Research Article

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# Cardiotrophin-1 eliminates hepatic steatosis in obese mice by mechanisms involving AMPK activation

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**Background & Aims**: Cardiotrophin-1 (CT-1) is a hepatoprotective cytokine that modulates fat and glucose metabolism in muscle and adipose tissue. Here we analyzed the changes in hepatic fat stores induced by recombinant CT-1 (rCT-1) and its therapeutic potential in non-alcoholic fatty liver disease (NAFLD).

**Methods**: rCT-1 was administered to two murine NAFLD models: *ob*/*ob* and high fat diet-fed mice. Livers were analyzed for lipid composition and expression of genes involved in fat metabolism. We studied the effects of rCT-1 on lipogenesis and fatty acid (FA) oxidation in liver cells and the ability of dominant negative inhibitor of AMP-activated protein kinase (AMPK) to block these effects.

**Results**: CT-1 was found to be upregulated in human and murine steatotic livers. In two NAFLD mouse models, treatment with rCT-1 for 10 days induced a marked decrease in liver triglyceride content with augmented proportion of poly-unsaturated FA and reduction of monounsaturated species. These changes were accompanied by attenuation of inflammation and improved insulin signaling. Chronic administration of rCT-1 caused downregulation of lipogenic genes and genes involved in FA import to hepatocytes together with amelioration of ER stress, elevation of $NAD^+$/NADH ratio, phosphorylation of LKB1 and AMPK, increased expression and activity of sirtuin1 (SIRT1) and upregulation of genes mediating FA oxidation. rCT-1 potently inhibited *de novo* lipogenesis and stimulated FA oxidation in liver cells both *in vitro* and *in vivo*. *In vitro* studies showed that these effects are mediated by activated AMPK.

**Conclusions**: rCT-1 resolves hepatic steatosis in obese mice by mechanisms involving AMPK activation. rCT-1 deserves consideration as a potential therapy for NAFLD.



Keywords: Cardiotrophin-1; Non-alcoholic fatty liver disease (NAFLD); Lipogenesis; Fatty acid oxidation; AMPK.

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*Abbreviations:* CT-1, Cardiotrophin-1; rCT-1, recombinant CT-1; NAFLD, non-alcoholic fatty liver disease; HFD, high fat diet; FA, fatty acid; ER, endoplasmic reticulum; DN, dominant negative; AMPK, AMP-activated protein kinase; SIRT1, sirtuin1; TG, triacylglycerol; NASH, non-alcoholic steatohepatitis; VLDL, very low density lipoproteins; PF, pair fed; FBS, fetal bovine serum; DAG, diacylglycerol; adipoR, adiponectin receptors; PPARα, peroxisome proliferator-activated receptor alpha; PGC-1α, peroxisome proliferator-activated receptor-gamma coactivator-1alpha; MCAD, medium-chain acyl-CoA dehydrogenase; CPT1, carnitine palmitoyltransferase 1; ACC2, acetyl CoA carboxylase 2; FAS, fatty acid synthase; SCD1, stearoyl-Coenzyme A desaturase 1; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SREBP-1c, sterol regulatory element binding protein 1c; CHREBP, carbohydrate-responsive element-binding protein; NAMPT, nicotinamide phosphoribosyltransferase; IRS1, insulin receptor substrate; LPL, lipoprotein lipase; IRE1, inositol-requiring enzyme; XBP1, X-box-binding protein1; LKB1, liver kinase B; CaMKKβ, calmodulin-dependent protein kinase kinase β; $^2H_2O$, deuterated water; Ad, adenovirus; GFP, green fluorescent protein; DNAMPK, dominant negative AMPK; ALT, alanine transferase; AST, aspartate transaminase.

## Introduction

In advanced societies overnutrition and sedentarism are promoting a striking increase in the prevalence of non-alcoholic fatty liver disease (NAFLD), a metabolic disorder which is closely associated with obesity, type 2 diabetes and insulin resistance [1]. In most cases the affected individuals show simple accumulation of triacylglycerol (TG) in hepatocytes (hepatic steatosis) with benign prognosis, but in about 20% of subjects with NAFLD the liver exhibits inflammatory changes and diverse degrees of fibrosis [2]. This condition is known as non-alcoholic steatohepatitis (NASH). Between 5% and 20% of patients with NASH evolve to liver cirrhosis, and of these 0.5% will develop hepatocellular carcinoma (HCC) in over 10 years [2]. Moreover, around 80% of cases with cryptogenic cirrhosis manifest histopathological changes suggestive of NASH [3].

The mechanisms determining the severity of hepatic damage in NAFLD are not well understood, but the progression of the liver

lesion appears to be linked to the development of insulin resistance. However, it is not known whether accumulation of TG or other lipid compounds causes hepatic insulin resistance or whether the latter is responsible for the development of hepatic steatosis [4].

Lipid accumulation in liver cells may be due to four main metabolic disturbances: (a) increased hepatocellular import of serum fatty acids (FA) derived from peripheral lipolysis or hydrolysis of circulating TG; (b) augmented *de novo* lipogenesis; (c) reduced fat consumption due to decreased FA oxidation or attenuated autophagy of lipid droplets; (d) impaired export from hepatocytes to blood of TG incorporated into very low density lipoproteins (VLDL). Therapies capable of modulating these processes may have a role in the management of NAFLD and associated co-morbidities.

Cardiotrophin-1 (CT-1) is a member of the IL-6 family of cytokines expressed mainly in skeletal muscle, myocardium, liver, and adipose tissue [5]. In the liver, CT-1 is produced both by hepatocytes and non-parenchymal cells [6]. CT-1 exerts robust hepatoprotective activities and has been proposed as a potential therapy for acute severe liver damage [6,7]. Recently, we have shown that CT-1 is a key molecule controlling energy metabolism in muscle and adipose tissue [5], but there is no information concerning the influence of CT-1 on liver lipid metabolism. In the present work we have investigated the effects of recombinant CT-1 (rCT-1) on hepatic fat stores and its potential to eliminate liver steatosis in two murine models of NAFLD. We observed that chronic administration of rCT-1 to obese mice stimulates FA oxidation in liver cells and reduces hepatic lipogenesis and fat import to the liver leading to marked reduction of hepatic steatosis, attenuation inflammation and improved insulin signaling. AMPK activation was found to be a key mediator of these events. Our findings reveal novel metabolic activities of rCT-1 in the liver, which make this molecule a potential therapy for NASH.

## Materials and methods

### *Experimental animals*

Eight weeks old *ob*/*ob* mice were obtained from the Janvier Laboratory (Le Genest St Isle). C57BL/6J mice were obtained from Harlan Laboratories (Barcelona, Spain) and were placed on high fat diet (HFD) (60% of kcal from fat, 20% from carbohydrates and 20% from protein, Research Diets, New Brunswick, NJ) *ad libitum*, and monitored for food intake and body weight for 12 weeks. *ob*/*ob* mice and HFD-fed animals were divided into three subgroups, one that received rCT-1 intravenously (0.2 mg/kg/day) for 10 days, another given saline instead of rCT-1 and a pair fed (PF) group given saline. Food intake and body weight were measured daily. Animals were sacrificed after an overnight fast, unless indicated otherwise. All experiments were carried out in compliance with our institution's ethical guidelines. rCT-1 was obtained as described [7].

### *Quantitative real-time reverse transcriptase polymerase chain reaction (Q-RT-PCR)*

Total RNA was extracted with TRIZOL (Invitrogen, Carlsbad, CA) and real-time PCR was performed using iCycler (Bio-Rad, Hercules, CA) and iQ SYBR Green Supermix (Bio-Rad). Primers were designed according to published complementary DNA or genomic sequences.

### *Isolation and treatment of primary murine hepatocytes*

Hepatocytes were isolated by Liberase Blendzyme 3 (Roche) perfusion as described previously [6]. Cultures were maintained in William's medium supplemented with 10% fetal bovine serum (FBS), nonessential amino acids, 2 mM glutamine, and antibiotics (all from Invitrogen). After 2 h the incubation medium was removed, and cells were re-fed the same medium with 1% FBS. Cells were then transduced with adenoviral vectors (100 MOI) and after 24 h post-infection glucose and insulin (Sigma) and rCT-1 (20 ng/ml) were added to the culture medium for 16 h and lipogenesis and FA oxidation assays were performed.

### *Generation of the GFP and DNAMPKα1 adenovirus*

Recombinant adenoviral constructs DNAMPKα1 (Viraquest; North Liberty, IA) and GFP recombinant adenovirus were produced in HEK293T cells and purified on cesium chloride gradients before use by the Gene Therapy Laboratory at CIMA.

### *Statistical analysis*

Data are presented as mean values ± SEM. Analyses were performed using GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA). Data were compared among groups using the Student *t* test. A $p$ value of <0.05 was considered significant.

Additional materials and methods are included as Supplementary Materials and methods.

## Results

### *Expression of CT-1 in steatotic livers*

The expression of CT-1 was studied by real-time PCR and western blots in steatotic livers from obese animals (*ob*/*ob* and HFD-fed mice). As depicted in Fig. 1A, the expression of *CT-1* mRNA and protein was significantly higher in steatotic livers from *ob*/*ob* and HFD-fed mice compared to wild-type mice and animals on control diet (CD), respectively. Also we found that *CT-1* mRNA levels were increased in the liver from patients with NAFLD in comparison to healthy subjects (Fig. 1B).

### *Chronic rCT-1 treatment resolves hepatic steatosis and attenuates hepatic inflammation in mice with NAFLD*

*ob*/*ob* mice constitute a useful animal model of NAFLD as they exhibit obesity, insulin resistance, intense liver steatosis, hepatomegaly and elevated serum transaminases. We found that treatment of 10 weeks-old *ob*/*ob* mice with rCT-1 at the dose of 0.2 mg/kg/day for 10 days resulted in a significant decrease of liver index and serum transaminases accompanied by a marked reduction in liver content of TG and diacylglycerol (DAG) compared to saline-treated controls. As rCT-1 possess anorexigenic properties we used as an additional control group mice given saline that were fed with the same amount of food consumed by rCT-1-treated animals (pair-fed, PF). The decrease in liver index, serum enzymes and liver TG and DAG content was more marked in rCT-1-treated mice than in PF animals (Fig. 1C and D) indicating that rCT-1 influenced liver lipid metabolism by mechanisms other than diminished food intake. In consonance with biochemical data, *ob*/*ob* mice that received rCT-1 showed a reduction of histological steatosis more intense than that observed in PF animals (Fig. 1E).

When we analyzed the liver inflammatory reaction we observed a smaller number of CD45+ cells and reduced expression of *TNFα* in both rCT-1-treated and PF mice compared to those that received saline (Fig. 1F and Supplementary Fig. 1A). Notably, animals treated with rCT-1, but not the PF group, showed a significant upregulation of the anti-inflammatory cytokine IL-10 in the liver. Moreover, the adiponectin receptors (adipoR1 and