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# Interaction between metformin and leucine in reducing hyperlipidemia and hepatic lipid accumulation in diet-induced obese mice☆

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ABSTRACT

*Background.* Leucine stimulates Sirt1 and AMPK signaling *in vitro* and *in vivo*. Since metformin converges on the same pathway, we have tested the ability of leucine to amplify the effects of metformin on AMPK-mediated hepatic lipid metabolism in diet-induced-obese insulin-resistant mice.

*Methods.* Mice were fed high leucine (24 g/kg diet) with or without sub-therapeutic levels of metformin (0.05–0.50 g/kg diet) or therapeutic levels of metformin (1.5 g/kg diet; ~300 mg/kg body weight).

*Results.* High-fat diet produced a 10-fold increase in inguinal fat pad weight and 25% increase in liver weight, histologically confirmed as steatosis. The leucine-metformin combinations reduced fat pad mass, normalized liver weight, liver and plasma lipids and inflammatory markers (interleukin 6, interleukin 1 beta, tumor necrosis factor alpha, monocyte chemotactic protein-1, C-reactive protein) comparable to the effects of therapeutic metformin. Moreover, the highest sub-therapeutic levels of metformin with leucine exerted significantly greater effects than therapeutic levels of metformin and fully reversed hepatic steatosis. These effects were mediated by upregulation of hepatic AMPK and associated changes in lipogenic gene expression (fatty acid synthase, stearoyl CoA desaturase, acetyl CoA carboxylase) in the liver.

*Conclusion.* A low-dose leucine-metformin combination exerts comparable effects on adiposity to therapeutic doses of metformin and fully reverses hepatic steatosis in diet-induced-obese mice.

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*Abbreviations:* ACC, acetyl CoA carboxylase; ALT, alanine aminotransferase; AMPK, 5' adenosine monophosphate-activated protein kinase; AST, aspartate transaminase; CRP, C-reactive protein; FAS, fatty acid synthase; HFD, high-fat diet; HMB, β-hydroxy-β-methyl-butyrate;IL6, interleukin 6; IL1β, interleukin 1 beta; Leu, leucine; LFD, low-fat diet; LKB1, liver kinase B1; MCP1, monocyte chemotactic protein 1; Met, metformin; NAD, nicotinamide adenine dinucleotide; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; Res, resveratrol; SCD1, Stearoyl-CoA carboxylase; Sirt1, sirtuin 1; TNFα, tumor necrosis factor 1 alpha.

☆ All authors have read and agree to the publication of the manuscript.

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## 1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a spectrum of disorders characterized by hepatic lipid accumulation and cellular degeneration in the absence of significant alcohol consumption which may progress to non-alcoholic steatohepatitis (NASH), cirrhosis or hepatocellular carcinoma [1]. The prevalence of NAFLD is 10–25% in the general population, and increases to ~75% in patients with obesity and diabetes [2]. The most widely accepted view of its pathogenesis is based on the "two-hit" or "multiple-hit" model with insulin resistance leading to hepatic lipid accumulation representing the first hit. Successive activation of multiple pathways resulting in oxidative and inflammatory stress could result in the second hit. However, despite the pivotal role of insulin resistance in the pathogenesis, insulin-sensitizing drugs have minimal effect on liver histology [3].

AMPK and Sirt1 are well known regulators of lipid and energy metabolism including hepatic lipid metabolism. High-fat diet (HFD) and excess energy intake have been shown to decrease Sirt1 and AMPK activity [4–6]. This in turn, may lead to mitochondrial loss or dysfunction, which plays a pivotal role in the development of metabolic diseases such as insulin resistance and diabetes. In contrast, both, Sirt1 and AMPK activation, prevents or attenuates glucose and lipid-induced increases in hepatic lipid accumulation, thus representing therapeutic targets [7–9].

We have previously demonstrated that the branched-chain amino acid leucine (Leu) has a unique role as an activator of the AMPK/Sirt1 pathway and thereby modulates lipid and energy metabolism. Leucine activates Sirt1, at least in part, by lowering the activation energy for $NAD^+$ and consequently also co-activates and amplifies the effects of other sirtuin activators [10]. For example, the combination of low dose resveratrol with leucine increased insulin sensitivity, muscle glucose utilization and palmitate oxidation *in vitro* and *in vivo* [11]. Moreover, Sirt1 activation leads to deacetylation of the protein kinase LKB1 which promotes AMPK phosphorylation [9]. Consistent with this concept, leucine, but not other amino acids such as alanine or valine, activated AMPK in a Sirt1-dependent manner [12].

Metformin (Met) is the first-line drug for treating type 2 diabetes, especially in overweight and obese patients [13]. Because of its insulin-sensitizing effects, it was also suggested as a treatment option for NAFLD/NASH. Although some studies have shown some beneficial effects on clinical markers such as improvement of alanine aminotransferase (ALT) and aspartate transaminase (AST) levels, it does not confer a consistent beneficial effect on liver histology [3] and is not recommended as a treatment for NAFLD or NASH [3,14]. However, since metformin also converges on the AMPK/Sirt1 pathway, and heterozygous Sirt1 knockout mice develop severe NASH [15], we tested the ability of leucine to amplify the effects of metformin on lipid metabolism and hepatic steatosis *in vitro* and *in vivo* in an obese mouse model of insulin resistance. Since we previously demonstrated synergy between leucine and resveratrol (Res) [11], we also sought to determine whether adding resveratrol to the Leu + Met combination would confer further benefit.

## 2. Material and Methods

### 2.1. Animals and Diets:

Six to eight weeks old male C57BL/6 J mice were purchased from Jackson Laboratories. Obesity and insulin resistance were induced via an HFD for 6 weeks. The animals were then randomized into one of the following groups with 10 animals/group and kept on their diet for 6 weeks.

#### 2.1.1. For Study 1 (Treatment Groups Without Resveratrol)

1) Control (low-fat diet (LFD), standard diet (LabDiet 5001), 2) HFD (Research Diets, 60% fat), 3) HFD + leucine (24 g/kg diet; Sigma Aldrich, St. Louis, MO) + metformin 0.15 g/kg diet (Sigma Aldrich, St. Louis, MO) (Leu + Met 0.15), 4) HFD + leucine + metformin 0.25 g/kg diet (Leu + Met 0.25), 5) HFD + leucine + metformin 0.5 g/kg diet (Leu + Met 0.5), 6) HFD + metformin control 1.5 g/kg diet (Met 1.5). The 1.5 g metformin/kg diet concentration was designed as a standard therapeutic dose to achieve a final dosing of ~300 mg/kg body weight. This concentration was used to compare the full therapeutic effects of the standard dose to the effects of the combinations of leucine with the lower doses of metformin, which are sub-therapeutic doses and previously found to exert no effect [16].

#### 2.1.2. For Study 2 (Treatment Groups With Resveratrol)

1) LFD, 2) HFD control, 3) HFD + leucine (24 g/kg diet), 4) HFD + leucine + resveratrol (12.5 mg/kg/diet; Sigma Aldrich, St. Louis, MO) (Leu + Res), 5) HFD + leucine + resveratrol + metformin (0.25 g/kg diet) (Leu + Res + Met 0.25), 6) HFD + leucine + resveratrol + metformin (0.15 g/kg diet) (Leu + Res + Met0.15), 7) HFD + leucine + resveratrol + metformin (0.05 g/kg diet) (Leu + Res +Met 0.05), 8) HFD + metformin control (1.5 g/kg diet) (Met 1.5).

Animals were housed in polypropylene cages at a room temperature of 22 °C and regime of 12 h light/dark cycle. The animals had free access to water and their experimental food throughout the experiment. Body weight was measured every week. At the end of the treatment period (6 weeks) all animals were humanely euthanized with $CO_2$ inhalation. Blood was collected via trunk bleed and tissues were collected for further experiments as described below.

### 2.2. Liver Histology

Liver tissues were fixed in 10% neutral formalin, embedded in paraffin and cut into 5 μm sections. Sections were processed for hematoxylin and eosin (H&E) staining and histological images were recorded using Nikon Eclipse E800 Microscopy with Zeiss AxioCam camera.

### 2.3. Gene Expression

Total RNA from liver was extracted using the Tri-Reagent kit (Molecular Research Center, Cincinnati, OH) and gene expression was assessed by quantitative reverse transcription (RT)-PCR (ABI Universal PCR Master Mix, Applied Biosystems, Foster City, CA) using a Stratagene Mx3000p thermocycler (Stratagene, La Jolla, CA). Cyclophilin was used to normalize the gene expression data. The primer and probe sets used in the assays were

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