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

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 EC50. 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).

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 EC50. 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).

Keywords: AMPK, Sirt1, Insulin sensitivity, Leucine, Metformin

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, Sir2 homologue 1; TNF-α, tumor necrosis factor alpha.

⁎ Corresponding author.
E-mail addresses: jbanerjee@nusirt.com, jheelambanerjee@yahoo.com (J. Banerjee).
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 NAD+, thus mimicking the effects of caloric restriction, and (Leu) to activate Sirt1 by lowering the activation energy for each other in a finely tuned network [6,7].

We previously found the branched-chain amino acid leucine (Leu) to activate Sirt1 by lowering the activation energy for Leu's full action, (typical of energy replete states). Sirt1 and AMPK activations are required for Met–Leu’s full action, showing anti-diabetic effects during the glucose fasting blood glucose and insulin as well as improved glycemic control, thus improving hyperglycemia and insulin sensitivity [8,9]. Accordingly, Sirt1 transgenic mice exhibit reduced levels of NAD+, thus mimicking the effects of caloric restriction, and (Leu) to activate Sirt1 by lowering the activation energy for each other in a finely tuned network [6,7].

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% CO₂ 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% CO₂ 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 mmol/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 10⁶ 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-Ct™ 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 MgCl₂) 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...
14,000g at 4 °C. The supernatant was aliquoted and stored at −80 °C for further experiments. Protein content of the cytoplasmic and nuclear fraction was measured with the BCA kit (Thermo Scientific, Grand Island, NY).

2.5. ELISA

Differentiated C2C12 muscle cells were treated for 2 h with Met (0.1 mmol/L)–Leu (0.5 mmol/L). 20 min before harvest they were stimulated with 100 nmol/L insulin. The ELISA assays (PathScan P-IRS1 (S307) and PathScan Total IRS1, Cell Signaling (Danvers, MA), cat #7287 and #7328, respectively) were performed according to the manufacturer’s instructions.

2.6. Western Blot

Phospho-AMPK (Thr172), AMPK, pACC (Ser79), ACC, pIRS1 (S318), IRS1, p-AKT (Thr308), AKT, pGSK, GSK, GS, TORC2/CRTC2 and β-actin antibodies were obtained from Cell Signaling (Danvers, MA). Protein levels of cell extracts were measured by BCA kit (Thermo Scientific, Pittsburgh, PA). For Western blot, 10–50 μg protein was resolved on 4–15% gradient polyacrylamide gels (Criterion precast gel, Bio-Rad Laboratories, Hercules, CA), transferred to either PVDF or nitrocellulose membranes, incubated in blocking buffer (3% BSA or 5% non-fat dry milk in TBS) and then incubated with primary antibody (1:1000 dilution), washed and incubated with horseradish peroxidase- or fluorescence-conjugated secondary antibody (1:10,000 dilution). Visualization was conducted using BioRad ChemiDoc instrumentation and software (Bio-Rad Laboratories, Hercules, CA) and band intensity was assessed using Image Lab 4.0 with correction for background and loading controls.

2.7. Glucose Utilization in C2C12 Muscle Cells

In the absence of a fatty acid source and oxidative metabolism, glycolysis and subsequent lactate production result in extracellular acidification. Extracellular acidification rate (ECAR) was measured using a Seahorse Bioscience XF24 analyzer (Seahorse Bioscience, Billerica, MA) in 24-well plates at 37 °C. C2C12 cells were seeded at 40,000 cells per well, differentiated as described above, treated for 24 h with or without metformin (0.1 mmol/L)–leucine (0.5 mmol/L). For SH-experiment, media was changed to 11 mmol/L glucose. After three baseline readings, insulin (0 to 500 nmol/L) was injected. After 20 min incubation, 14 mmol/L glucose was injected and extracellular acidification rate (ECAR) was measured over a two-hour period.

2.8. Sirt1 Activity

The Sirt1 FRET-based screening assay kit (Cayman Chemical Company, Ann Arbor, MI) was used for the measurement of Sirt1 activity in a cell-free system as well as in C2C12 muscle cells. For the cell-free experiment, Leu (0.5 mmol/L), Met (0.1 mmol/L) or combination was incubated with recombinant Sirt1 enzyme under different NAD+ concentrations (500 μmol/L, 100 μmol/L, 50 μmol/L and 10 μmol/L) for 30 min according to the manufacturer’s protocol. The protocol was modified for the measurements in cells as follows; cell lysate of differentiated C2C12 muscle cells treated with Leu (0.5 mmol/L), Met (0.1 mmol/L) or combination for 24 h was incubated with peptide substrate under low NAD+ (100 μmol/L) concentrations. The fluorescence was measured with excitation and emission wavelengths of 360 nm and 450 nm, respectively.

Fig. 1 – Met–Leu effect on Sirt1 activation. Recombinant human Sirt1 enzyme was incubated with Leucine (0.5 mmol/L) and/or metformin (0.1 mmol/L) for 45 min under different NAD+ concentrations (10 to 500 μmol/L) and fluorescence of deacetylated substrate was measured. (A and B) Representative fluorescence data with incubation of 50 μmol/L and 500 μmol/L NAD+, respectively. Data are expressed as means ± SEM (n = 5 to 6, p ≤ 0.05). (C) Summary of results represented as % change of Sirt1 activity ± SEM (n = 5 to 6, p ≤ 0.05). * indicates significantly different from control (p < 0.05).
The increase in fluorescence is proportional to the amount of deacetylated substrate and thus Sirt1 activity.

2.9. **C. elegans Maintenance**

Worms (N2 Bristol wild-type) were obtained from the Caenorhabditis Genetics Center (CGC) at the University of Minnesota and grown on standard NGM plates with *E. coli* (OP50) as food source at 21 °C. For treatments, adult worms were bleached to obtain eggs, which were allowed to hatch overnight in M9 buffer. Synchronized L1 larvae were transferred to *Escherichia coli* fed NGM plates containing indicated treatments for about 35 h to reach L4/young adult stage. All treatments were added to the agar in indicated concentrations.

2.10. **Glucose Content in C. elegans**

Glucose content of worms was measured using the Amplex® Red Glucose/Glucose Oxidase Assay Kit (Invitrogen, now ThermoFisher Scientific, Grand Island, NY).
Synchronized L1 worms were maintained in liquid media, as described in Ref. [13], containing 40 mmol/L glucose and indicated treatments until adulthood. Adult worms were washed off the plate. The supernatant was removed after centrifugation at 500g for 1 min. The worms were washed three times with M9 buffer. After the last wash, supernatant was removed except that ~150 μL and 50 μL of Reagent buffer from assay kit were added. Then worm solution was homogenized and lysate was used for assay according to the manufacturer’s instructions.

2.11. Life Span Study Protocol

50 synchronized stage L4 worms per group were placed on NGM agar plates seeded with E. coli strain OP50 (= day 1 of study). All treatments were added with the indicated concentrations to the agar plates. The worms were maintained at 21 °C throughout the duration of the study. Live worms were placed on new plates every day to eliminate progeny. Worms were scored as dead if they did not respond to repeated touches with the platinum pick and scored as censored if they crawled off the plate.

2.12. Palmitate-Induced Fatty Acid Oxidation

Cellular oxygen consumption was measured using a Seahorse Bioscience XF24 analyzer (Seahorse Bioscience, Billerica, MA) in 24-well plates with appropriate modifications for C. elegans or for cells, as follows. For the measurement of fatty acid oxidation in worms, treated L4/adult worms were washed off the plate with M9 buffer, and then washed (centrifuged at 500g for 1 min) three times. After final wash, the worm suspension was diluted to a worm count of 2 worms/20 μL worm suspension drop. Then 500 μL of the worm suspension was added to each well to reach a final worm count of ~50 worms/well with at least 5 replicates per group in each experiment. The Seahorse program was set up at 27 °C. Five successive baseline measures were taken prior to injection of palmitate (200 μmol/L final concentration). Three successive measurements of O2 consumption were then conducted, each followed by a 10-min waiting period. This measurement pattern was then repeated over a 2-h period. For the measurement of fatty acid oxidation in C2C12 muscle cells treated with the Met Leu combination on Sirt1 activity in a cell-free system (Fig. 1). Met-Leu synergistically activated human recombinant Sirt1 enzyme under low NAD+ conditions (50 μmol/L, Fig. 1A) but not under high NAD+ conditions (500 μmol/L, Fig. 1B), shifting the activation curve to the left and lowering the Michaelis constant (Km) value for NAD+ from 55.05 to 21.45 (Fig. 1C), the individual compounds exerted little or no significant independent effect. There was also a significant increase in the protein expression and activity of Sirt1 and of the ratio of Phospho-AMPK (Thr172)/AMPK in C2C12 muscle cells treated with the Met–Leu combination (Fig. 2A–C). Treatment of C2C12 muscle cells with the Sirt1 inhibitor Ex527 prevented the effects of Met–Leu on AMPK phosphorylation. However, the ratio of P-AMPK/AMPK was not changed with EX-527 compared to Met–Leu since there were small but statistically insignificant differences in total AMPK between the groups. AMPK knockdown blunted the effects of Met–Leu on

![Fig. 2 - Met-Leu effects on Sirt1 and AMPK signaling in C2C12 muscle cells.](image-url) Differentiated C2C12 muscle cells were treated with indicated treatments for 2 h and stimulated with insulin (10 nmol/L) 20 min prior to harvest. Protein expression of Sirt1 (A) and phospho-AMPK (Thr172) and total AMPK (B) were measured. Quantitative data and representative blots are shown. Data are represented as mean ± SEM (n = 3 to 4). (C) Sirt1 activity was measured in differentiated C2C12 muscle cells treated with Met (0.1 mmol/L), Leu (0.5 mmol/L) or the combination for 24 h. Fluorescence values were normalized to protein amount of cell lysate (n = 4 to 6). (D) Protein expression of phospho (Thr172)-AMPK and total AMPK: differentiated C2C12 muscle cells were treated with Met–Leu in presence or absence of the Sirt1 inhibitor EX527. Bar graphs were calculated from pooled densitometry measurements of two different blots (n = 3 to 6) and representative blots are shown. The bar graph of the ratio of P-AMPK/AMPK is shown as an insert (E) Protein expression of Ser79-ACC and ACC: differentiated C2C12 muscle cells were treated with Met–Leu in presence or absence of the Sirt1 inhibitor EX527 and AMPK inhibitor Compound C. Bar graphs of densitometry measurement and representative blot are shown (n = 2).
Sirt1 protein expression, indicating the interactive effects of Sirt1 and AMPK (Fig. 2D and E). To further explore whether AMPK and Sirt1 are both required for the full effect of the Met–Leu combination, we measured ACC phosphorylation as a ratio to total ACC and fatty acid oxidation as downstream targets of AMPK activation. Both, ACC phosphorylation and palmitate-induced oxygen consumption rate were increased by ~100% and ~20%, respectively, by Met–Leu and totally prevented by either AMPK and Sirt1 knockdown or inhibition (Figs. 2F and 3A to C). The level of AMPK or Sirt1 knockdown is shown in Fig. 3D and E.

We next examined the effects of the Met–Leu combination on insulin signaling. Stimulation with 10 nmol/L insulin 20 min prior to harvest produced the expected increase in phosphorylated (Ser318)-IRS in C2C12 muscle cells. Met–Leu treatment for 2 h in the absence of insulin resulted in a comparable (~25%) increase in IRS phosphorylation, and further significantly
enhanced IRS phosphorylation by an additional ~15% during insulin stimulation compared to insulin-treated controls (Fig. 4A). Similarly, Met-Leu treatment in the absence of insulin resulted in a significant increase in phosphorylated AKT expression in muscle cells comparable to that achieved with insulin, and Met-Leu further augmented insulin stimulation of P-AKT (Fig. 4B). However, IRS phosphorylation on Ser307 was not further stimulated by Met-Leu treatment when compared to insulin-stimulated control (Fig. 4C).

Next we measured ECAR, which is an indicator of the cellular glucose utilization, in C2C12 cells treated with Met-Leu for 2 h in response to different insulin concentrations via the Seahorse Bioscience XF24 analyzer. Fig. 5A shows a representative experiment with A, the time point of insulin injection (0.5 nmol/L, 5 nmol/L, 50 nmol/L or 500 nmol/L), and C, (20 min after A) the time point of 14 mmol/L glucose injection, to reach a final well concentration of 25 mmol/L glucose. Fig. 5B shows the calculated area under the curve of that experiment and Fig. 5C the summarized results of all four experiments. There was a shift in glucose utilization at the tested insulin concentrations with Met-Leu treatment. Met-Leu treatment increased glucose utilization comparable to 500 nmol/L insulin stimulation, and additional insulin to the Met-Leu treatment further enhanced this effect.

In liver HepG2 cells we found a trend ($p = 0.07$) for reduction in CRTC2 in the nucleus with Met-Leu treatment, as well as a significant increase in glycogen synthase kinase (GSK)-3β phosphorylation and an associated decrease in glycogen synthase (GS) phosphorylation treatment (Fig. 6).

Next we tested the effects on Met-Leu on glucose and fat metabolism in a simple model organism, C. elegans. Fig. 7A shows the oxygen consumption rate and the calculated area under the curve in worms treated with Met-Leu. There was a significant ~50% increase in fatty acid oxidation in C. elegans. Next, we measured the glucose content of worms kept either under normal (low) glucose conditions or under high (40 mmol/L glucose added to liquid media) glucose conditions. The high glucose conditions significantly increased the glucose content in the worms, which was prevented by simultaneous treatment with Met-Leu, indicative of higher glucose utilization in the worms (Fig. 7B). To test whether these effects modulate survival, we conducted life span studies under normal and high glucose conditions (2% glucose added to agar plates) with or without Met, Leu and the combinations (Fig. 8). High glucose shortened median survival by 22% compared to low glucose conditions. Leu combined with Met (0.1 mmol/L (Fig. 8A) and 0.5 mmol/L (Fig. 8B)) prolonged median and maximum life span under...
these high glucose conditions. The individual compounds exerted small but statistically insignificant effect (Fig. 8C).

4. Discussion

The major findings of this paper are that the Met–Leu combination increases insulin-independent stimulation of the insulin signaling pathway by activating the AMPK/Sirt1 pathway and increases insulin sensitivity and overall glucose and lipid metabolism.

Metabolic sensors such as AMPK and Sirt1 are crucial to the regulatory network for metabolic homeostasis by modulating pleiotropic effects in key metabolically relevant tissues such as liver, muscle and adipose tissue. SIRT1, a NAD+-dependent regulator of energy metabolism, has been reported to be activated through AMPK-mediated induction of nicotinamide phosphoribosyltransferase (NAMPT), the rate-limiting enzyme for NAD+ biosynthesis or changes in the NAD+/NADH ratio[14]. On the other hand, Sirt1-induced deacetylation of LKB1 causes its movement from the nucleus to the cytosol and subsequent activation of AMPK[14], suggesting reciprocal activation of these two energy sensing systems. Both systems represent targets for therapeutic intervention, as they are down-regulated during periods of over-nutrition and in obesity, diabetes and metabolic syndrome[15–18].

Metformin, acts in part via AMPK activation in liver to reduce hepatic gluconeogenesis as well as in muscle to increase glucose uptake[4,19]. In addition, metformin appears to increase both Sirt1 activity and protein expression via AMPK-dependent and independent pathways[20,21]. We have previously demonstrated that leucine amplifies the effects of metformin on AMPK and Sirt1 activation and enables a substantial dose reduction in vitro and in vivo[12,22]. Our previous data suggest that some of the effects of leucine are mediated by acting as a direct allosteric activator of Sirt1, reducing the $K_M$ for NAD+ and thereby allowing Sirt1 activation at lower NAD+ concentrations, which are characteristic for a metabolic replete state.[11]. Moreover, leucine facilitates the interaction of Sirt1 with other Sirt1 activating compounds such as resveratrol, which resulted in a further leftward shift of the activation curve[11]. Here we demonstrate that this ability of leucine can be extrapolated to metformin, which also has some direct effects on Sirt1 activation[23], as the Met–Leu combination further increased Sirt1 activity at low NAD+ concentrations (Fig. 1). Therefore, the activation of Sirt1 by the combination may be an early effect and AMPK independent, which leads to subsequent AMPK activation and further Sirt1 activation at later time points, as suggested by Liang et al.[24]. Moreover, it was demonstrated that Sirt1 acts upstream of AMPK to regulate hepatocellular lipid metabolism[25]. Consistent with this concept, the Sirt1 inhibitor EX527 blocked the effects of Met-Leu on AMPK phosphorylation, demonstrating that Sirt1 activation is necessary for AMPK activation (Fig. 2D). In contrast, AMPK knockdown only partially blunted the Met-Leu effect on Sirt1, suggesting both AMPK-dependent and independent activation of Sirt1 (Fig. 2E). However,
Fig. 6 – Met-Leu effects on enzyme of hepatic glucose metabolism in HepG2 liver cells. HepG2 liver cells were treated with indicated treatments for 24 h. Protein expression of P-GSK and GSK (A), and P-GS and GS (B) were measured. Quantitative data and representative blots are shown. Data are represented as mean ± SEM (n = 3 to 4). (C) Nuclear and cytosolic fraction of cell extract was separated as described under Materials and Methods. CRTC2 protein expression was measured in the cytosolic and nuclear fraction and normalized to β-actin. Data are represented as mean ± SEM (n = 3).
knockdown of either Sirt1 or AMPK prevented the effects of Met-Leu on downstream events such as ACC phosphorylation and palmitate-induced fatty acid oxidation (Fig. 2F and 3), implying that both are necessary for Met-Leu’s full action.

Since the Met-Leu combination increased AMPK and Sirt1 protein expression in C2C12 muscle cells (Fig. 2), we wanted to determine if this results in an insulin-independent increase in glucose muscle uptake. IRS-1 protein is a key player in the insulin signal transduction pathway to stimulate glucose uptake and is regulated by various kinases in response to metabolic stimuli at multiple serine, threonine and tyrosine phosphorylation sites [26]. AMPK and Sirt1 also regulate IRS-1 phosphorylation and muscle glucose uptake, although this may be a secondary response to inhibition of inflammatory mediators. For example, knockdown of Sirt1 led to increased inflammatory response accompanied by impaired IRS-1 signaling and decreased insulin-stimulated glucose transport in 3T3-L1 adipocytes whereas Sirt1 activators suppressed the inflammatory pathway and increased insulin sensitivity [27]. Similarly, treatment of 3T3-L1 adipocytes with AICAR, an AMPK activator, reduced TNF-α induced serine phosphorylation of IRS-1 through ERK and JNK and improved insulin resistance [28]. We found an upregulation of Ser 318 phosphorylation of IRS-1 with Met-Leu treatment comparable to insulin alone, and a further augmentation of insulin-induced IRS-1 phosphorylation. Although this phosphorylation site can be activated by PKC-zeta, JNK and other kinases which presumably disrupt the interaction of IR and IRS-1 and provide a negative feedback mechanism [26], there is also evidence that Ser 318 phosphorylation also occurs in the early phase of insulin signaling increasing AKT phosphorylation and glucose uptake and is required for an enhanced induction of insulin action [29]. However, in the late phase of insulin action, this phosphorylation site attenuates insulin-stimulated signal transduction and subsequently decreases glucose uptake in skeletal muscle. Therefore, this Ser318 phosphorylation may be necessary to maintain the physiological balance of cellular energy homeostasis [29]. In contrast, the Ser307 phosphorylation site is assumed to be an inhibitory phosphorylation site stimulated by factors such as free fatty acids, TNF-α and hyperinsulinemia and is involved in the development of insulin resistance [26,30,31]. In agreement with this concept, our data indicate that Met-Leu treatment did not affect the inhibitory Ser307 phosphorylation site. However, the increased Ser318 phosphorylation was associated with increased AKT phosphorylation and an increased glucose utilization in muscle (Figs. 4 and 5).

Diabetes and hyperglycemia are associated with increased gluconeogenesis and glycogen synthesis in the liver. Phosphoenolpyruvate carboxykinase (PEPCK) and glycogen synthase, key rate limiting enzymes of gluconeogenesis and glycogen synthesis, respectively, are tightly controlled by transcriptional regulation, phosphorylation and allosteric effectors in response to energetic signals [32,33]. Glycogen synthase kinase-3-β activates GS and is also negatively regulated by phosphorylation. AMPK activation reduces hepatic glucose production and glycogen production in the liver.
synthesis through phosphorylation of GSK-3β and of TORC2 (transducer of regulated CREB activity 2 or CRTC2), a co-activator of cAMP-responsive element binding protein (CREB) [34]. Phosphorylation of CRTC2 leads to its translocation from the nucleus to the cytosol and consequently to a reduced transcriptional activity of CREB, an important transcriptional regulator of hepatic PEPCK. CREB, in turn, is also directly phosphorylated by GSK-3β [34,35]. Therefore, AMPK-induced reduction of gluconeogenesis is mediated both by phosphorylation of GSK-3β and consequent reduction in CREB activity, and by decreased nuclear presence of TORC2 (CRTC2), which together reduce transcription of PEPCK.

Since we have recently demonstrated in mice treated with a Met–Leu combination that hepatic AMPK was activated associated with increased fat oxidation and decreased liver triglyceride accumulation [36], we wanted to explore the effects of AMPK activation with our treatment on hepatic glucose metabolism. Consistent with downstream effects of AMPK activation, we found trend for reduction (20%) of CRTC2 in the nucleus with Met–Leu associated with a significant increase in GSK-3β phosphorylation and an accompanying decrease in GS phosphorylation in liver HepG2 cells (Fig. 6).

The nematode C. elegans conserves 65% of the human disease-related genes, including pathways for fat and glucose metabolism, and is used as a well-defined simple model system for obesity and insulin resistance [37]. Worms kept on high glucose (40 mmol/L) agar plates reached an internal glucose concentration of 14 mmol/L, comparable to plasma glucose concentrations under poorly controlled diabetic conditions. This concentration shortened both median and maximum life span [38,39]. In another study [40], 2% glucose added to the agar plates shortened life span of C. elegans by ~20%, similar to data from the present study. Addition of the Met–Leu combination to the agar plates reversed the high glucose-induced impairment in fat and glucose metabolism in the worms and produced a corresponding increase in life span (Figs. 7 and 8).

The concentrations of metformin and leucine, used in the cell studies, were derived from our previous work for optimal Sirt1 activation and are pharmacologically relevant, as follows [11,12,36]. The fasting plasma concentration of leucine in humans is ~0.1 mmol/L and a plasma concentration of 0.5 mmol/L can be reached after ingestion of ~300 mg leucine/kg/day [41]. The peak systemic plasma concentration reached after a therapeutic dose of metformin (1000 mg) is ~2.4 mg/L (=18 μmol/L). However, it is higher in the portal vein where it reaches between 40 and 70 μmol/L [42,43]. Therefore, the 0.1 mmol/L concentration used in our cell studies is close to a pharmacologically achievable concentration, and is about 10 to 100 times lower than the metformin concentration used in typical cell studies, which usually range from 1 to 10 mmol/L [44–48]. In fact, it was demonstrated that metformin at low concentration (≤80 μmol/L) increases AMPK activity and suppresses dibutyryl-cAMP-stimulated glucose production while the inhibition of the respiratory chain complex 1 happens only at high concentrations (~5 mmol/L) [43].

There are some limitations to this study. While we mainly focus on the downstream targets of Sirt1 and AMPK, as they
are master regulators for energy metabolism, we did not further explore the bi-directional interaction of Sirt1 and AMPK. Previous reports demonstrate that Sirt1 can activate LKB1, the upstream kinase of AMPK, by lysine deactylation and thus promoting its translocation to the cytoplasm where it can interact with AMPK [6,14,21]. Although activation of AMPK depends primarily on phosphorylation of the alpha subunit on Thr172 by LKB1 [49], there is evidence that the AMPK enzymatic activity may also be stimulated without changes in Thr172 phosphorylation [50]. However, we did not measure AMPK phosphorylation directly. Instead we measured ACC (Ser79) phosphorylation, a direct target of AMPK, as a measure of AMPK activity. ACC phosphorylation was increased two-fold by Met-Leu, and this effect was prevented by either Sirt1 or AMPK inhibition.

5. Conclusion

In summary, these data demonstrate that leucine combined with a low dose of metformin stimulates the AMPK/Sirt1 pathway, especially under the low NAD+ concentrations characteristic of energy-replete conditions. This study also provides evidence for the interacting network of AMPK and Sirt1, with both being required for Met-Leu’s overall action. This AMPK/Sirt1 activation increases insulin-dependent and -independent glucose utilization in muscle, decreases hepatic glucose output and increases fat oxidation. These effects together restored high glucose utilization in muscle, decreases hepatic glucose output and increased median and maximum survival in the worms. These data provide mechanistic support for the therapeutic benefit of metformin–leucine combinations suggested by our previous preclinical studies [12,36].

Author Contributions

J.B. and A.B. were responsible for design and execution of the cell experiments. All authors were responsible for experimental design, data analysis, interpretation, writing and editing the manuscript. All authors read and approved the manuscript.

Funding

Financial support for this study was provided by NuSirt Biopharma, Nashville, TN.

Disclosure Statement

The authors A.B., J.B., and M.B.Z. are employees of NuSirt Biopharma. A.B. and M.B.Z. are stockholder of NuSirt Biopharma and have patents related to the reported work.

Acknowledgments

C. elegans strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440).

REFERENCES


