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Activation of the AMPK/Sirt1 pathway by a leucine–metformin combination increases insulin sensitivity in skeletal muscle, and stimulates glucose and lipid metabolism and increases life span in *Caenorhabditis elegans*

Jheelam Banerjee, Antje Bruckbauer, Michael B. Zemel*

NuSirt BioPharma Inc., 11020 Solway School Road, Knoxville, TN 37931, USA

ARTICLE INFO

Article history:
Received 28 January 2016
Accepted 29 June 2016

Keywords:
AMPK
Sirt1
Insulin sensitivity
Leucine
Metformin

ABSTRACT

Background. We have previously shown leucine (Leu) to activate Sirt1 by lowering its K_M for NAD^+, thereby amplifying the effects of other sirtuin activators and improving insulin sensitivity. Metformin (Met) converges on this pathway both indirectly (via AMPK) and by direct activation of Sirt1, and we recently found Leu to synergize with Met to improve insulin sensitivity and glycemic control while achieving ~80% dose-reduction in diet-induced obese mice. Accordingly, we sought here to define the mechanism of this interaction.

Methods. Muscle cells C2C12 and liver cells HepG2 were used to test the effect of Met–Leu on Sirt1 activation. *Caenorhabditis elegans* was used for glucose utilization and life span studies.

Results. Leu (0.5 mmol/L) + Met (50–100 μmol/L) synergistically activated Sirt1 ($p < 0.001$) at low (≤100 μmol/L) NAD^+ levels while Met exerted no independent effect. This was associated with an increase in AMPK and ACC, phosphorylation, and increased fatty acid oxidation, which was prevented by AMPK or Sirt inhibition or silencing. Met–Leu also increased P-IRS1/IRS1 and P-AKT/AKT and in insulin-independent glucose disposal in myotubes (~50%, $p < 0.002$) evident within 30 min as well as a 60% reduction in insulin EC_{50}. In addition, in HepG2 liver cells nuclear CREB regulated transcription coactivator 2 (CRTC2) protein expression and phosphorylation of glycogen synthase was decreased, while glycogen synthase kinase phosphorylation was increased indicating decreased gluconeogenesis and glycogen synthesis. We utilized *C. elegans* to assess the metabolic consequences of this interaction. Exposure to high glucose impaired glucose utilization and shortened life span by ~25%, while addition of Leu + Met to high glucose worms increased median and maximal life span by 29 and 15%, respectively ($p = 0.023$), restored normal glucose utilization and increased fat oxidation ~two-fold ($p < 0.005$), while metformin exerted no independent effect at any concentration (0.1–0.5 mmol/L).

Abbreviations: ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; AMPK, 5′ adenosine monophosphate-activated protein kinase; CREB, cAMP-responsive element binding protein; CRTC2, CREB regulated transcription coactivator 2; ECAR, extracellular acidification rate; ERK, extracellular signal regulated kinase; GS, glycogen synthase; GSK, glycogen synthase kinase; IR, insulin receptor; IRS1, insulin receptor substrate 1; JNK, c-Jun N-terminal kinases; Leu, leucine; LKB1, liver kinase B1; Met, metformin; NAD, nicotinamide adenine dinucleotide; NAMPT, nicotinamide phosphoribosyltransferase; PEPCK, phosphoenolpyruvate carboxykinase; pGS, phosphorylated glycogen synthase; pGSK, phosphorylated glycogen synthase kinase; PKC-ZETA, protein kinase C zeta; Sirt1, Sirtuin 1; TNF-α, tumor necrosis factor alpha.

* *Corresponding author*.
E-mail addresses: jbanerjee@nusirt.com, jheelambanerjee@yahoo.com (J. Banerjee).

http://dx.doi.org/10.1016/j.metabol.2016.06.011

Conclusion. Thus, Leu and Met synergize to enable Sirt1 activation at low NAD^+ concentrations (typical of energy replete states). Sirt1 and AMPK activations are required for Met–Leu's full action, which result in improvements in energy metabolism and insulin sensitivity.

1. Introduction

Metformin is considered the initial drug of choice for treating type 2 diabetes, as it is highly efficacious, exhibits an excellent safety profile, does not promote weight gain, does not increase the risk for hypoglycemia and has been shown to reduce the risk of diabetes-related comorbidities and death [1]. However, metformin monotherapy often fails to achieve optimal glycemic control due to inter-individual variability in response to drug initiation and maintenance, failure to achieve optimal dose titration secondary to drug-induced gastrointestinal discomfort, or drug discontinuation due to the development of adverse effects [2]. Although there are multiple other antihyperglycemic drug classes which can be used as second choice monotherapy or add-on treatments, they still show some disadvantages in comparison to successful metformin monotherapy [3,4] Accordingly, there is still a need to optimize metformin efficacy at lower doses [5].

The AMP-activated protein kinase (AMPK) and the NAD^+-dependent sirtuins (Sirt1 and Sirt6) are key sensors of energy status and regulators of glucose and lipid metabolism, activating each other in a finely tuned network [6,7].

While insulin resistance and diabetes are associated with impairment of this pathway, activation of the AMPK-Sirt1 axis improves hyperglycemia and insulin sensitivity [8,9]. Accordingly, Sirt1 transgenic mice exhibit reduced levels of fasting blood glucose and insulin as well as improved glycemic control, showing anti-diabetic effects, during the glucose tolerance test [10].

We previously found the branched-chain amino acid leucine (Leu) to activate Sirt1 by lowering the activation energy for NAD^+, thus mimicking the effects of caloric restriction, and enabling co-activation and amplification of the effects of other AMPK/sirtuin activators at low concentrations [11]. As a result of metformin and leucine converging on this AMPK-Sirt1 axis [1], we found leucine and metformin to exhibit synergistic effects to improve insulin sensitivity and glycemic control in diet-induced obese mice while achieving a ~80% dose-reduction of metformin [12]. Therefore, this study was designed to further define the mechanism of the interaction of leucine and metformin on AMPK/Sirt1 activation, and to assess the metabolic consequences on glucose utilization and life span in *Caenorhabditis elegans*.

2. Materials and Methods

2.1. Cell Culture

Murine C2C12 muscle cells were grown in the absence of insulin in Dulbecco's modified Eagle's medium (DMEM, 25 mmol/L glucose) containing 10% fetal bovine serum (FBS) and antibiotics (1% penicillin–streptomycin) at 37 °C in 5% CO_2 in air. They were then seeded into plates for the final treatments and then induced to differentiate with a DMEM containing 2% horse serum and 1% Pen-Strep. The cells were maintained in this differentiation medium for 5 days before treatment. HepG2 cells were either grown in low glucose DMEM (5 mmol/L glucose) or high glucose DMEM (25 mmol/L glucose) containing 10% fetal bovine serum (FBS) and antibiotics (1% penicillin–streptomycin) at 37 °C in 5% CO_2 in air. C2C12 cells were treated with metformin (0.1 mmol/L), leucine (0.5 mmol/L) and a combination of both drugs for 2 h, followed by 20 min insulin (10 nmol/L) stimulation. Proteins were then extracted using NP40 buffer. HepG2 cells were treated with a combination of Met–Leu for 24 h to 48 h and then cells were harvested. Protein concentrations were quantitated with the BCA kit (Thermo Scientific).

2.2. siRNA Transfection

C2C12 muscle cells were seeded with 10^4 cells/well on 96-well plate or 24-well Seahorse plate (confluence, ~50 to 60%). ~24 h later, cells were transfected with siRNA against Sirt1 (Ambion ID# 174219) or AMPK (Ambion ID# 221537) complexed to Lipofectamine RNAiMAX reagent (ThermoFisher Scientific, Cat# 13778-030) according to manufacturer's instructions for 24 to 48 h. Then they were differentiated in 2% horse serum for an additional 4 days prior to treatment.

2.3. Gene Expression Analysis

Cells were grown in a 96-well plate. Cell Lysis, reverse transcription and RT-PCR were performed using the TaqMan® Gene Expression Cells-to-C_T™ Kit (Life Technologies, Cat #4399002) according to manufacturer's instructions. Gene expression was assessed by RT-PCR using StepOnePlus™ PCR system (Thermo Fisher Scientific) and TaqMan® Gene expression assays for AMPK (Life Technologies, Cat #Mm01264789) and Sirt1 (Life Technologies, Cat #Mm01168521).

2.4. Nuclear and Cytosolic Extraction

Treated HepG2 cells were scraped from culture flasks and washed with cold PBS twice. Cell pellet was re-suspended in 500 μL of hypotonic buffer (20 mmol/L Tris–HCl, pH 7.4; 10 mmol/L NaCl; 3 mmol/L $MgCl_2$) and incubated on ice for 15 min. Then 25 μL detergent (10% NP40) was added and vortexed for 10 s. The homogenate was centrifuged for 10 min at 3000 rpm at 4 °C, and the supernatant containing the cytoplasmic fraction was then aliquoted and stored at −80 °C for further experiments. The pellet, containing the nuclear fraction, was re-suspended in 50 μL of Cell Extraction buffer (Life Technologies, Grand Island, NY; Cat #FNN0011) supplemented with 1 mmol/L PMSF and Protease Inhibitor Cocktail (Sigma, St. Louis, MO; Cat #P-2714, 1:100 dilution) for 30 min on ice with vortexing at 10 min intervals. Then the homogenate was centrifuged for 30 min at