Pharmacological Stimulation of NADH Oxidation Ameliorates Obesity and Related Phenotypes in Mice

Jung Hwan Hwang1*, Dong Wook Kim1*, Eun Jin Jo4, Yong Kyung Kim1, Young Suk Jo1, Ji Hoon Park3, Sang Ku Yoo4, Myung Kyu Park4, Tae Hwan Kwak4, Young Lim Kho6, Jin Han6, Hueng-Sik Choi7, Sang-Hee Lee2, Jin Man Kim2, InKyu Leq8, Taeyoon Kyung9, Cholsoom Jang9, Jongkyeong Chung9, Gi Ryang Kweon9, Minho Shong1

1Department of Internal Medicine, 2Department of Pathology, 3Department of Biochemistry, Chungnam National University School of Medicine, Daejeon 301-721, Korea.
4Mazence Inc. R&D Center, Suwon 443-813, Korea.
5Department of Environmental Health, Seoul Health College, Sungnam 461-713, Korea.
6Department of Physiology and Biophysics, Inje University College of Medicine, Busan 614-735, Korea.
7Hormone Research Center, Chonnam National University, Kwangju 500-757, Korea
8Section of Endocrinology, Department of Internal Medicine, Kyungpook National University School of Medicine, 50 Samduk-2Ga, Junggu, Daegu, 700-721, Korea
9Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon 305-701, Korea.

*These authors contributed equally to this work.

To whom correspondence should be addressed:
E-mail: MS (minhos@cnu.ac.kr) and GRK (mitochondria@cnu.ac.kr)

ABSTRACT

Objective—Nicotinamide adenine dinucleotides (NAD\textsuperscript{+} and NADH) play a crucial role in cellular energy metabolism, and dysregulated NAD\textsuperscript{+}/NADH ratio is implicated in metabolic syndrome. However, it is still unknown that modulating intracellular NAD\textsuperscript{+}/NADH ratio is beneficial in treating metabolic syndrome. Thus, we tried to determine whether pharmacological stimulation of NADH oxidation provides therapeutic effects in rodent models of metabolic syndrome.

Research Design and Methods—We used β-lapachone (βL), a natural substrate of NADH:quinone oxidoreductase 1 (NQO1), to stimulate NADH oxidation. The βL-induced pharmacological effect on cellular energy metabolism was evaluated in cells derived from NQO1-deficient mice. In vivo therapeutic effects of βL on metabolic syndrome were examined in diet-induced obesity (DIO) and ob/ob mice.

Results—NQO1-dependent NADH oxidation by βL strongly provoked mitochondrial fatty acid oxidation in vitro and in vivo. These effects were accompanied with activation of AMP-activated protein kinase (AMPK) and carnitine palmitoyltransferase (CPT) and suppression of acetyl-CoA carboxylase (ACC) activity. Consistently, systemic βL administration in rodent models of metabolic syndrome dramatically ameliorated their key symptoms such as increased adiposity, glucose intolerance, dyslipidemia, and fatty liver. The treated mice also showed higher expressions of the genes related to mitochondrial energy metabolism (PGC-1α, NRF-1) and caloric restriction (Sirt1), consistent with the increased mitochondrial biogenesis and energy expenditure.

Conclusions—Pharmacological activation of NADH oxidation by NQO1 resolves obesity and related phenotypes in mice, opening the possibility that it may provide the basis for a new therapy for the treatment of metabolic syndrome.
Metabolic syndrome comprises a constellation of specific cardiovascular disease risk factors whose underlying pathophysiology is related to insulin resistance (1). All the components of metabolic syndrome, such as dyslipidemia, high blood pressure, glucose intolerance, liver and muscle fat infiltration, are related to central obesity. It has been reported that the imbalance between energy intake and expenditure is clearly related to obesity and metabolic disorders (2). Accordingly, less calorie intake accompanied by more energy expenditure through exercise is the most effective modalities for the prevention of metabolic syndrome (3). In fact, calorie restriction (4) and increased physical activity have been known to prevent and reverse the phenotypes of metabolic syndrome by activating metabolic regulator proteins such as Sirt1, AMPK, and PPARγ coactivator-1α (PGC-1α) (5-8). Interestingly, the protective effects of these proteins against metabolic syndrome are closely linked to increased mitochondrial functions (9; 10).

Previous studies have indicated that NAD⁺ and NADH are fundamental mediators of energy metabolism (11; 12). Increased intracellular level of NAD⁺ activates Sirt1-dependent metabolic control, mediating the effects of CR in mammals (5). Moreover, pharmacological activation of Sirt1 prevents development of metabolic syndrome through the synergistic activation of AMPK and PGC-1α, which results in elevated mitochondrial respiration (10). Mitochondrial NADH is a critical electron donor in mitochondrial electron transport chain, and cytosolic NADH can be shuttled into mitochondria, thereby affecting mitochondrial oxidative phosphorylation. Based on these ideas, we hypothesized that transiently increased NAD⁺ levels coupled with decreased NADH levels in cytosol provoke mitochondrial oxidative phosphorylation and long-term induction of higher NAD⁺/NADH ratio mimics the effects of CR.

NQO1 is a cytosolic antioxidant flavoprotein that catalyzes the reduction of highly reactive quinone metabolites by using NADH as an electron donor (Figure 1A) (13). A naturally occurring compound βL is a substrate of NQO1 and its treatment to cancer cells results in depletion of NADH and consequent apoptosis (14). ArQule (ARQ) 501, a synthetic version of βL, is a promising anti-cancer drug currently in multiple phase II clinical trials. And its anti-cancer action is based on the findings that cancer cells usually exhibit high level of NQO1 expression. In this study, we investigate the effect of βL on the metabolic syndrome of rodent models to examine whether pharmacological induction of high NAD⁺/NADH ratio is beneficial in treating the key components of metabolic syndrome.

RESEARCH DESIGN AND METHODS

Animal models. All animal procedures were in accordance with the guidelines issued by the Institutional Animal Care and Use Committee of the Chungnam National University School of Medicine. OLETF rats were from the Otsuka Research Institute. Male ob/ob and C57BL/6 mice were from The Jackson Laboratory and housed 4 per cage in a room maintained at constant temperature (25°C) on a light:dark 12:12 h schedule. Four-week-old male C57BL/6 mice were fed a high fat diet (HFD, Research Diets, Inc., 24% (w/w), 45% calories as fat) ad libitum for seven weeks. Groups of mice were untreated, vehicle-treated (calcium silicate), pair-fed, and βL-treated (p.o., micronized particles of βL coated with calcium silicate). Body weight and food intake were measured daily. At the end of experiments, one mouse from each group was anesthetized and examined by MRI. Other mice were dissected and tissue weight was measured.
Antibodies, plasmids and reagents. Anti-AMPKα antibody was purchased from Cell Signalling Technology (for immunoblotting) and Upstate (for immunoprecipitation). Anti-phospho-T172 AMPKα, anti-ACC, and anti-phospho-S79 ACC antibodies were from Cell Signalling Technology. Anti-NQO1 antibody was from Santa Cruz Biotechnology. Anti-OxPhos Complex II subunit antibody was from Molecular Probes. Anti-α-tubulin antibody was from Sigma. pEFires HA-NQO1 and pEFires HA-NQO1C609T plasmids were kindly gifted from Dr. Dad Asher (Weizmann Institute of Science, Israel). Other reagents were purchased from Sigma, Calbiocam, or Amresco.

LC-MS/MS analysis. Cell extracts were prepared in 200 µL lysis buffer containing 80% ice cold methanol/water (MtOH/H2O). The cells were subjected to ultrasonication with a sonic Dismembator (Fisher Scientific, Fairland, NJ) on power setting 3 for 30 S. After centrifugation at 12,000 rpm for 10 min, the supernatant was filtered through a Microcon YM-3 filter (Millipore, Bedford, MA) at 4 °C for 40 min and evaporated down to dryness using a Vacufuge Concentrator before reconstitution in 50 µL of water. The chromatographic system was set up as described in a previous work (15) with minor modifications. The mass spectrometer was operated in the negative ion mode with an electrospray voltage of 4000 V at 300 °C and was supplied by auxiliary gas (30 psi) (15). Quantification was performed with Xcalibur™ software (Thermo Fisher Scientific, USA) using the standard-addition method. Liver samples were extracted by perchloric acid (HClO4) or potassium hydroxide (4) solution to determine adenine, and oxidized and reduced pyridine nucleotide contents, respectively. Electrospray-ionization mass spectrometry was performed in positive ion mode using MDS Sciex API 4000 Triple Quadrupole Mass Spectrometer (Applied Biosystems, Ontario, CA) followed by chromatographic separation on an Agilent 1100 series HPLC system (Agilent technologies, Palo Alto, CA) equipped with an X-Terra MS C18 2.1 × 150 mm, 3.5 µm column (Waters, Milford, Massachusetts, USA) as previously described (16).

Histochemistry and electron microscopy. Mouse tissues were fixed with formalin, dehydrated with ethanol, embedded in paraffin, and cut at a thickness of 5 µM. Sections of epididymal fat, liver, soleus muscle and extensor digitorum longus (EDL) were prepared for haematoxylin and eosin staining. Liver and muscle tissues were prepared as described previously (17) for transmission electron microscopy [Tecnai G2 Spirit Twin (FEI company, USA), Korea Basic Science Institute].

Enzyme assays. Cytoplasmic extracts were prepared from mouse tissues for enzyme assays. NQO1 activity was measured as described previously (14). Briefly, this method measures the decrease in absorbance at 600 nm due to the reduction of 2,6-dichlorophenolindophenol (DCPIP). Total AMPK activity was measured using a synthetic “SAMS” peptide substrate and [γ-32P]ATP as described previously (18). ACC activity was estimated by quantifying the fixation of 14CO2 to acid-stable products (19). CPT activity was measured by the transfer of 14C-carnitine into mitochondria matrix in L6 myoblasts and soleus muscle as described previously (19). HAD activity was measured in L6 myoblasts by monitoring the conversion of acetoacetyl-CoA to L-3-hydroxybutyryl CoA and the concomitant oxidation of NADH to NAD+. The reaction was monitored at 340 nm as described previously (20).

Analysis of fatty acid oxidation and malonyl-CoA concentration. 14C-palmityl-CoA (Perkin Elmer) oxidation by βL was measured in L6 myoblasts and soleus muscle using 14CO2 and 0.2 mL of enhenthonium solution as described previously (20).
Malonyl-CoA was measured by HPLC as described previously (21).

**Analysis of physiological indicators.** O$_2$ consumption was estimated as described previously (22). For indirect calorimetry, individual mice were placed in calorimetry chambers (Oxymax OPTO-M3 system; Columbus Instruments, Columbus, OH) and allowed to adapt for 48 h. During the first 24 h, mice had free access to food and water, and during the second 24 h, the mice had access to water only. Energy expenditure was calculated by measuring O$_2$ consumption and CO$_2$ production every 30 min for 24 h. For cold resistance test, DIO mice were exposed to 4°C ambient temperature for 12 h and rectal temperature was measured for 12 h. Blood samples were collected in heparinized tubes and separated by centrifugation and stored at -20°C for future use. Enzymatic colorimetry was used to quantify triglyceride, total cholesterol, free fatty acid, and glucose (Beckman Instruments, CA). Plasma insulin (Linco Research, MO), TNF$\alpha$ (R&D System), adiponectin (Linco Research, MO), resistin (KOMED) and leptin (Linco Research, MO) were quantified by ELISA as described previously (4; 23-25).

**DNA microarray and quantitative RT-PCR.** Microarray analysis was performed with pooled adipose, liver or muscle tissue from adult male mice treated with vehicle or $\beta$L for 4 weeks. Total RNA was prepared from homogenized tissues using Trizol reagent (Invitrogen, Carlsbad, CA). Probes for microarray analysis were prepared from 10 $\mu$g of total RNA and hybridized to mouse 430A GeneChips (Affymetrix, Santa Clara, CA). The hybridized arrays were scanned and raw data extracted using Microarray Analysis Suite 5.0 (Affymetrix). For quantitative PCR, cDNA was reverse-transcribed from 1 $\mu$g of total RNA with Superscript II and oligo primer (26). The resulting cDNAs were amplified using LightCycler FastStart DNA Master SYBR Green I kit and LightCycler according to the manufacturer's instructions (Roche Diagnostics, Indianapolis, IN). Expression data were normalized to $\beta$-actin

**Immunoblotting.** Total proteins from liver, muscle (EDL and soleus) and indicated cells were extracted in RIPA lysis buffer (500 mM Tris-HCl pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 0.25% Na-deoxycholate, 1 mM PMSF) and content was determined using the Bio-Rad dye binding microassay (Bio-Rad Laboratories, Inc., Hercules, CA). 20 $\mu$g of protein per lane were electrophoresed on a sodium dodecyl sulfate (SDS)-polyacrylamide gel after boiling for 5 min in SDS sample buffer. Proteins were blotted onto Hybond enhanced luminescence membranes (Amersham Pharmacia Biotech, Arlington Heights, IL). After electroblotting, the membranes were blocked with Tris-buffered saline (TBS) and Tween 20 (10 mM Tris-HCl pH 7.4, 150 mM NaCl, and 0.1% Tween 20) containing 5% nonfat dry milk and incubated with the primary antibody diluted in blocking buffer for overnight at 4°C. Membranes were then washed, incubated with the appropriate second antibodies for 1 hour at a room temperature, and rewashed. Blotted proteins were detected by using ECL.

**Statistical analyses.** Results were expressed as the mean ± SD. Differences between groups were examined for statistical significance using Student's $t$ test and analysis of variance (ANOVA). The difference was considered to be significant if $P<0.05$.

**RESULTS**

**Enhanced cytosolic NADH oxidation by NQO1 stimulates cellular energy metabolism.** We screened natural compounds that induce NADH oxidation in the presence of NQO1. Interestingly, $\beta$L and its structurally related compounds; tanshinone 2A and cryptotanshinone rapidly induced NADH oxidation in vitro (Figure S1A). In particular, $\beta$L strongly decreased the fluorescence intensity of NADH in NQO1-
expressing HepG2 cells but not in NQO1-deficient HEK293 cells, suggesting that NQO1 is required for βL-induced NADH oxidation (Figure S1B). Consistently, HEK293 cells transfected with wild-type NQO1 but not catalytically-inactive NQO1 became responsive to βL (Figure S1B). We measured total NAD⁺ and NADH by the LC-MS/MS method (15) in L6 muscle cells treated with different doses of βL. As expected, the NAD⁺/NADH ratio was increased in L6 cells following βL treatment (Figure 1B). These results indicate that βL induces NADH oxidation only in the presence of NQO1 activity.

Because the intracellular NAD⁺/NADH ratio indicates the energy status of cells (27; 28), βL-induced NADH oxidation may stimulate AMPK signaling pathway, a well-known energy sensing pathway activated under energy depletion (6; 7; 29). In fact, βL treatment dramatically induced the activating phosphorylation in the catalytic subunit of AMPKα (AMPKα T172) within 30 min (Figure 1C, S2A). Moreover, AMPK-dependent inhibitory phosphorylation of acetyl-CoA carboxylase (ACC S79) was also strongly induced in βL-treated cells (Figure 1C, S2A). To examine whether βL-induced AMPK activation requires NQO1 activity in vivo, we generated NQO1 knockout mice and treated βL into MEFs isolated from the heterozygous (+/-) and homozygous (-/-) knockout mice. Strikingly, βL-induced phosphorylations of AMPK and ACC were not observed in NQO1-deficient MEFs but observed in NQO1+/− MEFs (Figure 1D, E). Consistent with these immunoblot results, βL treatment modulated AMPK and ACC activities only in NQO1+/− MEFs but not in NQO1−/− MEFs (Figure 1D, E). Furthermore, NQO1-specific inhibitors, dicoumarol and ES936, completely blocked βL-induced AMPK phosphorylation (Figure S2B). These results collectively indicate that βL induces AMPK activation specifically via NQO1.

Because AMPK activation and concomitant ACC inhibition stimulate fatty acid oxidation (30; 31), we measured the activities of two representative metabolic enzymes regulating mitochondrial fatty acid oxidation. Intriguingly, the activities of carnitine palmitoyltransferase (CPT) and 3-hydroxyacyl-CoA dehydrogenase (HAD) were significantly increased in βL-treated L6 myoblast cells (Figure 1F, G). Consistently, mitochondrial fatty acid oxidation measured by 13C-palmitoyl-CoA oxidation was substantially elevated in βL-treated cells (Figure 1H). These results suggest that βL-induced NADH oxidation activates mitochondrial fuel metabolism by stimulating AMPK signaling pathway.

**Pharmacological NADH oxidation stimulates activation of AMPK and fatty acid oxidation in vivo.** To investigate the in vivo effects of βL, we examined βL-induced metabolic changes in diet-induced obesity (DIO) mice. Consistent with the cell data, βL treatment highly increased NAD⁺/NADH ratio in the liver of DIO mice (Figure 2A). Moreover, βL treatment substantially increased the phosphorylations of AMPK and ACC, and the activity of AMPK in the liver, extensor digitorum longus (EDL), and soleus muscle of DIO mice (Figure 2B-E). We also monitored several enzymatic activities involved in mitochondrial fatty acid oxidation in the soleus muscle of βL-treated DIO mice. Consequently, βL treatment significantly reduced the activity of ACC and the level of its metabolic product malonyl-CoA (32; 33) in the soleus muscle (Figure 2F, G). In contrast, the activities of CPT and HAD were highly increased in βL-treated mice compared to those of vehicle-treated mice (Figure 2H, I). Consistently, the rate of mitochondrial fatty acid oxidation was much higher in βL-treated mice (Figure 2J). These data indicate that increased NADH oxidation by βL treatment stimulates AMPK signaling pathway and mitochondrial fatty acid oxidation in vivo.
βL reverses the obesity and related phenotypes in DIO mice and ob/ob mice.

To examine whether βL-induced NADH oxidation has a therapeutic potential in treating metabolic syndrome, we systematically treated DIO mice with βL for 8 weeks (Figures 3, S3). Surprisingly, the body weight of βL-treated mice continuously decreased throughout the treatment, while that of the control mice slightly increased (Figure 3A). Interestingly, the food intake of βL-treated mice decreased during the first 2 weeks of treatment and then returned to the normal state (Figure 3B). Because βL-treated mice exhibited progressive weight loss even after returning to the normal state of food intake (Figure 3A, B). To observe the role of decreased food intake in the early phase of βL administration, we compared the body weight of the βL-treated group with that of the pair-fed group. In Fig. 3C, the treated group (n=8) and pair-fed group (n=8) showed 24.9 ± 7.36 % and -3.4 ± 7.86 % weight loss compared to the base line weight. A comparison of the weights of the treated and pair-fed groups to the vehicle group, showed that βL-treated and pair-fed groups had weight loses of 33.6 ± 5.78 % and 10.5 ± 3.00 %, respectively; thus, about 23% of total weight loss can be attributed to the peripheral actions of βL (Figure 3C, S3). These finding suggest that a major factor governing body weight loss in βL-treated mice is likely to be increased energy expenditure rather than decreased food intake.

Consistent with the decreased body weight, magnetic resonance imaging (MRI) of the coronal and transverse sections of βL-treated DIO mice showed dramatic decreases in subcutaneous and visceral adipose tissues (Figure 3D). Moreover, the weights of subcutaneous, mesenteric, perirenal, and gonadal fats in βL-treated DIO mice were considerably decreased (Figure S4), implicating that their body weight loss is due to the reduced adipose tissues. After analysing various indicators of glucose and fat metabolism in the sera of βL-treated DIO mice, we found overall decreased amounts of triglyceride, cholesterol, free fatty acid, glucose, insulin, adiponectin, resistin, and leptin in βL-treated DIO mice (Table 1). Notably, the adipocyte marker perilipin staining revealed that the size of lipid droplets in the epididymal fat cells was much smaller in βL-treated DIO mice (Figure 3E). Moreover, oil red O staining in the liver showed that βL treatment considerably suppressed the liver steatosis in DIO mice (Figure 3F). All these dramatic metabolic changes, including reduced body weight and adipocyte size, and decreased hepatic steatosis were similarly observed in βL-treated ob/ob mice (Figure 4A-D). Consistently, βL treatment resulted in enhanced glucose disposal rates and improved insulin sensitivity in both DIO mice and Otsuka Long Evans Tokushima Fatty (OLETF) type 2 diabetic model rats (Figure S5). Therefore, we conclude that βL treatment can alleviate the key symptoms of metabolic syndrome in the rodent models with obesity and diabetes.

Pharmacological NADH oxidation modulates expression of genes involved in metabolism and mitochondrial functions.

To further understand the underlying mechanisms of βL-induced metabolic improvements, we performed genome-wide microarray analyses in the liver, muscle, and adipose tissues of βL-treated DIO mice. Intriguingly, genes involved in metabolism and mitochondrial functions were differentially expressed in DIO mice exposed to βL, and quantitative RT-PCR experiments also verified these results (Table 2). For example, βL treatment strongly induced PGC-1α and nuclear respiratory factor-1 (NRF-1), the master regulators of mitochondrial biogenesis (34; 35), in the liver and muscle of DIO mice. In addition, mitochondrial metabolic genes (COX4 and COX7) were
also upregulated in the muscle of βL-treated DIO mice. Interestingly, Sirt1 and Sirt3, which are known to be activated in response to CR (5; 36; 37), were also highly induced in the muscle of βL-treated mice. Moreover, genes related to lipolysis (LPL and ATGL) and glucose uptake (GLUT4) were upregulated in the adipose tissue of βL-treated DIO mice. However, fatty acid synthase (FASN) and peroxisome proliferator-activated receptor gamma (PPARγ) that play key roles in adipogenesis and fatty acid synthesis (38) were downregulated in βL-treated DIO mice. These results strongly suggest that βL-induced NADH oxidation stimulates mitochondrial biogenesis and functions, and also enhances energy metabolism by controlling global gene expression related to cellular metabolism.

**Long-term pharmacological NADH oxidation stimulates mitochondrial biogenesis and energy expenditure.** Since several genes affected by βL treatment take parts in mitochondrial remodelling and biogenesis (34; 39; 40), we examined the physiological and ultrastructural changes of mitochondria in βL-treated DIO mice. As a result, mitochondria in the liver of untreated DIO mice appeared to be swollen, distorted, and deficient in cristae and matrix (Figure 5A, B). However, DIO mice treated with βL for 8 weeks showed an increased number of mitochondria with normal morphologies, such as well-organized cristae and matrix structures (Figure 5A, B). Furthermore, the mitochondria collected from the soleus muscle in βL-treated DIO mice exhibited higher levels of mitochondrial DNA and complex II subunit expression compared to those in untreated control, suggesting that βL treatment actively induced mitochondrial biogenesis (Figure 5C). Consistently, the number of fibers in the soleus muscle that showed strong ATPase staining intensity and intermyofibrillar mitochondria was increased in βL-treated DIO mice (Figure 5D).

To further characterize the physiological effects of mitochondrial restoration in βL-treated DIO mice, we measured two metabolic indices: respiratory oxygen consumption and cold-resistance. The quantity of resting oxygen uptake (VO₂) was markedly increased after adjusting for body weight in βL-treated DIO mice (Figure 5E), whereas the ratio of night respiratory exchange was decreased (0.77, βL-treated mice; 0.82, control mice; P<0.005, data not shown). However, VO₂ that had not been adjusted for body weight showed no statistical significance between control and βL-treated groups. Thus, adipose tissue mass, which is profoundly decreased by βL treatment, may be an important denominator for the analysis of resting VO₂. In addition, βL-treated DIO mice displayed much stronger cold-resistance than the control mice (Figure 5F). More importantly, βL treatment significantly increased energy expenditure of DIO mice (Figure 5G). Overall, these results propose that increases in mitochondrial integrity, oxygen consumption, and energy expenditure are the underlying mechanisms of βL in assuaging the metabolic symptom of DIO mice.

**DISCUSSION**

NAD⁺ and its derivatives are classic metabolites involved in energy metabolism (11). Recent evidence suggests that NAD⁺ is a central regulator in cellular energy metabolism. Moreover, the importance of cellular NAD⁺ levels has been highlighted by the discovery of SIR2-related proteins (sirtuins) that mediate CR responses in yeast and mammals (41). Yeast Sir2 and a mammalian orthologue sirtuin, Sirt1, are NAD⁺-dependent deacetylases that are regulated by the NAD⁺/NADH ratio (5). Furthermore, accumulating evidence has suggested that NAD⁺/NADH ratios are altered in various pathological conditions, such as neurodegeneration, diabetes and oxidative
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stress (28; 42). In fact, the pharmacological activation of NAD⁺-dependent SirT1 compensates for the effects of a high calorie diet (10). Based on these observations, pharmacological induction of higher NAD⁺/NADH would be expected to ameliorate metabolic syndrome.

NQO1 is a flavoenzyme that utilizes NAD(P)H as an electron donor to catalyze the reduction of substrates. Previous studies and our data suggest that βL is a specific and high affinity substrate of NQO1 in vitro and in vivo (14). Therefore, βL would have the propensity to induce a higher NAD⁺/NADH ratio. More interestingly, the expression of NQO1, a target of βL, is highly upregulated in liver, muscle and fat tissues which are important for the regulation of whole body energy metabolism and insulin sensitivity (Fig. S6). The promoter of the NQO1 gene has an antioxidant response element that serves as the binding motif for the factor Nrf2 which is activated by ROS stimuli. The increased ROS stimulation in metabolically active tissues under obesity and diabetic conditions may activate Nrf2 and NQO1 gene expression. Consistently, Palming et al (43) found that human adipocytes have high NQO1 expression and that NQO1 expression levels were positively correlated with adiposity, glucose tolerance and markers of liver dysfunction. Taken together, NQO1 would appear to be a useful pharmacological target for the induction of higher NAD⁺/NADH ratios in obesity and diabetic models.

In this study, we have shown that the enhanced NADH oxidation induced by βL in NQO1-present cells increases phosphorylation and activation of AMPK. The reduced cytosolic NADH levels induced by βL treatment may disturb the proper distribution of NADH reducing equivalents in mitochondrial complexes for energy production, suggesting that βL may transiently increase AMP levels and activate AMPK to compensate for cellular energy depletion (10; 36; 44). Two upstream kinases, LKB1 and CaMKK, are involved in the phosphorylation of the catalytic subunit of AMPK (45; 46). We tested the effects of βL on AMPK phosphorylation in LKB1-deficient MEFs. AMPK phosphorylation was only observed at very early time points (30 seconds to 5 min) after treatment with βL in LKB1-deficient cells (data not shown). This pattern of AMPK activation is quite different from that observed in cells with normal LKB1 expression (e.g., Fig. S2A), in which βL resulted prolonged AMPK phosphorylation. The CaMKK inhibitor (STO609) completely abolished the early phase of AMPK phosphorylation (data not shown). These findings strongly suggest that both LKB1 and CaMKK are required for full activation of AMPK under βL-induced AMPK phosphorylation conditions. Activation of AMPK led to increased fatty acid oxidation by inactivating ACC phosphorylation. Consistently, βL-mediated AMPK activation induces ACC phosphorylation and activation of CPT and HAD, resulting in beneficial fatty acid oxidation. Taken together, these findings suggest that pharmacological stimulation of NADH oxidation leads to increased mitochondrial fatty acid oxidation.

We additionally examined whether βL-induced NADH oxidation could be used as therapy for treating obesity, a major component of metabolic syndrome. Pair-fed studies (Fig. 3C, S3) clearly demonstrated that loss of weight induced by βL is mediated by both central and peripheral actions. However, although a decrease of food intake after βL treatment was noted in the early phase of treatment, food intake recovered with continued βL administration. We found that intracranial administration of βL in mice resulted in a dramatic decrease in feeding behaviour (data not shown); however it is unclear which mechanism in the hypothalamus mediates the βL-induced decrease in food intake. Long-term weight
loss induced by βL is mainly due to the peripheral action of βL and its weight reducing effect, which are accompanied by a smaller fat mass, lower fatty infiltration in the liver, decreased levels of plasma insulin, triglyceride and glucose, and increased glucose disposal rates following insulin treatment. Collectively, these results suggest that the increased NADH oxidation induced by βL could lead to an amelioration of key components of metabolic syndrome in rodent models with obesity and diabetes.

The reversal of obesity and other phenotypes of DIO and ob/ob mice by βL is associated with the normalization of disorganized mitochondrial ultrastructure and increased numbers of mitochondria. Mitochondrial dysfunctions are frequently associated with obesity and diabetes, and improvements in obesity and glucose tolerance are associated with enhanced mitochondrial oxphos functions and biogenesis (47; 48). Interestingly, an examination of the gene expression patterns following βL treatment showed increased expression of PGC-1α and SirT family genes in liver, muscle, and adipose tissues. Prolonged AMPK activation is known to increase PGC-1α expression, SirT1 activity, and mitochondrial biogenesis. Taken together, it is plausible that AMPK activation by βL induces PGC-1α, a master regulator of mitochondrial biogenesis, and Sirt1, a mediator of CR effects, which promote higher mitochondrial biogenesis and energy expenditure in mice. The resting VO2 was found to be higher in the βL-treated group than in the vehicle group after adjusting for body weight. However, this finding does not necessarily indicate that βL treatment increases resting metabolic rate because changes of body weight, including fat mass, may affect resting VO2. To find out the effects of βL treatment on metabolic rate, additional analysis of submaximal and maximal VO2 with or without adjustments for body composition will be required. Therefore, the consequences of long-term physiological adaptations on enhanced NADH oxidation following βL treatment will require further study to prove the potential of βL as a new treatment for obesity and diabetes.

Previous studies showed that βL inhibits tumorigenesis, and that NQO1 mediates this effect of βL (13; 14). Interestingly, the cytotoxic effect of βL was prominently observed in cultured cancer cells, whereas it was barely detectable in normal primary cultured cells (Figure S7) (49). Although primary cultured MEFs showed strong resistance to βL cytotoxicity, approximately 10% of the MEFs underwent cell death (Figure S7, S8). However, this phenomenon was completely suppressed by adding palmitate to the culture medium (Figure S8), suggesting that increased palmitate oxidation compensates βL cytotoxicity. Taken together, these data suggest that βL-induced cell death occurs selectively in cancer cells that exhibit reduced mitochondrial oxidative phosphorylation. Even though the systemic toxicity of βL in humans has not been fully established, ARQ501, a synthetic version of βL, has been subjected to phase I and II clinical trials and only mild adverse effects such as anemia and fatigue have been observed. Nonetheless, it is indispensable to evaluate the systemic toxicity of βL using different administration routes, formulas and exposure times.

ACKNOWLEDGEMENTS

We thank Young Mi Kang, Ki Nam Min, and Jong Kuk Park for technical assistances and animal cares. This work was supported by KOSEF(M10753020001-07N5302-00110), Ministry of Education, Science and Technology, and MarineBio Research grant(B10400207A290100210), Ministry of Maritime and Fisheries and KT&G grant, Korea. JHH, and YKK were supported by the second phase of BK21 program, Ministry of
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Education, Korea. IKL was supported by National Research Laboratory program from KOSEF, Ministry of Education, Science and Technology, Korea. JC was supported by a National Creative Research Initiatives grant (M01080206) from KOSEF, Ministry of Education, Science and Technology, Korea.

REFERENCES

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Table 1. Metabolic parameters in mice treated with vehicle or βL

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle</th>
<th>βL</th>
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<tr>
<td>Triglyceride (mg/dL)</td>
<td>80.8±10.57</td>
<td>66.3±7.66*</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>219.8±52.84</td>
<td>140±19.34**</td>
</tr>
<tr>
<td>Free fatty acid (uEq/L)</td>
<td>1085±174.1</td>
<td>487±69.26**</td>
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<tr>
<td>Adiponectin (ng/mL)</td>
<td>21.74±4.63</td>
<td>8.4±1.02**</td>
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<tr>
<td>Resistin (ng/mL)</td>
<td>1.458±0.23</td>
<td>1±0.27**</td>
</tr>
<tr>
<td>TNFa (pg/mL)</td>
<td>19.88±4.03</td>
<td>22.82±7.1</td>
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<tr>
<td>Leptin (ng/mL)</td>
<td>128.8±13.85</td>
<td>33.47±17.56**</td>
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<tr>
<td>Glucose (mg/dL)</td>
<td>232.8±15.78</td>
<td>133.3±33.66**</td>
</tr>
<tr>
<td>Insulin (ng/dL)</td>
<td>4.1±1.49</td>
<td>1.68±0.95**</td>
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After oral administration with vehicle (n=5) or βL (n=6) for 4 weeks, metabolic parameters were analyzed. The values indicate the average of each group. *P<0.05, **P<0.005 for βL-treated group versus vehicle control group.
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Table 2. Expression of genes related to energy metabolism and mitochondrial functions in the liver, muscle, and white adipose tissue.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Genes</th>
<th>Relative mRNA levels (βL/vehicle group)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>PGC1α (peroxisome proliferative activated receptor, gamma, coactivator 1 alpha)</td>
<td>3.46±2.1*</td>
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<tr>
<td></td>
<td>NRF-1 (nuclear respiratory factor-1)</td>
<td>1.88±0.38*</td>
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<td></td>
<td>mtTFA (transcription factor A, mitochondrial)</td>
<td>1.32±0.32</td>
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<tr>
<td></td>
<td>CPT1 (carnitine palmitoyltransferase 1)</td>
<td>1.56±0.29*</td>
</tr>
<tr>
<td></td>
<td>UCP2 (uncoupling protein 2)</td>
<td>3.06±0.67**</td>
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<tr>
<td></td>
<td>AMPKα1 (AMP-activated protein kinase 1 alpha)</td>
<td>1.84±0.64*</td>
</tr>
<tr>
<td></td>
<td>AMPKα2 (AMP-activated protein kinase 2 alpha)</td>
<td>1.42±0.39</td>
</tr>
<tr>
<td></td>
<td>PPARγ (peroxisome proliferator activated receptor gamma)</td>
<td>1.00±0.25</td>
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<tr>
<td>Muscle</td>
<td>PGC1α</td>
<td>1.70±0.31*</td>
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<tr>
<td></td>
<td>NRF-1</td>
<td>1.60±0.42*</td>
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<tr>
<td></td>
<td>mtTFA</td>
<td>0.81±0.45</td>
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<tr>
<td></td>
<td>CPT1</td>
<td>1.07±0.48</td>
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<td></td>
<td>COX4 (cytochrome c oxidase subunit IV isoform)</td>
<td>2.18±0.45*</td>
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<td></td>
<td>COX7 (cytochrome c oxidase subunit VII)</td>
<td>1.69±0.36</td>
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<td>GLUT2 (glucose transporter 2)</td>
<td>5.08±4.55</td>
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<tr>
<td></td>
<td>GLUT4 (glucose transporter 4)</td>
<td>1.45±0.89</td>
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<tr>
<td></td>
<td>PPARα (peroxisome proliferator activated receptor alpha)</td>
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<td>AMPKα1</td>
<td>2.80±1.71</td>
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<td>AMPKα2</td>
<td>1.69±1.33</td>
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<td></td>
<td>SIRT1 (sirtuin 1)</td>
<td>2.05±0.44*</td>
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<td>SIRT3 (sirtuin 3)</td>
<td>3.33±0.84*</td>
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<td></td>
<td>UCP2</td>
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<td>UCP3 (uncoupling protein 3)</td>
<td>2.78±1.75</td>
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<td>Gonadal fat</td>
<td>PGC1α</td>
<td>2.02±0.3**</td>
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<tr>
<td>(WAT)</td>
<td>NRF-1</td>
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<td>FASN (fatty acid synthase)</td>
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<td>LPL (lipoprotein lipase)</td>
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<td>ATGL (adipose triglyceride lipase)</td>
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<td>SCD1 (stearoyl-Coenzyme A desaturase 1)</td>
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<td>AOX (acyl-CoA oxidase)</td>
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<td>GLUT2</td>
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<td>GLUT4</td>
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<td>PPARα</td>
<td>2.36±0.32**</td>
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<td>PPARγ</td>
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<td>SIRT1</td>
<td>1.45±0.18**</td>
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<tr>
<td></td>
<td>SIRT2 (sirtuin 2)</td>
<td>1.33±0.15**</td>
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<tr>
<td></td>
<td>SIRT3</td>
<td>4.33±0.91*</td>
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</table>

After oral administration with vehicle (n=5) or βL (n=6) for 4 weeks, relative mRNA levels in the indicated tissues were analyzed by quantitative RT-PCR. The values indicate relative mRNA levels of βL-treated groups compared to those of vehicle control groups. *P<0.05, **P<0.005 for βL-treated group versus vehicle control group.
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Figure 1. βL-induced NADH oxidation stimulates AMPK signaling pathway and mitochondrial fuel metabolism in NQO1-expressing cells.

(A) Schematic illustration of NQO1-mediated chemical reaction. NADH is oxidized by providing two electrons to βL, which is catalyzed by NQO1.

(B) L6 myoblasts were treated with indicated concentration of βL for 30 min and extracted in lysis buffer containing 80% ice cold methanol/water (MeOH/H2O). Representative NAD+/NADH ratios were determined by LC-MS/MS analysis as materials and methods. All experiments were performed in triplicate.

(C) βL-induced phosphorylations of AMPKα and ACC. Immunoblot analyses of the lysates from MEF treated with βL (5 μM) for the indicated time periods were performed with antibodies against the indicated proteins.

(D) Phosphorylation and activation of AMPK by βL in NQO1+/+ and NQO1−/− MEFs. Phosphorylation of AMPKα was estimated by immunoblot analyses of the lysates from NQO1+/+ and NQO1−/− MEF treated with βL (5 μM) or DMSO for 30 min, and the activity of AMPK was measured by using the SAMS peptide assay. Error bars indicate SD.

(E) Phosphorylation of ACC was estimated by immunoblot analyses of the lysates from NQO1+/+ and NQO1−/− MEF treated with βL (5 μM) or DMSO for 30 min and the activity of ACC was determined by quantifying the fixation of 14CO2 to acid-stable products.

(F to H) Comparison of CPT (F), HAD (G) activities, or 14C-palmitoyl-CoA oxidation (H) between DMSO- and βL-treated L6 myoblasts. Error bars indicate SD (*P <0.05; **P<0.005).
Figure 2. βL activates AMPK signaling pathway and fatty acid oxidation in vivo.

(A) NAD⁺/NADH ratio was calculated in the liver of male mice treated intravenously with vehicle (n=5; open bars) or 5 mg/kg βL (n=5; close bars) for the indicated time (*P<0.05).

(B to D) Effects of βL on phosphorylations of AMPK and ACC in vivo. DIO mice were dosed orally with vehicle (n=3) or 50 mg/kg βL (n=3). After treatment of βL for 2 hours, extracts from liver (B), extensor digitorum longus (EDL) (C), and soleus muscle (D) were immunoblotted with antibodies against the indicated proteins.

(E) Comparison of the AMPK activity in the three indicated tissues of DIO mice dosed orally with vesicle- and 50 mg/kg βL for 2 hours (n=5, respectively).

(F and G) Comparisons of the ACC activity (F) and malonyl-CoA quantity (G) in the soleus muscle of DIO mice dosed orally with vesicle- and 50 mg/kg βL for 2 hours (n=5, respectively).

(H to J) βL stimulates mitochondrial energy metabolism in DIO mice. Comparisons of the CPT (H), HAD activities (I), or 14C-palmityl-CoA oxidation (J) in vehicle- and 50 mg/kg βL-treated DIO mice (n=5, respectively). Mice used for all the experiments were male (*P<0.05; **P<0.005).
Figure 3. βL treatment ameliorates the metabolic symptoms of DIO mice.

(A and B) The body weights (A) and the ratios of food intake to body weight (B) of the three DIO mice groups, untreated (□; n=30), vehicle-treated (△; n=42), or βL-treated (▼; n=48) group, were monitored during the oral administration of 50 mg/kg/day βL for 8 weeks.

(C) The body weight of three mouse groups, untreated (□; n=6), pair-fed (△; n=8), and 50 mg/kg/day βL-treated (▼; n=8) group, were monitored for 8 weeks after oral administration of βL (*P<0.05; **P<0.005).

(D) Representative MRI of the coronal (upper) and transverse (lower) sections in the four indicated mice groups. DIO mice were orally administrated with vehicle or 50 mg/kg/day βL for 8 weeks. Lean mice were used as control for a normal fat concentration.

(E) The sizes of lipid droplets in gonadal fat (GF) were compared between DIO mice treated with vehicle (left) and 50 mg/kg/day βL (right) for 4 weeks. Immunostaining with anti-perilipin antibody (red) was used to measure the size of lipid-droplets.

(F) Oil-red O staining (red) in the liver from DIO mice treated with vehicle (upper) or 50 mg/kg/day βL (lower) for 4 weeks was used to evaluate hepatic steatosis. Mice used for all the experiments were male (*P<0.05; **P<0.005).
Figure 4. The effects of βL on various metabolic symptoms in ob/ob mice
(A and B) The body weight (A) and the ratio of food intake to body weight (B) of three mouse groups, untreated (□; n=4), vehicle-treated (△; n=8), and 200 mg/kg/day βL-treated (▽; n=8) group, were monitored for 8 weeks after oral administration of βL (*P<0.05; **P<0.005).
(C) The photographic images of lean, vehicle-treated, and 200 mg/kg/day βL-treated ob/ob mice after 8 weeks of treatment.
(D) Tissue sections of the liver (upper) and gonadal fat (GF, lower) of ob/ob mice treated with vehicle (left) or 200 mg/kg/day βL (right) for 8 weeks were stained with haematoxylin and eosin (upper) and anti-perilipin antibody (lower), respectively.
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Figure 5. Long-term treatment of βL stimulates mitochondrial remodelling and biogenesis and increases energy expenditure.

(A and B) Transmission electron microscopic images of the liver (A) and soleus muscle (B) of DIO mice treated with vehicle or 50 mg/kg/day βL for 8 weeks. N: nucleus, L: lipid droplets, C: cytosol, M: mitochondria.

(C) Immunoblot analysis against complex II subunit (upper) and quantitative RT-PCR analysis for mitochondrial DNA (mtDNA) contents (lower) were performed in the soleus muscle of DIO mice treated with vehicle (n=5) or 50 mg/kg/day βL (n=5). α-Tubulin was used as loading control.

(D) pH-sensitive ATPase staining (pale purple) of histological sections was performed in the soleus muscle of DIO mice treated with vehicle (left) and βL (right).

(E and F) Oxygen consumption (VO₂) (E) and cold-resistance (F) were compared between DIO mice treated with vehicle (□; n=5) and 50 mg/kg/day βL (◇; n=5) for 8 weeks.

(G) Comparison of the energy expenditure between DIO mice treated with vehicle and βL. Mice used for all the experiments were male (*P<0.05; **P<0.005).