Original Article
Leucine-nicotinic acid synergy stimulates AMPK/Sirt1 signaling and regulates lipid metabolism and lifespan in Caenorhabditis elegans, and hyperlipidemia and atherosclerosis in mice

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Abstract: Background/Aims: Nicotinic acid (NA), a lipid-lowering drug, serves as a source of NAD^+, the cofactor for Sirt1. Leucine (Leu) stimulates the AMPK/Sirt1 axis and amplifies the effects of other AMPK/Sirt1 activating compounds. Therefore, we tested the interactive effects of leucine and low dose NA on AMPK/Sirt1 signaling and downstream effects of lipid metabolism in cell culture, C. elegans and mice. Methods: LDL-receptor knockout mice were fed an atherogenic Western diet supplemented with leucine (24 g/kg diet) and sub-therapeutic NA combinations (50 mg/kg diet and 250 mg/kg diet) or low therapeutic NA (1000 mg/kg diet) for 8 weeks to evaluate markers of hyperlipidemia and atherosclerosis. Results: NA-Leu increased P-AMPK and Sirt1 in adipocytes and myotubes. In C. elegans, NA-Leu increased P-AMPK and DAF-16 (FOXO), reduced lipid accumulation and increased median survival under mild oxidative stress conditions. In the mice, NA-Leu reduced total cholesterol, cholesterol esters, plasma triglycerides, atherosclerotic lesion size, lipid area, and aortic macrophage infiltration, similar to the therapeutic NA dose. Conclusion: Leu amplifies the effects of NA on lipid metabolism, hyperlipidemia and atherosclerosis in mice, at least in part by activation of the AMPK/Sirt1 axis. This combination may be a potential therapeutic alternative for hyperlipidemia and atherosclerosis.

Keywords: AMPK, atherosclerosis, *C. elegans*, leucine, lipid metabolism, nicotinic acid, Sirt1

Introduction

Atherosclerosis is a slowly progressive disease which is the main cause of morbidity and mortality in developed countries [1, 2]. It is caused by the accumulation of cholesterol and triglycerides in the arterial wall which initiates an inflammatory and cell death signaling cascade, resulting over time in reduced wall elasticity and decreased blood flow through the arteries. Therefore, dyslipidemia and hypercholesterolemia are considered major risk factors for disease development. Consequently, lipid lowering treatments are one of the most successful strategies to reduce cardiovascular disease risk [2]. Statins are the most widely used drugs and have significantly reduced the number of major cardiovascular events; however sub-optimal dosing or drug discontinuation occurs in 10

to 20% of patients due to the development of side effects. In addition, accumulating data shows a relationship between statin use, elevation of blood glucose levels and incidence of new-onset diabetes [3, 4].

Nicotinic acid (NA) is a form of vitamin B3 (niacin) and has been used for decades as an effective treatment of dyslipidemias, as it lowers total and LDL-cholesterol, triglycerides and lipoprotein, raises HDL-cholesterol and reduces atherosclerotic plaque progression [5]. In addition, its anti-inflammatory, anti-thrombotic and anti-oxidant activities contribute to its overall beneficial effects [4]. However, therapeutic doses (1-3 g/day) are generally poorly tolerated because of severe vasodilation and flushing response [5]. In addition, the use of niacin is constrained in diabetic patients due to its

potential hyperglycemic adverse effects [4, 6]. While side effects are milder with the sustained and extended release preparations, they are still sufficient to limit patient acceptance.

The effects of niacin have been widely thought to be mediated by activation of the G-protein-coupled hydroxycarboxylic acid receptor 2 (HC-AR2 or GPR109A), thereby suppressing adipocyte lipolysis and reducing substrate availability for hepatic lipid synthesis [7]. However, recent evidence suggests that niacin's lipid lowering effects are mediated by GPR109A independent mechanisms since treatment with GPR-109A agonists in mice lowered plasma free fatty acids comparable to niacin without any effects on triglycerides and other lipid parameters [7]. Moreover, GPR109A receptors are not expressed in liver cells and therefore can not exert niacin's effect on lipoproteins.

NA is also a substrate for NAD^+ biosynthesis, the cofactor for Sirt1, and treatment with NA increases cellular NAD^+ levels and Sirt 1 activity [8, 9]. Sirt1 is a well-known regulator of glucose and lipid metabolism. In addition, it deacetylates and thereby activates the liver X receptor (LXR), a regulator of cholesterol and lipid homeostasis [10]. Moreover, Sirt1 has direct beneficial effects on vascular endothelium-dependent function such as vasodilation via eNOS derived nitric oxide (NO), reversal of cholesterol transport in macrophages, prevention of foam cell production, as well as anti-thrombotic and anti-inflammatory effects by downregulation of the NFκB pathway [10]. Therefore, it is likely that some effects of NA are mediated by Sirt 1 activation.

We have previously demonstrated that leucine activates Sirt1, at least in part, by lowering the activation energy for NAD^+, thereby co-activating and amplifying the effects of other sirtuin activators while reducing their required effective concentration [11, 12]. Therefore, we assessed in this study whether leucine also amplifies the effects of NA on lipid metabolism and atherosclerosis and enables a meaningful dose reduction.

Materials and methods

Cell culture

Murine 3T3-L1 pre-adipocytes (Zenbio, Research Triangle Park, NC, USA) were grown in the absence of insulin in Dulbecco's modified Eagle's medium (DMEM, 25 mM glucose) containing 10% fetal bovine serum (FBS) and antibiotics (1% penicillin-streptomycin) (= adipocyte medium) at 37°C in 5% CO_2 in air. Confluent pre-adipocytes were induced to differentiate with a standard differentiation medium (DM2-L1, Zen-Bio Inc., NC). Pre-adipocytes were maintained in this differentiation medium for 3 days and subsequently cultured in adipocyte medium for further 8 to 10 days to allow at least 90% of cells to reach full differentiation before treatment. Medium was changed every 2-3 days; differentiation was determined microscopically via inclusion of fat droplets.

Human hepatoma HepG2 cells (ATCC, Manassas, VA, USA) were grown in Dulbecco's modified Eagle's medium (DMEM, 5.5 mM glucose) containing 10% fetal bovine serum (FBS) and antibiotics (1% penicillin-streptomycin) at 37°C in 5% CO_2 in air. Medium was replaced with fresh medium every 2 to 3 days. Cells were split at a 1:4 ratio at 70 to 80% confluency.

C2C12 muscle cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotics (1% penicillin-streptomycin) at 37°C in 5% CO_2 in air. Cells were grown to 100% confluence, changed into differentiation medium (DMEM with 2% horse serum and 1% penicillin-streptomycin), and fed with fresh differentiation medium every other day until myotubes were fully formed (6 days).

Caenorhabditis elegans (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. Then synchronized L1 larvae were transferred to E. coli fed NGM plates containing indicated treatments for about 35 hours to reach L4/young adult stage. All treatments were added to the agar in indicated concentrations.

C. elegans Oil Red O staining

Treated L4/young adult worms were washed off from plates three times with PBS and collected

in a 15 ml conical tube, then centrifuged at 1000 g for 30 sec. The supernatant was discarded and the pellet was washed with 10 ml PBS. After centrifugation, the supernatant was discarded except 400 µl, which was transferred to a new 1.5 ml eppendorf tube. Then 500 µl of 2x MRWB (160 mM KCl, 40 mM NaCL, 14 mM Na$_2$EGTA, 1 mM Spermidine HCl, 0.4 mM Spermine, 30 mM NaPIPES pH 7.4, 0.2% b-Mercaptoethanol) and 100 µl of 20% Paraformaldehyde was added and gently rocked for 60 min at room temperature. Then tubes were centrifuged at 1500 g for 30 sec, then aspirated and washed with PBS once, centrifuged again and aspirated to 300 µl. Then 700 µl of isopropanol was added, mixed by inverting the tube and incubated with gentle shaking for 15 min at room temperature. Tubes were centrifuged, and isopropanol was removed. Then 1 ml of 60% filtered Oil Red O dye solution (0.5 g Oil Red O in 100 ml anhydrous isopropanol, equilibrated for 2 days by stirring at RT, then 4 vol ddH$_2$O was mixed with 6 vol dye solution and equilibrated for 15 min at RT, then filtered with 0.2 M pore size) was added to worms and rotated on shaker overnight. Worms were pelleted by centrifugation (1200 g for 30 sec), dye was removed and washed 4 times with ddH$_2$O to remove any unbound stain. For quantification, the Oil Red O was eluted from the cells by addition of 100% isopropanol and the optical density of 200 µl aliquots (triplicates/sample) was determined at a wavelength of 540 nm using a Biotek Synergy HT Microplate Reader (BioTek, Winooski, VT, USA). Data were normalized to protein content using the Pierce BCA protein assay kit.

Lifespan study protocol

50 to 60 synchronized adult worms per group were placed on NGM agar plates seeded with E. coli strain OP-50 (= counted as day 4 of study). To study lifespan under mild oxidative stress, 2 mM paraquat was added to the NGM agar in addition to the treatments (10 nM NA, 0.5 mM Leu, 10 µM NA or NA (10 nM)-Leu (0.5 mM) combination). For the lifespan study under normal condition, the worms were maintained on regular NGM plates (control) or NGM plates containing NA (10 nM)-Leu (0.5 mM). 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 plati-num pick and scored as censored if they crawled off the plate.

Protein preparation for western blot

To obtain worm lysate, treated L4/young adult worms were washed off from plates with M9 buffer and collected into microcentrifuge tubes. After centrifugation (500 g for 5 min) supernatant was removed to about 100 µl. Then 250 µl RIPA buffer plus Protease and Phosphatase inhibitor mix was added.

To obtain cell lysate, cells were grown on 6-well plate and treated as described above. Cells were washed with PBS and then lysed with RIPA buffer plus Protease and Phosphatase inhibitor mix, incubated on ice for 10 minutes. Cell or worm lysates were homogenized, then centrifuged at 16,000 g for 10 min at 4°C. The clear supernatant was used for further experiments. Protein content was determined using the Pierce BCA protein assay kit.

Western blot

AMPK, Phospho-AMPKα (Thr172)- and Sirt1 antibodies were obtained from Cell Signaling (Danvers, MA, cat # 2532, 2535 and 9475, respectively), Sir2 and DAF-16 antibody were obtained from MyBiosource Inc. (San Diego, CA, cat # MBS621556) and Santa Cruz Biotechnology Inc. (Dallas, TX, cat # sc-9229), respectively. Protein levels of cell extracts were measured by BCA kit (Thermo Scientific, Pittsburgh, PA). Equal amounts of protein (10 to 30 ug) were resolved on 10% Tris/HCL polyacrylaminde gels (Criterion precast gel, Bio-Rad Laboratories, Hercules, CA), transferred to PVDF membranes, incubated in blocking buffer (3% BSA in TBS) and then incubated with primary antibody (1:1000 dilution for AMPK, Phospho-AMPK and Sirt1, 1:3000 dilution for Sir2, and 1:200 dilution for DAF-16), washed and incubated with secondary (anti-rabbit for AMPK, Phospho-AMPK and Sirt1, and anti-goat for DAF-16) horseradish peroxidase-conjugated antibody. Visualization and chemiluminescent detection was conducted using BioRad Chemi-Doc instrumentation and software (Bio-Rad Laboratories, Hercules, CA) and band intensity was assessed using Image Lab 4.0 (Bio-Rad Laboratories, Hercules, CA), with correction for background. Band intensities were normalized to stain-free blots to control for loading, as described [13, 14].

Seahorse fatty acid oxidation

The palmitate-stimulated oxygen consumption rate (OCR) was measured with the Seahorse XF 24 analyzer (Agilent Technologies Inc., Santa Clara, CA, USA) as previously described [15]. Cells were seeded at 40,000 cells per well, differentiated as described above, treated for 24 hours with the indicated treatments, washed twice with non-buffered carbonate-free pH 7.4 low glucose (2.5 mM) DMEM containing carnitine (0.5 mM), equilibrated with 550 mL of the same media in a non-CO_2 incubator for 30 minutes, and then inserted into the instrument for 15 minutes of further equilibration. O_2 consumption was measured in three successive baseline measures at eight-minute intervals prior to injection of palmitate (200 µM final concentration). Post-palmitate-injection measurements were taken over a 3-hour period with cycles consisting of 10 min break and three successive measurements of O_2 consumption.

Sirt1 activity

The Sirt1 FRET-based screening assay kit (Cayman Chemical Company, Ann Arbor, MI, USA) was used for the measurement of Sirt1 activity in 3T3L1 adipocytes. The protocol was modified for the measurements in cells as follows; cell lysate (5 uL) of differentiated 3T3L1 treated with Leu (0.5 mM), NA (10 nM) or combination for 24 hours was incubated with peptide substrate under low NAD^+ (250 uM) concentrations. The fluorescence was measured with excitation and emission wavelengths of 360 nm and 450 nm, respectively. The increase in fluorescence is proportional to the amount of deacetylated substrate and thus Sirt1 activity.

Animals

This study and all animal procedures were performed under the auspices of an Institutional Animal Care and Use Committee-approved protocol of the Georgia State University and in accordance with PHS policy and recommendations of the Guide (Protocol number A12024).

LDL receptor knockout (LDLRKO) mice were obtained from Jackson Laboratories (Bar Harbor, ME), and housed in groups under room temperature in a humidity-controlled environment with a regular light and dark cycle. All mice were provided free access to an atherogenic western diet (WD) containing 0.21% cholesterol (by weight) and 40% calories from fat and water for 4 weeks prior to treatment. After this induction period, the mice were randomized to one of the following treatment groups (n=12/group): (a) the WD diet alone, (b) WD and 24 g of leucine/kg diet, (c) WD and 24 g of leucine/kg diet and 50 mg nicotinic acid/kg diet, (d) WD and 24 g of leucine/kg diet and 250 mg nicotinic acid/kg diet, or (e) WD and 1000 mg nicotinic acid/kg diet. The treatments were administered for an additional eight weeks (total study duration 12 weeks); then the mice were humanely euthanized with CO_2 inhalation. Blood and tissues were collected for further experiments as described below.

Plasma cholesterol and triglyceride levels

After four and eight weeks of treatment, mice were fasted for 4 hours and blood samples in all groups were obtained from the tails and collected into EDTA-coated tubes to analyze plasma lipid and cholesterol profiles. Plasma total cholesterol (TC, Pointe Scientific, Canton, MI), free cholesterol (FC, Wako, Richmond, VA) and triglyceride (TG, Wako, Richmond, VA) concentrations were measured using enzymatic assays according to manufacturer's instructions. Cholesterol ester (CE) was calculated by multiplying the difference between TC and FC by 1.67.

Atherosclerosis assessment

Following euthanasia, the circulatory system was perfused with phosphate-buffered saline (PBS), then the heart and aorta were removed. The upper one-third of the heart was dissected and embedded in Optimal Cutting Temperature Compound (Sakura Tissue-Tek, Torrance, CA), frozen, and stored at -80°C. Blocks were serially cut at 8 µm intervals and stained with hematoxylin and 0.5% Oil Red O (Sigma-Aldrich) to evaluate aortic sinus atherosclerotic intimal area. Atherosclerotic lesion area and Oil Red O positive area were quantified using Image-Pro Plus software (Media Cybernetics, Bethesda, MD). Whole aorta (from sinotubular junction to iliac bifurcate) was dissected and fixed in 10% formalin, and the adventitia was cleaned. Aortas were opened along the longitudinal axis and pinned onto black silicon elastomer (Rubber-Cal, Santa Ana, CA) for the quantification of atherosclerotic lesion area. The percent-
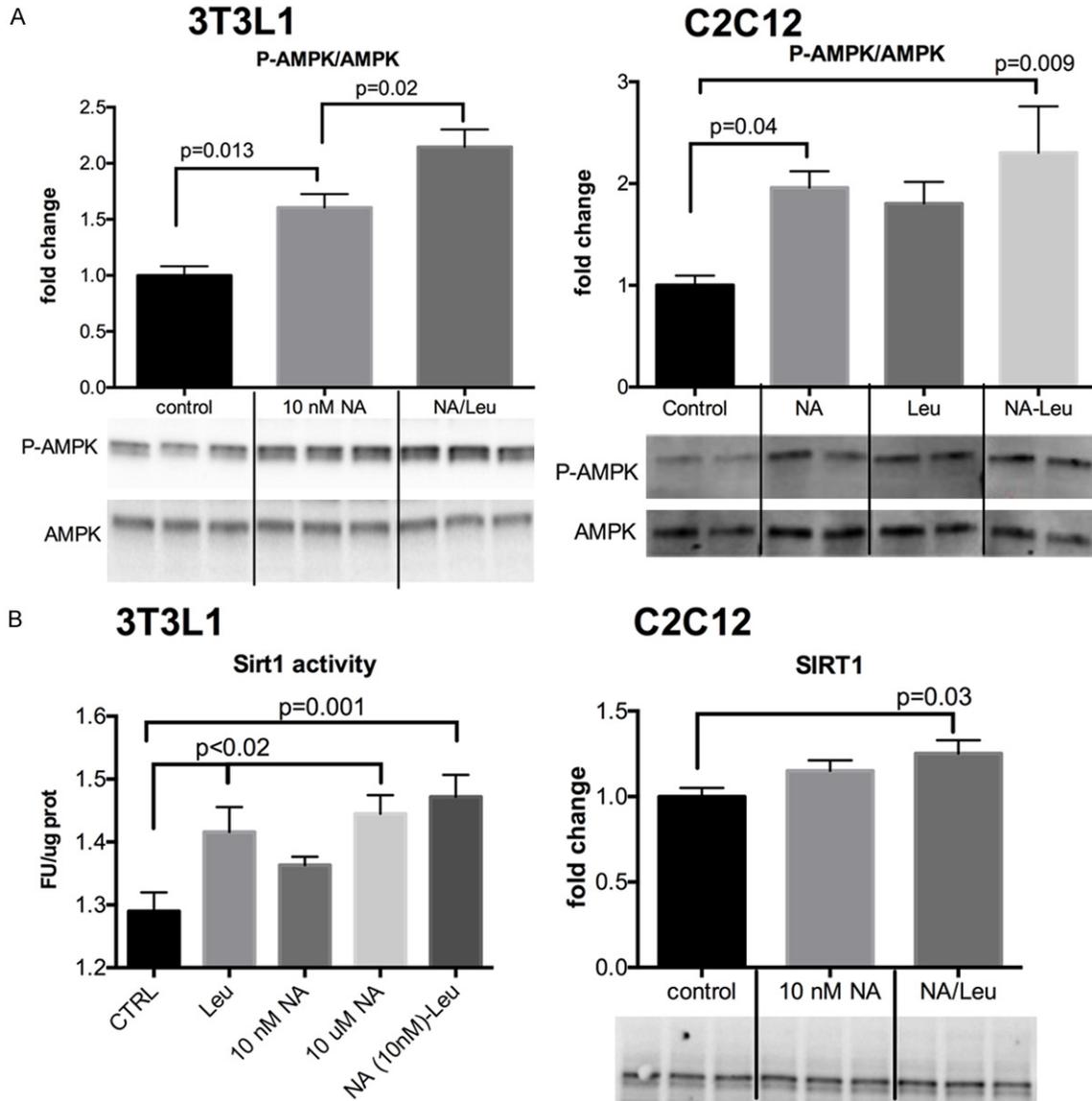
Figure 1. NA-Leu combination increases AMPK and Sirt1 activity in muscle cells and fat cells. Differentiated 3T3L1 adipocytes and C2C12 muscle cells were treated with indicated treatments for 4 or 24 h. Protein expression of Phospho-AMPK and total AMPK (A) and Sirt1 activity in 3T3L1 and Sirt1 protein expression in C2C12 (B) were measured, as described under Methods. Quantitative data and representative blots are shown. Data are represented as mean ± SEM (n=3 to 6).

age of total aortic surface covered with atherosclerotic lesions was quantified by Image-Pro Plus software (Media Cybernetics, Bethesda, MD).

Macrophage infiltration

Sections of the aortic sinus were immunostained with rat monoclonal antibody against macrophage-specific CD68 (Clone FA11, 1:75, AbD Serotec, Raleigh, NC) followed by staining

with alkaline phosphatase-conjugated mouse anti-rat (for CD68, 1:50) secondary antibodies (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Control slides contained no primary antibody. The CD68-positive areas were analyzed using Image-Pro Plus software (Media Cybernetics, Bethesda, MD).

Statistical analysis

Data were analyzed via one-way analysis of variance and least significant difference test
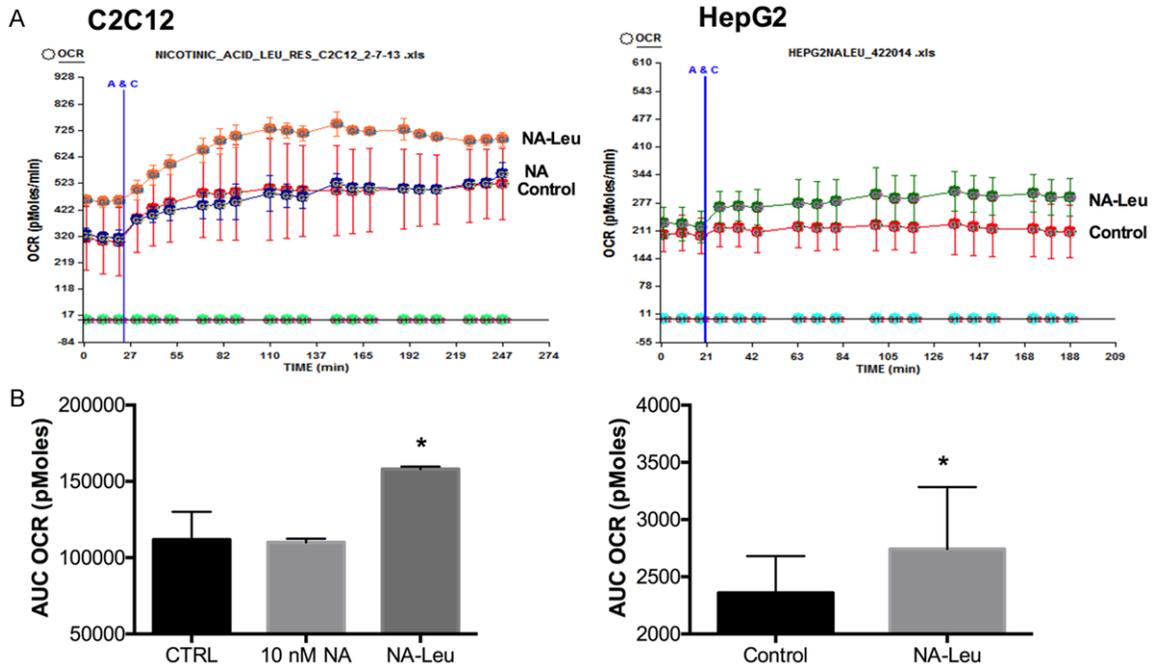
Figure 2. NA-Leu combination increases palmitate-induced fat oxidation in muscle cells and liver cells. Differentiated C2C12 muscle cells (n=3) (A) and HepG2 liver cells (n=10) (B) were treated with indicated treatments for 24 h. Oxygen consumption rate (OCR) after 200 μM palmitate injection (A&C) was measured and the area under the curve (AUC) was calculated. Data are represented as mean ± SEM. *significantly different from control (P<0.05).

was used to separate significantly different group means using GraphPad Prism version 6 (GraphPad Software, La Jolla California USA, www.graphpad.com). All data are expressed as mean ± SEM.

For the statistical analysis of the lifespan study with *C. elegans*, data were analyzed via the standard approach for survival analysis using Kaplan-Meier estimate, as described [16, 17]. Statistical significance between curves was determined with the Gehan-Breslow Wilcoxon test using Prism 6 (GraphPad Software, La Jolla California, USA, www.graphpad.com); this test gives more weight to deaths at early time points [18]. The hazard ratio describes the rate of deaths in the treatment group compared to the control group, assuming that the rate is consistent over time.

Results

We previously demonstrated that leucine synergizes with other Sirt1/AMPK activators, such as resveratrol or metformin, to increase muscle, adipocyte and liver fat oxidation *in vitro* and *in vivo* [12, 19]. To test whether these effects could be also achieved with a combination of a low concentration of NA, an indirect Sirt1 activator, we explored Sirt1 and AMPK signaling in C2C12 muscle cells, 3T3L1 adipocytes and HepG2 liver cells. As demonstrated in **Figure 1**, leucine combined with low dose NA increased AMPK phosphorylation and Sirt1 activity in 3T3L1 adipocytes, and C2C12 muscle cells more than the individual components, although leucine and NA (10 nM) also had modest effects on AMPK and Sirt1. Since fat oxidation is a downstream effect of this signaling pathway, we measured the oxygen consumption rate (OCR) after palmitate stimulation in muscle and liver cells (**Figure 2**), and found them to be significantly increased by the NA-Leu combination by 41% and 16% in C2C12 and HepG2 cells, respectively, compared to control untreated cells.

Next, we explored the overall effects of the NA-Leu combination in the simple organism *C. elegans*, which is considered a well-defined model for obesity and lipid metabolism [20]. **Figure 3** demonstrates that Sir2, the ratio of Phospho-AMPK/AMPK as well as DAF-16, the ortholog of the transcription factor FOXO, were increased in *C. elegans* by 85%, 43% and 100%, respectively. This was associated with a
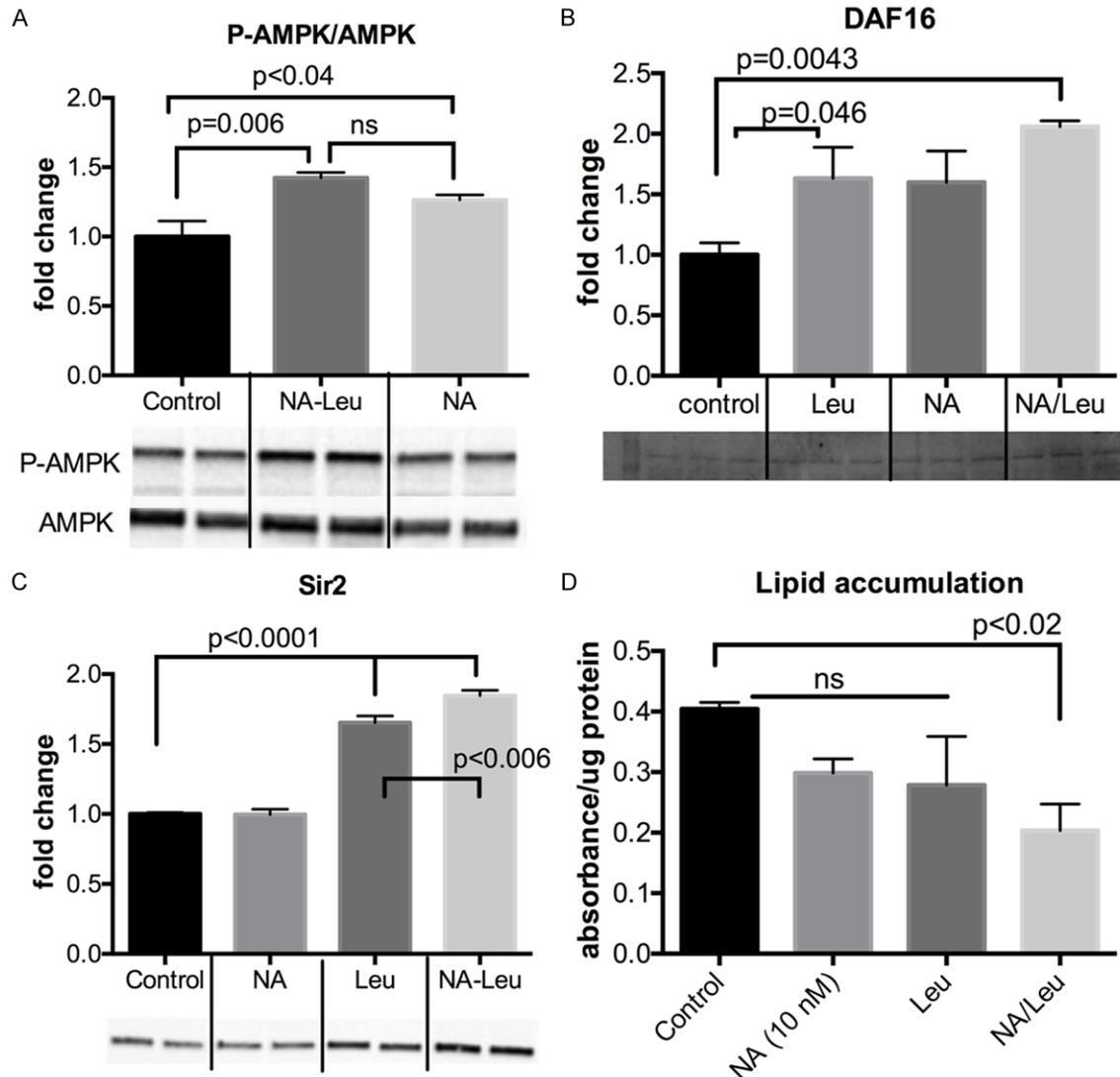
Figure 3. NA-Leu combination increases P-AMPK and DAF-16 protein expression and reduces lipid accumulation in *C. elegans*. *C. elegans* L1 larvae were maintained on agar plates containing indicated treatments until adulthood. Protein expression of P-AMPK/AMPK (A), DAF-16 (B) and Sir2 (C) are shown as quantitative data and representative blots (n=2-4). (D) Quantitative data of lipid accumulation after Oil Red O Staining (n=4). Data are represented as mean ± SEM.

50% decrease in lipid accumulation in the worms (**Figure 3D**). Although the individual compounds exhibited a small effect on these parameters, only the NA-Leu combination resulted in a significant reduction of lipid accumulation. To test whether these effects can influence overall life expectancy, we conducted lifespan studies in *C. elegans*. Since metabolic diseases such as obesity and diabetes are associated with an increase in oxidative stress, we conducted the lifespan studies under normal and mild oxidative stress conditions (2 mM

paraquat) (**Figure 4**). There was no difference in the survival curves under normal conditions (**Figure 4D**). However, under mild oxidative conditions, the NA-Leu combination significantly increased median survival by 25% and reduced the hazard ratio by 20% (**Figure 4A** and **4C**).

Since NA has been used for decades as a therapeutic drug for dyslipidemia and atherosclerosis, we next tested the effects of the NA-Leu combination on lipid metabolism and atherosclerosis in LDLRKO mice, a mouse model of
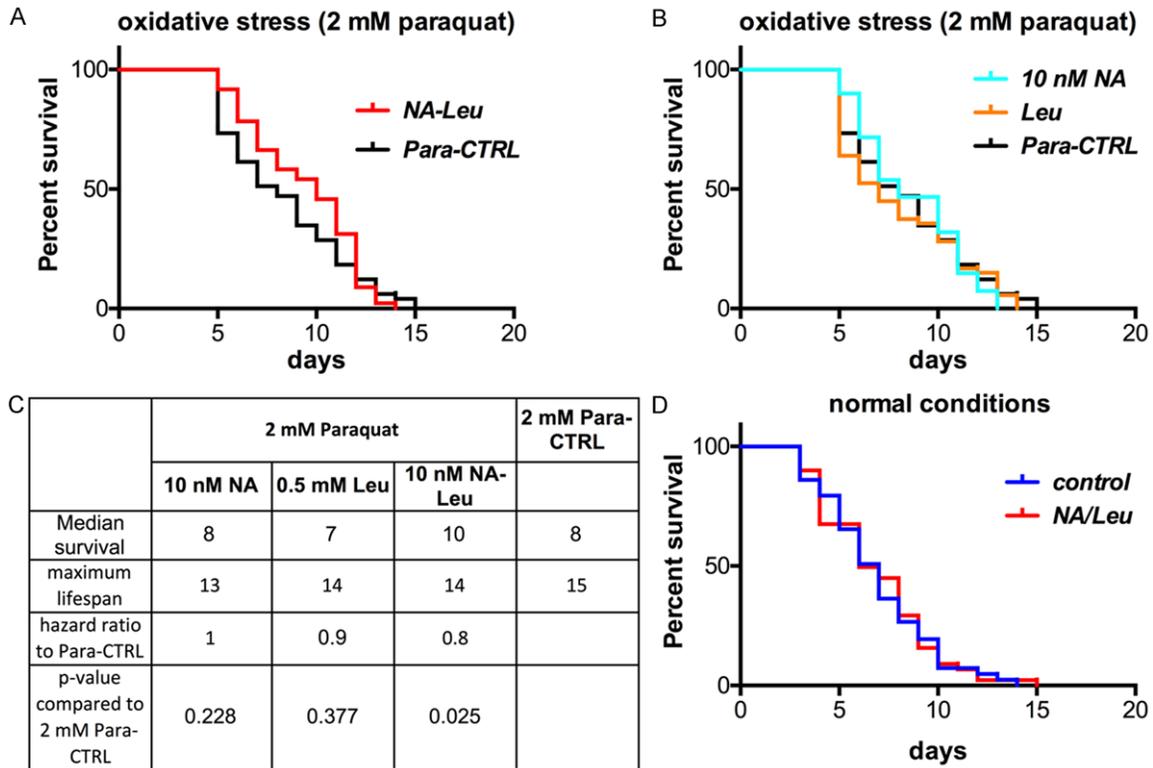
Figure 4. NA-Leu increases the median lifespan in *C. elegans* under mild oxidative conditions. Survival curves of synchronized L4 worms maintained on NGM agar plates until death with or without NA-Leu under mild oxidative conditions (2 mM paraquat (A and B), n=60 per group) or without paraquat (= normal conditions (D), n=50 per group). (B) Survival curves of individual treatment groups under mild oxidative conditions (n=60 per group). (C) Lifespan table summarizing median survival, maximum lifespan and hazard ratio using the Kaplan-Meier survival analysis and Gehan-Breslow-Wilcoxon test to determine statistical significance between curves (P<0.05), as described under Material & Methods.

atherosclerosis. Concentrations of about 100-300 mg/kg body weight are used in the literature to achieve niacin effects on lipid metabolism and atherosclerosis [21-24]. Therefore, we selected a concentration of 1000 mg/kg diet as the therapeutic dose; this is equivalent to about 100 mg NA/kg body weight (based on a 30 g mouse and a food intake of 3 g/day) and approximately equivalent to a low therapeutic dose of nicotinic acid in hypercholesterolemic humans (~1,500 mg/day). A four-times and twenty-times lower dose of NA was used for the combination with leucine which we assumed to have no effect by itself. As expected, the atherogenic western diet resulted in profound elevations in plasma cholesterol, cholesterol esters, and trigylceyrides in the LDLRKO mice. Addition of a therapeutic dose of NA (1,000 mg/kg diet) for four weeks resulted in 20% and 28% decreases in total cholesterol and cholesterol esters, respectively (P<0.01) (**Figure 5A**).

Although leucine exerted no independent effect on these parameters, addition of leucine to sub-therapeutic doses of NA (50 or 250 mg/kg diet) resulted in comparable decreases in total cholesterol and esterified cholesterol to that found with the therapeutic dose (P<0.01). These differences were sustained at eight weeks time point (**Figure 5B**). Similarly, addition of a therapeutic dose of NA (1,000 mg/kg diet) resulted in a 32% decrease in plasma triglycerides (P<0.01) (**Figure 5A**) at four weeks, and a statistically comparable decrease in triglycerides was found when leucine was added to sub-therapeutic doses of NA (50 or 250 mg/kg diet) (P<0.01). However, these reductions were not sustained at 8 weeks, although there was still a trend for the NA-Leu 250 mg combination (P=0.058) (**Figure 5B**).

To assess the degree of atherosclerosis, sections of the aorta were histologically examined
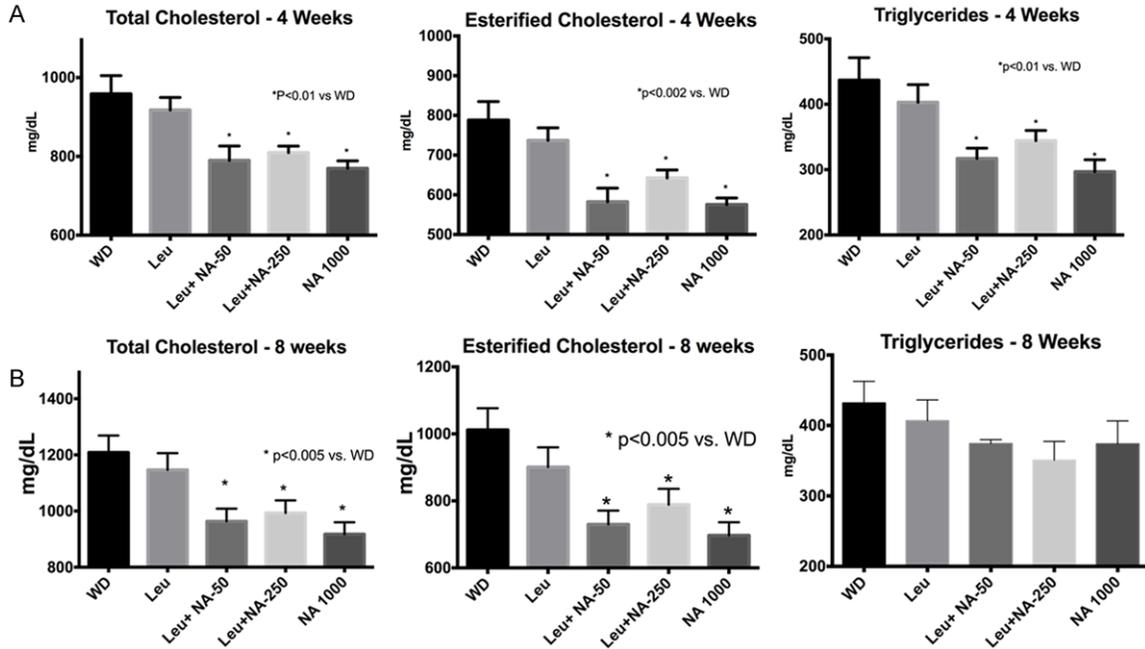
Figure 5. Plasma cholesterol and triglycerides in LDLRKO mice. LDLRKO mice were fed an atherogenic western diet (WD) for eight weeks supplemented with different sub-therapeutic doses NA (50 or 250 mg NA) combined with leucine, or a low full-therapeutic dose of NA (1000 mg NA). Plasma lipids (total cholesterol, esterified cholesterol and triglycerides) were measured at 4 weeks (A) and 8 weeks (B) of treatment (n=12).

and stained for lipid deposition (Oil Red O staining) and macrophage infiltration (CD68 immunostaining). Addition of a therapeutic dose of NA (1,000 mg/kg diet) to the Western diet resulted in a ~50% decrease in atherosclerotic lesion size, as illustrated in the representative Oil Red O stained aortic histology slides and quantified as total lesion and lipid area (**Figure 6**). Also addition of leucine to a sub-therapeutic dose of NA (50 mg/kg diet) resulted in a comparable decrease in lesion and lipid area to that found with the therapeutic dose (P<0.0001). Although leucine exerted an independent effect on plaque area, this effect was significantly less than that of the sub-therapeutic NA-Leu combination. Similarly, addition of a therapeutic dose of NA (1,000 mg/kg diet) to the Western diet also resulted in a ~50% decrease in aortic macrophage infiltration, represented as CD68-positive area in the histology slides and quantified as % CD68 positive area in the lesion (**Figure 7**). Leucine combined to the sub-therapeutic dose of NA (50 mg/kg diet) resulted in a comparable decrease in macrophage infiltration to that found with the therapeutic dose (P<0.0001). However, leucine also exerted a significant independent effect on reducing macrophage infiltration; this effect was not as great as the therapeutic dose of NA, but was also not significantly different from the NA-Leu combination.

Discussion

In this study we demonstrate that the combination of a low dose of NA with leucine results in effects on lipid metabolism and atherosclerosis *in vitro* and *in vivo* comparable to a full therapeutic dose of NA alone.

The pathophysiology of atherosclerosis is a multi-step process. Since the accumulation of lipoprotein-derived lipids in the arterial wall is considered the first step, normalization of hypercholesterolemia and dyslipidemia are important therapeutic goals to stop progression of atherosclerotic plaque development. Clinical evidence over the last decades have clearly demonstrated a robust relationship between LDL-cholesterol reduction and the decrease in cardiovascular disease (CVD) with every 1 mmol/L (~40 mg/dl) reduction in LDL-cholesterol resulting in a ~22% decrease in CVD events [25]. Therefore, lipid-lowering drugs are considered first-line treatment options when other lifestyle changes are not sustain-
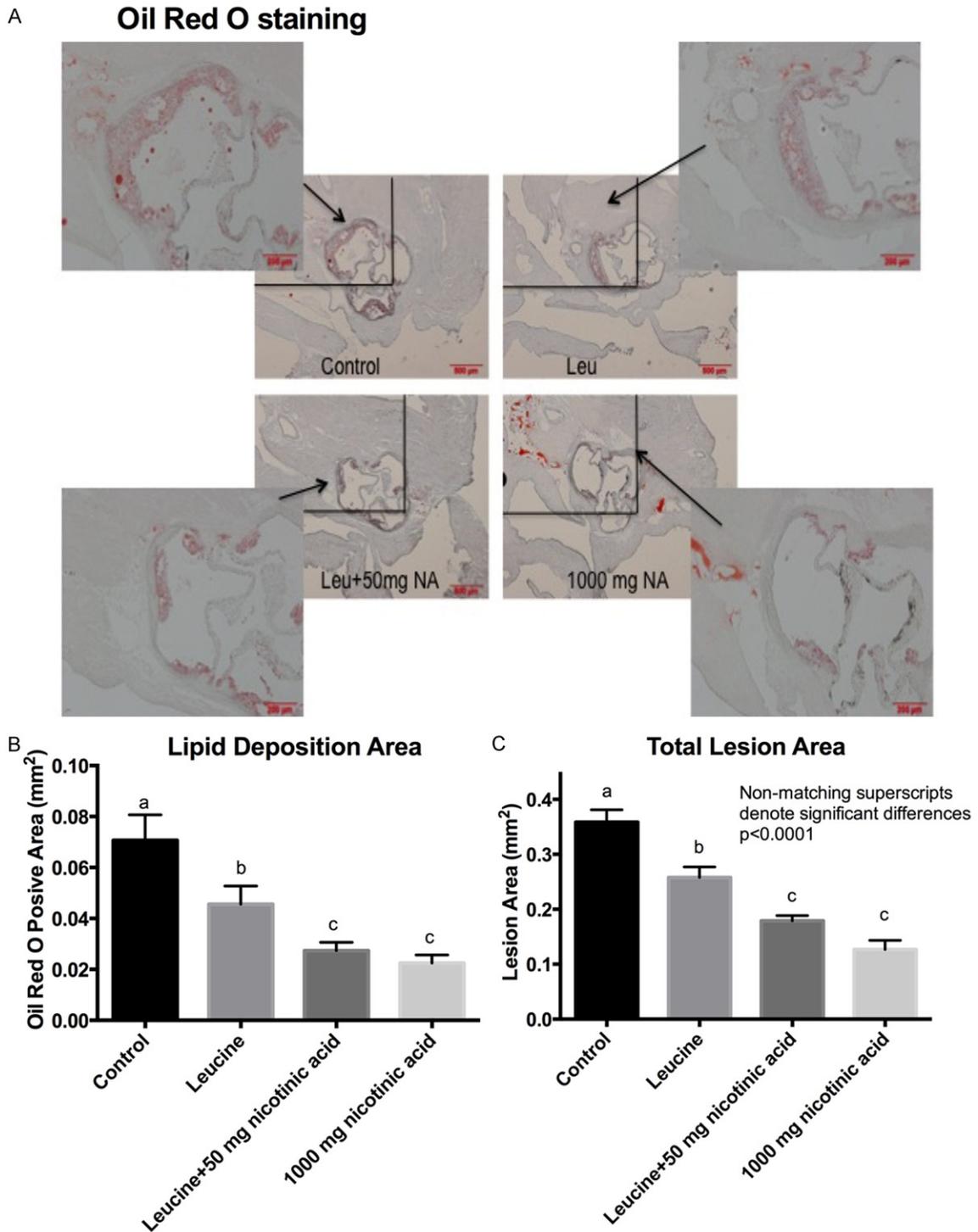
Figure 6. Lipid deposition in aortic plaques of LDLRKO mice. LDLRKO mice were fed an atherogenic western diet (WD) for eight weeks supplemented with different sub-therapeutic doses NA (50 or 250 mg NA) combined with leucine, or a low full-therapeutic dose of NA (1000 mg NA). After eight weeks of treatment, a subset of six mice per group was euthanized and three sections of the aortic sinus from each animal were evaluated for atherosclerotic plaque development after Oil Red O staining. A. Representative images of aortic plaque lipid deposition with 10x magnification of the left upper corner. B. Quantification of aortic lipid deposition (Oil Red O positive area). C. Quantification of atherosclerotic lesion area of the whole aorta (from sinotubular junction to iliac bifurcate). Differing letters above the bars denote significant differences between groups, $P \leq 0.05$ (n=6).

A immuno-staining with CD68 antibody
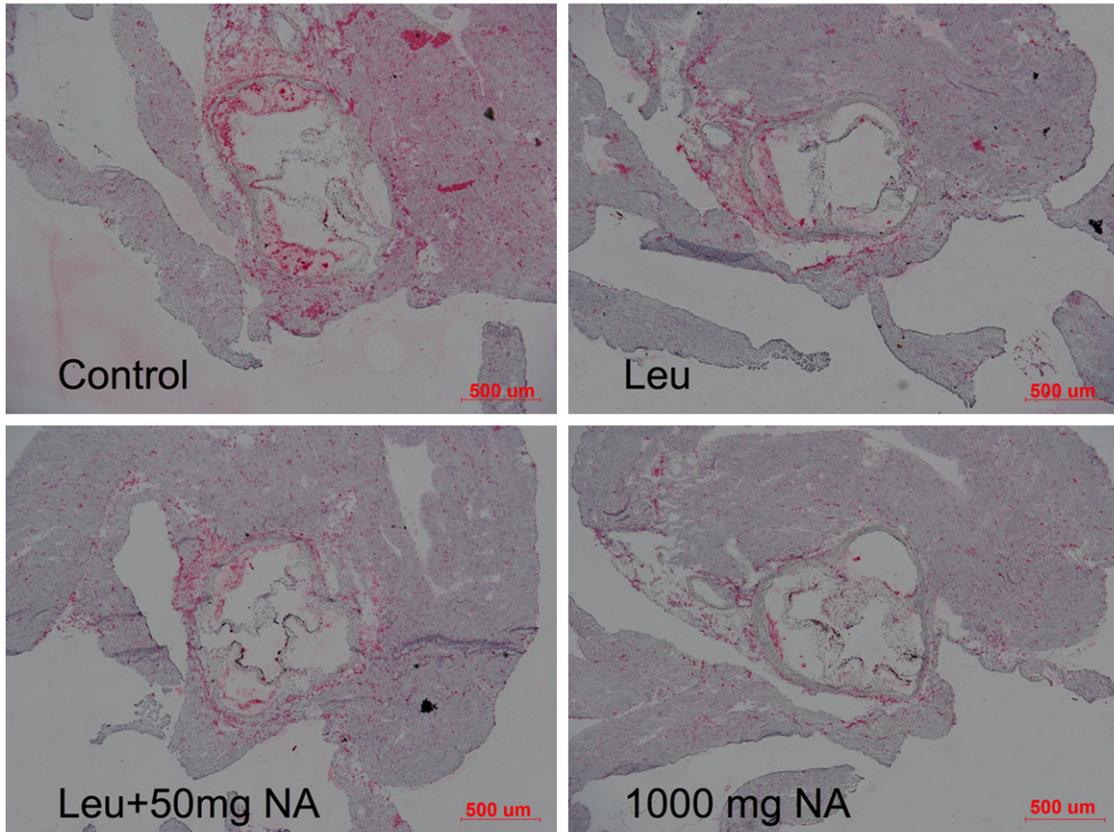
B aortic macrophage infiltration
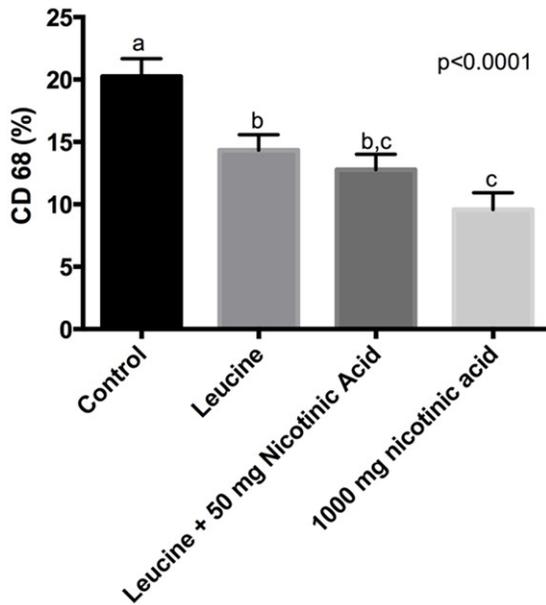
Figure 7. Aortic macrophage infiltration in LDLRKO mice. LDLRKO mice were fed an atherogenic western diet (WD) for eight weeks supplemented with different sub-therapeutic doses NA (50 or 250 mg NA) combined with leucine, or a low full-therapeutic dose of NA (1000 mg NA). After eight weeks of treatment, a subset of six mice per group was euthanized and three sections of the aortic sinus from each animal were immuno-stained with rat monoclonal antibody against macrophage-specific CD68. (A) Representative images of CD68 immune-stained aortic slides and (B) quantification of the CD68-positive areas. Differing letters above the bars denote significant differences between groups, P≤0.05 (n=6).

able. Most lipid lowering drugs achieve a reduction of 15% (fibrates) to 50% (potent statins) in LDL-cholesterol [3]. However, despite their potent effects on LDL-cholesterol reductions, statins often need to be combined with other drugs to achieve improvement on other lipid
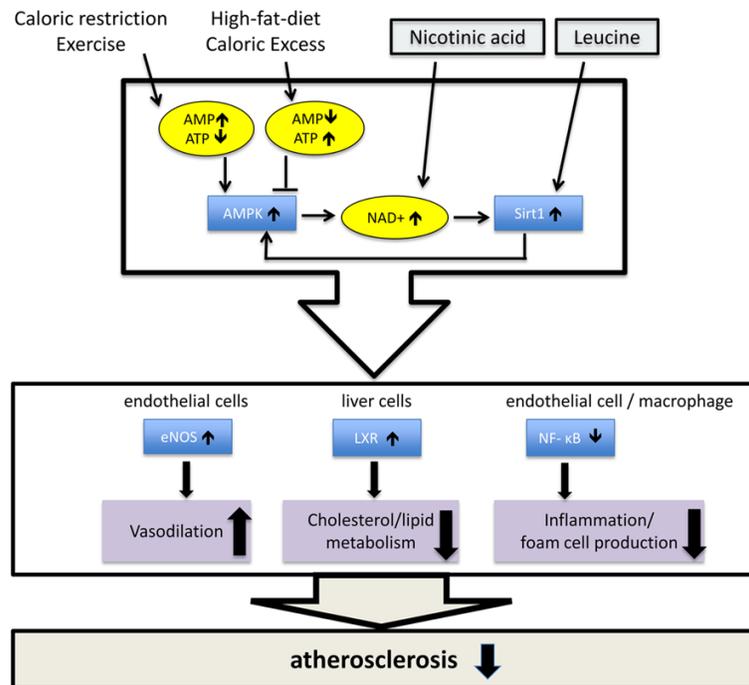
Figure 8. Proposed model of the synergistic effects of NA-Leu combination. AMPK and Sirt1 are nutrient sensors and bidirectionally interacting with each other. Caloric restriction or exercise increases AMP, thus activating AMPK, while high-fat diet or caloric excess increases ATP and consecutively inhibits AMPK. Low dose nicotinic acid in combination with leucine synergistically activates Sirt1, counteracting the inhibitory effects of overnutrition. AMPK/ Sirt1 activation improves atherosclerosis by acting in multiple ways; activation of LXR in liver cells reduces hypercholesterolemia and hyperlipidemia, inhibition of NF-κB signaling in endothelial cells and macrophages prevents inflammation and foam cell production, and activation of eNOS improves endothelium-dependent vasodilation [10, 42].

associated with decreased Sirt1 activation [30, 31]. Also leucine enables co-activation with other AMPK/Sirt1 activators thereby reducing their necessary concentration [12, 30]. Since NA acts as substrate for NAD$^+$ biosynthesis and activates Sirt1 by increasing cellular NAD$^+$ levels, we hypothesized that leucine would also amplify the effects of NA on Sirt1 activation. In accordance with this, Leu combined with a low concentration of NA activated Phospho-AMPK and Sirt1 significantly in adipocytes, muscle cells and *C. elegans*, while the individual components had little or no effect. This resulted in increased fatty acid oxidation in muscle cells and liver HepG2 cells as well as in a reduction in lipid accumulation in *C. elegans*.

Inflammation plays also a major role in the development and progression of atherosclerosis. Oxidized lipids and lipoproteins induce an immune response in the endothelial cells with the production of adhesion molecules, consecutively recruiting and activating monocytes and finally transforming them into cholesterol-laden foam cells [32, 33]. Sirt1 exerts an anti-inflammatory effect by deacetylating RelA/ p65, thus reducing NF-κB signaling and the expression of endothelial adhesion molecules such as ICAM-1 and VCAM-1 [34]. Si et al. reported that niacin attenuated aortic vascular inflammation induced by high fat diet in guinea pigs and lowered the number of macrophages in the arterial wall by ~66%, measured as CD68 positive stained cells. This was also associated with a reduction in NF-κB signaling and other inflammatory markers such as IL-6, TNF-α and CRP [35]. Accordingly, we saw in our study about a 50% reduction in aortic macrophage infiltration (CD68 staining) with the NA-Leu combination, comparable to the full dose NA.

parameters and an overall reduction in arteriosclerotic lesion size [26]. For example, although 100 mg/kg/day atorvastatin feeding in mice for 2 months resulted in a ~45% reduction in total cholesterol, LDL-cholesterol and inflammation, the reduction of atherosclerotic lesion size was only mild and non-significant [26]. The NA-Leu combination in the present study achieved a ~20% reduction in total cholesterol; however, this was also associated with a significant reduction (~50%) in total lipid deposition and total lesion area.

Sirt1 and AMPK are well-known regulators of glucose and lipid metabolism and, via a bidirectional interaction, they stimulate fatty acid oxidation and inhibit hepatic lipid accumulation [27-29]. We have demonstrated that leucine activates Sirt1 by reducing the required concentration for the cofactor NAD$^+$, thus enabling Sirt1 activation particularly under replete metabolic conditions such as obesity which are

Oxidative stress is a causative or exacerbating factor for many human metabolic diseases including atherosclerosis, and thus influences

aging [36]. *C. elegans* is considered a good model to study metabolic disorders since it contains many key components related to metabolic and oxidative stress networks [37]. Paraquat is an oxidative stressor which interferes with the electron transfer in the mitochondrial respiratory chain and thus increases ROS production. The 2 mM paraquat concentration caused a mild oxidative stress in the worms without significantly changing the median survival and total survival curve, although the maximum survival was slightly (but not significantly) increased. Under these conditions, the NA-Leu combination increased significantly the median lifespan and reduced the hazard ratio by 20% in *C. elegans* while there was no difference under normal culture conditions. The NA-Leu combination also increased significantly the protein expression of DAF-16 in *C. elegans*, a transcription factor regulating insulin signaling and leading to lifespan extension under oxidative stress conditions [38, 39].

In vitro experiments with nicotinic acid are generally conducted with concentrations in the µM to mM range [8, 40]. Based on dose-response curves (data not shown), concentrations of NA ≤ 100 nM exerted no effect on the variables studied, and our experimental cell culture concentrations were therefore set below this level at 10 nM to be tested in combination with leucine at a concentration (0.5 mM) that we have previously demonstrated to be attainable in diet or supplement and which has no independent effect. Similarly, we reduced the nicotinic acid concentration in the leucine combination by up to 95% (50 mg/kg diet) in the animal diet with no loss of efficacy on the tested variables compared to the therapeutic dose (1000 mg/day). Moreover, the dose was sixty-fold lower compared to a similar study [23], which used a concentration of 0.3% NA (3 g NA/kg diet) to evaluate the effects of NA on atherosclerosis in LDLRKO mice fed a high fat-atherogenic diet. In this study, ~30% reduction in atherosclerotic lesion size was achieved, while the 50 mg NA-Leu combination in our study resulted in a ~50% reduction.

Limitations

Although the LDLRKO mouse is considered to be a good mouse model for atherosclerosis, it has some limitations. Development of marked

hypercholesterolemia and atherosclerosis in this model requires the feeding of an atherogenic diet. However, the components of these diets are not standardized, a great variety with different concentrations of fat and cholesterol exists, which makes comparisons of results to other studies sometimes difficult [41]. In addition, we did not provide control groups for the NA 50 and 250 mg/kg diet in the absence of leucine; instead, we assumed that these concentrations would not have significant independent effects on the measured parameters since they are far below the therapeutic range of NA in mice, as described above [21-24].

Conclusion

These data demonstrate the amplifying effects of leucine on NA induced effects on atherosclerosis, as depicted in **Figure 8**. This allows a dose reduction of NA of ~95% without loss of efficacy which will lower the incidence of adverse effects. The effects of this combination are associated with activation of the AMPK/Sirt1 pathway.

Disclosure of conflict of interest

None.

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