15} effectively decreases body weight, body fat mass, food intake, and circulating insulin and glucose levels (1), and these effects persist several days after discontinuation of treatment. The pathway mediating the effects of CNTF have been suggested to be downstream of the putative point of leptin resistance (1). In addition, it has been shown previously that CNTF significantly and dose-dependently decreases insulin and glucose concentrations in leptin- and insulin resistant mouse models of obesity and that it improves glucose tolerance following 7 days of treatment (1).

Serum adiponectin concentrations are inversely associated with obesity, insulin resistance and type 2 diabetes in rodents and humans, whereas increased serum adiponectin concentrations are associated with improved insulin sensitivity (5,6). Adiponectin mediates its effects through at least two cell-membrane receptors, adiponectin receptor 1 (AdipoR1), most abundantly expressed in skeletal muscle, and adiponectin receptor 2 (AdipoR2), predominantly expressed in liver (5). Both receptors may regulate insulin sensitivity (6,7). Binding and activation of these receptors by adiponectin stimulates phosphorylation of AMPK and acetyl-CoA-carboxylase (ACC) in myocytes and hepatocytes *in vitro* as well as in rodents *in vivo* (7,8), suggesting that activation of the pleiotropic AMPK is part of the signaling pathway downstream of adiponectin (9).

The weight reducing effects of CNTF result from direct effects in the periphery and may be, in contrast to leptin, independent of central signalling. In skeletal muscle, CNTF signals through the CNTFR α -IL-6R-gp130 β receptor complex to increase fatty-acid oxidation and reduce insulin resistance by activating AMP-activated protein kinase (AMPK) (3). Thus, the signaling pathway activated by CNTF_{AX15} is the AMPK pathway, i.e. the signaling pathway also activated by adiponectin (3,4). The net effects of both adiponectin and CNTF are to

enhance oxidation of fatty acids and to decrease the synthesis and deposition of lipids and metabolites in muscle, thereby enhancing insulin action (4). Thus, both CNTF and adiponectin, an adipokine with insulin sensitizing effects, play a significant role in the pathogenesis of obesity-associated insulin resistance and type 2 diabetes, but whether their actions are independent of each other or whether there is cross talk between these two systems has not been adequately studied.

Because AMPK can be activated by both, CNTF and adiponectin, we investigated whether the metabolic effects of CNTF_{AX15} could be mediated through altered regulation of adiponectin or its receptors. We found that CNTF_{AX15} may increase serum adiponectin levels independently of its effect on weight loss, while both CNTF_{AX15} and pair feeding increase liver and muscle AdipoR1 expression in a similar pattern in DIO-C57Bl/6J mice. These findings suggest that increased serum adiponectin levels and/or AdipoR1 expression may, at least in part, mediate the observed metabolic effects of CNTF_{AX15}. In contrast, no effect of hypocaloric feeding or CNTF_{AX15}-treatment on liver AdipoR2 expression could be observed in the study presented here, suggesting that AdipoR2 may not be directly associated with the insulin sensitizing effects of CNTF_{AX15} in mice. These findings are in accordance with a recent study in humans, demonstrating a strong correlation between AdipoR1 expression in muscle and insulin sensitivity as well as metabolic parameters, but no association between AdipoR2-mRNA expression in muscle and insulin sensitivity (10).

The study presented herein suggests that circulating adiponectin and AdipoR1-mRNA are increased in response to CNTF_{AX15} administration at the 0.3 µg/g/d dose in diet-induced-obesity, findings which may be of importance in the pathophysiology of insulin resistance. To investigate further whether changes in the expression pattern of adiponectin receptors could be attributable to transactivation of additional genes following hypocaloric diet or CNTF_{AX15}-treatment for this and/or different periods of time, further physiology studies and micro-array experiments are warranted. In addition, more detailed studies on the potential interaction or crosstalk of signaling pathways downstream of leptin, adiponectin and CNTF are needed.

In summary, we demonstrate for the first time that CNTF-induced hypophagia and weight loss results in significantly upregulated serum adiponectin levels and AdipoR1-mRNA expression in muscle and liver. These changes may mediate the effects of CNTF_{AX15} on activating the AMPK signaling pathway and thus increasing fatty-acid oxidation and improving insulin sensitivity in skeletal muscle.

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Table 1

Effect of 7 days of CNTF_{Ax15} administration (0.1 and 0.3 µg/g/day sc.) or pair-feeding on weight loss, cumulative and caloric food intake in DIO mice. Values are expressed as means ± SEM, analysed by ANOVA with post-hoc tests.

Group	Initial Weight (g)	Weight Loss (g)	Food Intake (g)	Caloric Intake (kcal)
PBS	44.02 ± 2.02	1.32 ± 0.37	37.97 ± 1.40	172.02 ± 6.33
CNTF_{Ax15} 0.1	44.23 ± 1.88	9.05 ± 1.31 ****	17.80 ± 2.70 ****	80.64 ± 12.25 ****
CNTF_{Ax15} 0.3	44.23 ± 1.77	12.15 ± 0.91 **** ⁺	13.16 ± 1.39 ****	59.62 ± 6.30 ****
Pair fed to CNTF_{Ax15} 0.3	44.60 ± 2.51	8.36 ± 0.60 ****	13.72 ± 0.01 ****	62.18 ± 0.03 ****

p < 0.0001 vs. the PBS treated group

⁺p < 0.01 vs. the pair-fed group

Effect of 7 days of CNTF_{Axis15} administration (0.1 and 0.3 µg/g/day sc) or pair-feeding on circulating leptin and adiponectin levels, adiponectin mRNA expression in WAT, AdipoR1 mRNA expression in muscle and liver, and AdipoR2 mRNA expression in liver in DIO mice. Data are expressed as means ± SE by ANOVA with post-hoc tests.

Table 2

Group	Leptin (ng/ml)	Adiponectin (µg/ml)	Adiponectin- mRNA (WAT)	AdipoR1- mRNA (Muscle)	AdipoR1 mRNA (Liver)	AdipoR2 mRNA (Liver)
PBS	21.9 ± 2.0	6.9 ± 1.3	100.0 ± 17.1	100.1 ± 8.2	100.1 ± 3.6	100.0 ± 13.1
CNTF _{Axis15} 0.1	11.2 ± 1.3 **	7.9 ± 0.9	93.5 ± 11.9	122.3 ± 9.4	103.4 ± 1.5 ^^	108.5 ± 12.3
CNTF _{Axis15} 0.3	8.5 ± 1.5 ****^	10.1 ± 0.8 *^	81.2 ± 9.4	142.1 ± 9.2 **	124.5 ± 3.5 ***	87.1 ± 10.3
Pair-fed to CNTF _{Axis15} 0.3	16.8 ± 2.8	6.8 ± 0.6	70.0 ± 6.5	150.5 ± 8.8 **	130.7 ± 6.5 ****	99.0 ± 9.6

* p<0.05

** p<0.01

*** p<0.001

**** p<0.0001 vs. the PBS treated group

^ p<0.05

^^ p<0.001 vs. the pair-fed group