

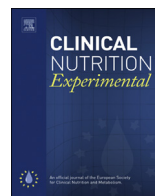


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Unacylated ghrelin does not alter mitochondrial function, redox state and triglyceride content in rat liver in vivo

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SUMMARY

Changes in liver mitochondrial function with more oxidized redox state and enhanced inflammation may contribute to the onset of obesity- and insulin resistance-associated hepatic complications, including non-alcoholic fatty liver disease and steato-hepatitis. Unacylated ghrelin (UnAG) is a gastric hormone reported to be associated with lower oxidative stress in different cell types, but its potential effects on liver mitochondrial function, redox state and inflammation in vivo remains undetermined. We investigated the impact of chronic UnAG overexpression (Tg Myh6/Ghrl) leading to systemic upregulation of circulating hormone on mitochondrial ATP production, redox state (oxidized-to-total glutathione) and inflammation markers in lean mice. Compared to wild-type animals (wt), Tg Myh6/Ghrl had superimposable liver weight, triglyceride content and plasma lipid profile. Liver mitochondrial enzyme activities and ATP production as well as oxidized-to-total glutathione were also similar in the two groups. In addition, no differences were observed in tissue inflammation marker TNF-alpha between wild-type and Tg Myh6/Ghrl animals. Thus, chronic systemic UnAG upregulation does not alter liver triglyceride content, mitochondrial function, redox state and inflammation markers in lean mice. These findings do not support a major

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role of UnAG as a physiological modulator of *in vivo* liver oxidative-lipid metabolism and inflammation.

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1. Introduction

Changes in liver mitochondrial function, more oxidized redox state and enhanced inflammation are involved in the onset of hepatic metabolic diseases including non-alcoholic fatty liver disease (NAFLD) and steato-hepatitis (NASH) [1–3]. Recent findings indicate that upregulation of hepatic mitochondrial function is associated with enhanced oxidative stress and inflammation in human early NASH [1]. Potential hormonal regulators of liver oxidative metabolism and inflammation remain only partly understood.

Ghrelin is a gastric hormone circulating in acylated (AG) and unacylated (UnAG) forms [4–7]. AG is a major orexigenic regulator of appetite and may induce hepatic lipogenesis and fat accumulation [8–10]. A seemingly paradoxical beneficial impact of AG to limit liver triglyceride content, oxidative stress and inflammation in high-fat diet-induced obesity has however been recently reported [11] and lower total plasma ghrelin has been observed in NASH and NAFLD patients [12,13]. UnAG has no direct impact on appetite but independent metabolic effects of UnAG are rapidly emerging and could be mediated by yet unidentified independent receptor(s). In particular, UnAG is reportedly associated with lower insulin resistance in humans [14–16] and it may lower glucose output from hepatocytes [17] as well as reduce oxidative stress in non-liver cells *in vitro* [18–21].

Potential *in vivo* effects of UnAG on mitochondrial oxidative metabolism, oxidative stress and inflammation as well as triglyceride content remain unknown. In the current study we therefore investigated the impact of chronic UnAG overexpression (Tg Myh6/Ghrl) with systemic upregulation of circulating hormone [22] on hepatic mitochondrial ATP production, redox state (oxidized-to-total glutathione) and inflammation markers, as well as their association with tissue triglyceride content in lean mice.

2. Research design and methods

2.1. Experimental design and protocols

2.1.1. Animal model

Transgenic mice overexpressing UnAG (Tg Myh6/Ghrl) and related metabolic and hormonal characteristics were previously described [22], with selective ghrelin overproduction in the heart resulting in approximately 40-fold increment in circulating UnAG due to negligible acylating activity in both heart and circulation. 6 Tg Myh6/Ghrl and 6 matched wild-type male mice underwent 16-week feeding with standard diet (10% calories from fat; Research Diets, New Brunswick, NJ). All animals were subsequently anesthetized with Tiobutabarbital 100 mg/kg, Tiletamine/Zolazepam (1:1) 40 mg/kg IP and liver was dissected, weighed, aliquoted and quickly processed for *ex-vivo* analyses or frozen in liquid nitrogen. Blood was then collected through cardiac puncture, plasma was separated and both plasma and liver were stored at –80 °C until analyses. All animal procedures were compliant with 2010/63/EU Directive.

2.2. Analytical methods

2.2.1. Plasma insulin, non-esterified fatty acids (NEFA) and thiobarbituric acid reactive substances (TBARS)

Plasma insulin concentration was measured by ELISA (Ultrasensitive Insulin ELISA, DRG, Springfield, NJ). Plasma glucose and NEFA were determined by standard enzymatic-colorimetric assays. The plasma lipid peroxidation marker TBARS was measured using a commercially available kit (Oxitek, Zepto-metrix Co, Buffalo, NY) as referenced [23].

2.2.2. Mitochondrial enzyme activities and ATP synthesis

Activities of the Krebs cycle and respiratory chain flux marker enzymes citrate synthase (CS) and cytochrome c oxidase (COX) were measured spectrophotometrically as referenced [24]. Ex-vivo ATP synthesis rate in freshly isolated mitochondria was measured using different combinations of respiratory substrates [8,25]. Final composition and reaction concentrations (mmol/l) were: 0.25 pyruvate, 0.0125 palmitoyl-L-carnitine, 2.5 α -ketoglutarate, 0.25 malate (PPKM); 0.025 palmitoyl-L-carnitine, 0.5 malate (PCM).

2.2.3. Liver total and oxidized glutathione and TNF-alpha

Total and oxidised glutathione levels were determined using the method by Rahman et al. [25] on ~30 mg of liver sample homogenised in ice-cold 5% (wt/vol.) metaphosphoric acid (20 ml/g tissue). Reduced glutathione (GSH) was calculated as total glutathione minus its oxidised fraction (GSSG). For TNF-alpha measurement, total tissue protein was extracted from tissue homogenates as referenced [11] and TNF-alpha was then measured using a commercially available kit (Pierce Biotechnology, Rockford, IL, USA).

2.2.4. Liver triglycerides

Liver triglyceride content was measured from 35 to 40 mg liver and each muscle after lyophilisation [11]. Briefly, dry tissue samples were homogenized in 2:1 chloroform-methanol solution in a 20:1 volume-to-weight ratio and kept at 4 °C overnight with gentle shaking. Tissue triglycerides were measured after phase separation using a commercially available colorimetric reagent (TG; Roche Diagnostics, Indianapolis, IN).

2.2.5. Statistical analysis

Groups were compared using Student t-test or one-way ANOVA followed by post hoc tests, as appropriate. Bonferroni correction for multiple comparisons was applied. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Animal characteristics and liver weight and triglyceride content (Fig. 1)

Up-regulation of circulating UnAG by myocardial overexpression of the ghrelin gene (Tg Myh6/Ghrl) [22] did not modify body weight (WT: 30.2 ± 0.5 ; Tg Myh6/Ghrl: 28.5 ± 1.1 g; $P=NS$) or caloric intake (WT: 13.7 ± 0.2 ; Tg Myh6/Ghrl: 14.2 ± 0.4 kcal/d; $P=NS$). Blood glucose (WT: 109 ± 7 ; Tg Myh6/

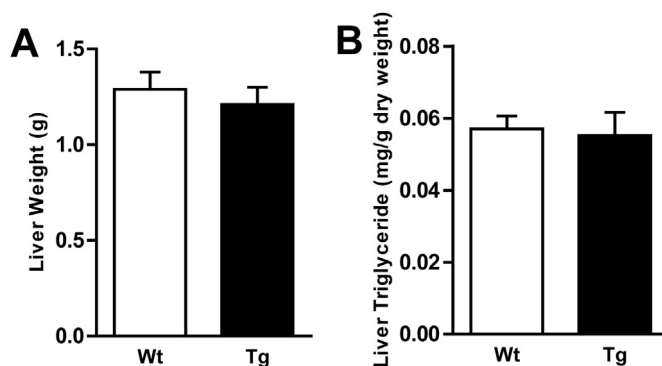


Fig. 1. Systemic UnAG upregulation and liver weight and triglyceride content. Effects of UnAG overexpression in transgenic Myh6/Ghrl (Tg) vs. wild type (Wt) mice on (A) liver weight and (B) liver triglyceride content. $P=NS$ between groups, mean \pm SEM, $n = 7$ /group.

Ghrl: 102 ± 9 mg/dL; $P=NS$), plasma insulin (WT: 13.2 ± 1.5 ; Tg Myh6/Ghrl: 12.8 ± 2.0 μ U/mL; $P=NS$) and NEFA (WT: 0.30 ± 0.05 ; Tg Myh6/Ghrl: 0.32 ± 0.08 mmol/L; $P=NS$) concentrations also were comparable in both groups. Liver weight was not different in WT and Tg Myh6/Ghrl mice, and no differences were also observed between groups in liver triglyceride content.

3.2. Systemic circulating UnAG up-regulation does not modify liver mitochondrial ATP production, redox state markers and tissue TNF- α (Figs. 2–4)

Upregulation of circulating UnAG did not modify liver CS and COX enzyme activities, and mitochondrial ATP production was also unchanged in Tg Myh6/Ghrl (Fig. 2). Oxidized-to-total glutathione, a marker of tissue redox state, was comparable in wild-type and Tg Myh6/Ghrl mice and plasma TBARS concentration, a systemic marker of lipid peroxidation, was also not modified (Fig. 3). Consistent with the above observations, upregulation of circulating UnAG also did not change liver content of the proinflammatory cytokine and tissue inflammation marker TNF- α (Fig. 4).

4. Discussion

This study demonstrated that systemic UnAG upregulation through cardiac UnAG overexpression in lean mice does not modify liver mitochondrial-energy metabolism, redox state, inflammation markers and triglyceride content. The current findings therefore do not support a role of UnAG as a modulator of hepatic mitochondrial-lipid metabolism under the current experimental conditions.

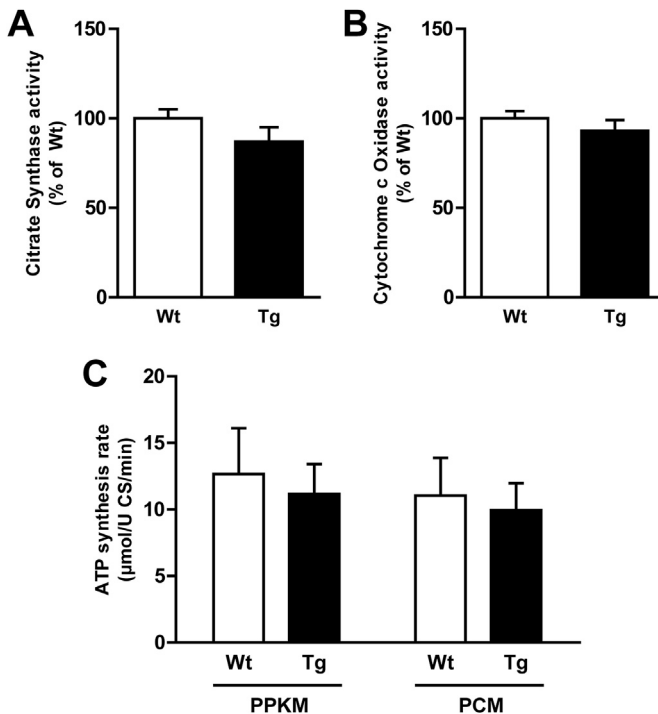


Fig. 2. Systemic UnAG upregulation and mitochondrial liver enzyme activities and ATP production. Effects of UnAG overexpression in transgenic Myh6/Ghrl (Tg) vs. wild type (Wt) mice on (A) liver mitochondrial citrate synthase and (B) cytochrome c oxidase activities and on (C) isolated mitochondria ATP synthesis rate with different respiratory substrates (A, PPKM: Pyruvate + Palmitoyl-L-Carnitine + α -Ketoglutarate + Malate; PCM: Palmitoyl-L-Carnitine + Malate). $P=NS$ between groups, mean \pm SEM, $n = 7$ /group.

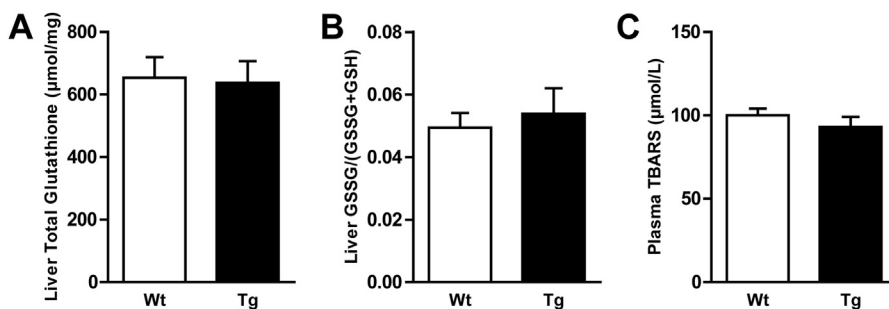


Fig. 3. Systemic UnAG upregulation and liver and plasma redox state markers. Effects of UnAG overexpression in transgenic Myh6/Ghrl (Tg) vs. wild type (Wt) mice on (A) total and (B) oxidized (GSSG) over total (GSH: reduced) glutathione and on (C) plasma thiobarbituric acid reactive substances. $P=NS$ between groups, mean \pm SEM, $n = 7$ /group.

A protective association between higher total plasma ghrelin and reduced prevalence of hepatic fat accumulation and non-alcoholic fatty liver disease has been previously described in humans [12,13], but potential differential contributions of circulating ghrelin forms to the regulation of liver metabolic pathways remain largely to be determined. Acylated ghrelin was reported to exert complex, nutritional status-dependent hepatic effects [26]. In particular, AG activities include enhanced hepatic lipogenesis leading to higher tissue triglyceride content in lean rodents [8], while potentially beneficial anti-oxidative and anti-inflammatory effects of AG were reported in experimental obesity and models of liver injury including streptozotocin-induced diabetes [11,27,28].

Metabolic effects of UnAG have been less extensively studied, and no data have been available on its potential effects on hepatic metabolic pathways *in vivo*. UnAG is however an emerging independent contributor to the regulation of whole-body insulin sensitivity, is clinically associated with favorable metabolic profiles and may exert anti-oxidative effects in different cell types [15,16,18–21]. Although no independent UnAG receptor has been yet identified, several lines of evidence directly support its existence [14]. Most importantly, opposite effects of AG and UnAG have been indeed reported on hepatocytes glucose output *in vitro* [17] thereby directly supporting independent hepatic activities of UnAG. In the current study, we therefore studied potential effects of UnAG on liver oxidative metabolism and redox state, inflammatory molecules and liver triglyceride content in lean rodents. Our findings strongly indicate that UnAG does not contribute to regulate these parameters under basal physiological metabolic and nutritional conditions. It should be pointed out that lack of UnAG activities cannot be assumed to apply under different experimental settings and, potentially, in the presence of primary hepatic alterations due to nutritional, metabolic or toxicity-induced derangements. The

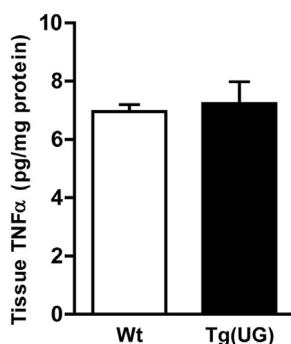


Fig. 4. Systemic UnAG upregulation and liver TNF-alpha. Effects of UnAG overexpression in transgenic Myh6/Ghrl (Tg) vs. wild type (Wt) mice on tissue TNF α levels. $P=NS$ between groups, mean \pm SEM, $n = 7$ /group.

current findings however suggest that reported positive associations between circulating UnAG and higher insulin sensitivity with favorable metabolic profile in humans [16] are not related to primary effects on hepatic energy-lipid metabolism. Future studies should investigate specific changes in UnAG and their potential mechanistic role in pathological conditions.

In conclusion, our study provides novel *in vivo* data on potential hepatic effects of UnAG. Our findings do not support a major role of UnAG as an *in vivo* physiological modulator of liver oxidative-lipid metabolism and inflammation.

Author contributions

GGC performed experiments, researched and analyzed data and contributed to study design and writing of the manuscript, MZ contributed to discussion and reviewed/edited the manuscript, AS performed experiments and contributed to data analysis and discussion, PV contributed to data discussion, GR and MDN performed experiments and contributed to data discussion, NF contributed to data discussion, GG contributed to data discussion, MG reviewed and discussed data and reviewed/edited the manuscript, AG reviewed and discussed data and reviewed/edited the manuscript, RB designed the study, reviewed data and wrote the manuscript, and acts as guarantor for the article. All authors gave final approval to the submitted manuscript.

Conflict of interest

None.

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