Leucine-Metformin Synergy Activates the AMPK/Sirt1 Pathway to Increase Insulin Sensitivity in Skeletal Muscle and Glucose and Lipid Metabolism and Lifespan in C. elegans

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1237-P Abstract

We have previously shown leucine (Leu) to activate Sirt1 by lowering it's Km 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. Leu (0.5 mM) + Met (50-100 μM) synergistically activated Sirt1 ($p<0.001$) at low (≤100 μM) NAD^+ levels while Met exerted no independent effect. This was associated with an increase in AMPK and IRS1 phosphorylation and in insulin-independent glucose disposal in myotubes (~50%, $p<0.002$) evident within 30 minutes as well as a 60% reduction in insulin EC_{50}. We utilized *C. elegans* to assess the metabolic consequences of this interaction. Exposure to high glucose impaired glucose utilization and shortened lifespan by ~25%, while addition of Leu + Met to high glucose worms increased median and maximal lifespan 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 – 2.0 mM). Thus, Leu and Met synergize to enable Sirt1 activation at low NAD^+ concentrations (typical of energy replete states), resulting in improvements in energy metabolism and insulin sensitivity.

Introduction

AMPK and Sirt1 are key sensors of energy status and regulators of glucose and lipid metabolism. While insulin resistance and diabetes are associated with impairment of this pathway [1,2], activation of the AMPK-Sirt1 axis improves hyperglycemia and insulin sensitivity [3,4].The branched-chain amino acid leucine (Leu) activates Sirt1 by lowering the activation energy for NAD^+, thus mimicking the effects of caloric restriction, and enabling coactivation and amplification of the effects of other AMPK/sirtuin activators at low concentrations [5]. Metformin, the first-line anti-diabetes drug, also acts on the AMPK-Sirt1 axis [6], thus synergizing with leucine to improve insulin sensitivity and glycemic control in diet-induced obese mice while achieving a ~80% dose-reduction of metformin [7, 8].

This study was designed to define the mechanism of the interaction of leucine and metformin on AMPK/Sirt1 activation, and to assess the metabolic consequences on glucose utilization and lifespan in C. elegans.

Materials & Methods

Cell culture:

C2C12 muscle cells were differentiated in DMEM containing 2% horse serum and 1% Pen-Strep for 5 days, then treated with indicated treatments

- ***Western blot:*** Met-Leu treatment for 2 hours, then 20 min insulin (10 nM) stimulation. P-IRS, IRS, P-AMPK, AMPK : Cell Signaling (1:1000 dilution)
- ***Glucose utilization:*** C2C12 cells were differentiated on Seahorse (SH) 24-well plate and treated for 24h with or without Met (0.1 mM)-Leu (0.5 mM). For SH-experiment, media was changed to 11 mM glucose. After three baseline readings, insulin (0 to 500 nM) was injected. After 20 min incubation, 14 mM glucose was injected and extracellular acidification rate (ECAR) was measured over a two-hour period.

C. elegans:

- ***Lifespan:*** 50 young adult worms per group were placed on NGM agar plates containing indicated treatments and seeded with E.coli strain OP-50. Alive worms were counted and placed on new plates every day to eliminate progeny. Data were analyzed with the Kaplan-Meier survival curves using Prism 6 (GraphPad Software) and statistical significance was determined by Log-rank (Mantel-Cox) test
- ***Glucose measurement:*** C. elegans was maintained on NGM agar plates with or without 2% glucose added for 48 hours. Worms were washed, homogenized and lysate was used for determination of glucose content normalized to total protein content.
- ***Fatty acid oxidation:*** C. elegans were treated for 24 hours. 200 worms/well were placed in SH-plate. After 6 baseline readings, 200 μM palmitate (A&C) was injected and the oxygen consumption rate (OCR) was measured over 2 hours. (n=10/group)

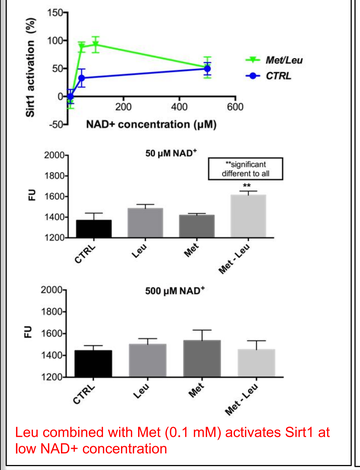
Cell-free Sirt1 activity:

Sirt1 FRET-based screening assay kit (Cayman Chemical Company, Ann Arbor, MI, USA).
Leu (0.5 mM), Met (0.1 mM) or combination were incubated with recombinant Sirt1 enzyme under different NAD+ concentration (500 μM, 100 μM, 50 μM and 10 μM) for 30 minutes. Then fluorescence was measured which is proportional to the amount of deacetylated substrate.

Results

Cell-free

Met-Leu effects on Sirt1 activation

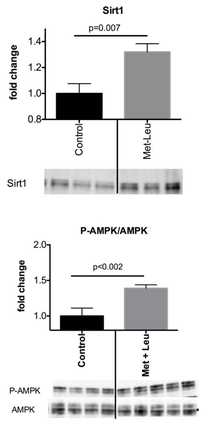


Leu combined with Met (0.1 mM) activates Sirt1 at low NAD+ concentration

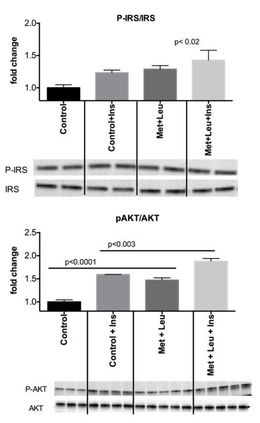
Myotubes

Met-Leu effects on insulin signaling

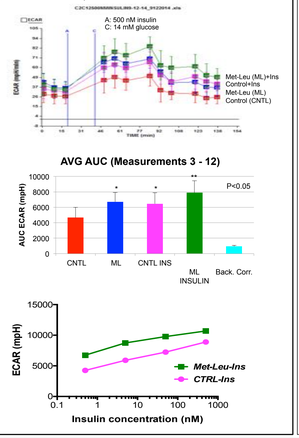
Effects on Sirt1/AMPK signaling (Western blots)



Effects on IRS and AKT signaling (Western blots)

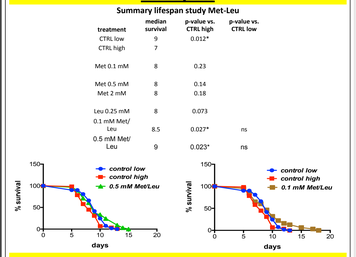


Glucose utilization in response to insulin (0.5 – 500 nM)

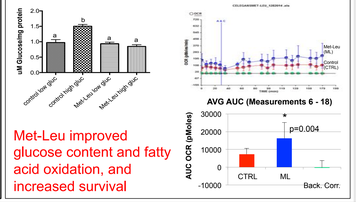


C. elegans

Lifespan



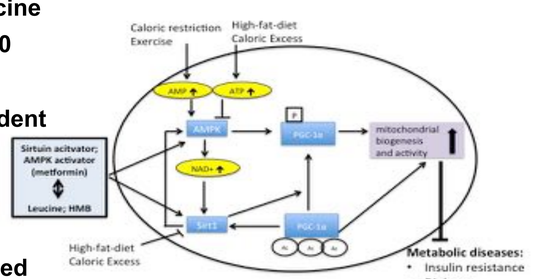
Glucose and fat metabolism



Met-Leu improved glucose content and fatty acid oxidation, and increased survival

Conclusions

- **Low dose metformin (50-100 μM) combined with leucine synergistically activate Sirt1 at low NAD^+ levels (<100 μM), characteristic of replete states**
- **The Met-Leu combination increases insulin-independent glucose disposal in myotubes, associated with an increase in AMPK and IRS phosphorylation**
- **Met-Leu restored high-glucose induced impaired glucose and fat utilization in C. elegans, and increased median and maximal lifespan**



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