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Long-term treatment with nicotinamide induces glucose intolerance and skeletal muscle lipotoxicity in normal chow-fed mice: compared to diet-induced obesity☆

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Abstract

Nicotinamide (NAM), or vitamin B3, is an essential coenzyme for ATP synthesis and an inhibitor of sirtuin 1. Recently, conflicting results were reported regarding the treatment of NAM in type 2 diabetes and obesity. The aim of this study was to determine whether and how long-term treatment with NAM at lower dose would affect insulin sensitivity in mice fed chow diet. We treated mice with NAM (100 mg/kg/day) and normal chow for 8 weeks. Strikingly, NAM induced glucose intolerance and skeletal muscle lipid accumulation in nonobese mice. NAM impaired mitochondrial respiration capacity and energy production in skeletal muscle, in combination with increased expression of the mediators for mitophagy (p62, PINK1, PARK2 and NIX) and autophagy (FOXO3, Bnip3, CTSL, Beclin1 and LC-3b). Next, we treated mice with high-fat diet (HFD) and resveratrol (RSV; 100 mg/kg/day) for 8 weeks. RSV protected against HFD-induced insulin resistance and obesity. HFD increased skeletal muscle lipid content as well as NAM, but this increase was attenuated by RSV. In contrast to NAM, HFD enhanced fatty acid oxidative capacity. Muscle transcript levels of genes for mitophagy and autophagy were largely suppressed by HFD, whereas RSV did not rescue these effects. These differences suggest that skeletal muscle autophagy may represent adaptive response to NAM-induced lipotoxicity, whereas reduced autophagy in skeletal muscle may promote HFD-induced lipotoxicity. Our results demonstrate that chronic NAM supplementation in healthy individuals, although at lower dose than previously reported, is still detrimental to glucose homeostasis and skeletal muscle lipid metabolism.

Keywords: Nicotinamide (NAM); Sirtuin 1; Insulin resistance; Lipid accumulation; Skeletal muscle; Mitochondrial; Mitophagy

1. Introduction

Sirtuin 1 (SIRT1), a NAD-dependent histone deacetylase, plays a positive role in metabolic pathway through its deacetylase activity for a number of substrates involved in glucose or lipid metabolism [1,2]. As a known activator of SIRT1, resveratrol (RSV) improves mitochondrial function and protects against diet-induced obesity and insulin resistance [3]. SIRT1 is required for the beneficial effects of RSV on mitochondrial function [4]. Nicotinamide (NAM), a form of vitamin B3 and a NAD+ precursor, is a potent inhibitor of SIRT1 [5]. NAM physically inhibits SIRT1 deacetylation activity and increases the levels of acetylated histone and p53 [6]. Previously, NAM was observed to have preventive and therapeutic effects on type 1 diabetes and protected β-cell against the toxic effects of alloxan and streptozocin [7–9]. Evidence from animal and human studies suggested that NAM can be used as a dietary micronutrient to protect against the development of insulin-dependent diabetes mellitus (IDDM). Recent studies indicated that NAM treatment (100 mg/kg, 4 weeks) improved glucose metabolism in a rodent model of obesity and type 2 diabetes [10]. Nicotinamide riboside (NR) can be used as a natural vitamin to enhance oxidative metabolism, protect against diet-induced obesity and ameliorate metabolic and age-related disorders characterized by defective mitochondrial function [11]. These findings indicate that NAM is an antidiabetic vitamin for both type 1 and type 2 diabetes. If so, there may be a paradox in the regulation of SIRT1. In contrast to NAM as an inhibitor of SIRT1, RSV is a SIRT1 activator used for improving insulin resistance and mitochondrial function [3]. It is noteworthy that NAM is also a substrate for nicotinamide *N*-methyltransferase (NNMT), which methylates NAM to produce *N*(1)-methylnicotinamide (MNAM). Recent studies have suggested that NNMT activity and plasma MNAM levels are linked to diet-induced obesity and diabetes in rodents and humans [12–14]. Thus, the metabolic effects of NAM supplementation are very likely not only the result of the inhibition of SIRT1 activity. Another paradox is due to

Abbreviations: BSA, bovine serum albumin; DAG, diacylglycerol; FA, fatty acids; FFA, free fatty acids; GTT, glucose tolerance tests; HFD, high-fat diet; IDDM, insulin-dependent diabetes mellitus; IPGTT, intraperitoneal glucose tolerance tests; ITT, insulin tolerance tests; MNAM, *N*(1)-methylnicotinamide; MRI, magnetic resonance imaging; mtDNA, mitochondrial DNA; NAM, nicotinamide; NNMT, nicotinamide *N*-methyltransferase; NR, nicotinamide riboside; RSV, resveratrol; SIRT1, sirtuin 1; T2DM, type 2 diabetes mellitus.

☆ Disclosure Statement: The authors have nothing to disclose.

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the dosage of NAM treatment. Previous studies showed that NAM administration (2 g/day, 2 weeks) caused a decrease in insulin sensitivity in IDDM patients despite increased insulin secretion, suggesting that the use of NAM in IDDM may lead to insulin resistance in humans [15]. Animal study also reported that NAM supplementation (4 g/kg, 8 weeks) induced detrimental metabolic changes in young rats, as evidenced by increased hepatic DNA damage and impaired glucose tolerance and insulin sensitivity [16]. Cumulative administration of NAM (2 g/kg) promoted the development of type 2 diabetes mellitus (T2DM) [17]. Excess NAM intake leads to homocysteine-related cardiovascular disease [18]. The antidiabetic role of NAM as a vitamin and the associated underlying mechanisms need to be further understood.

Lipotoxicity has been defined as the accumulation of lipid in nonadipose tissue, leading to cellular dysfunction and death. However, triglyceride (TAG) within skeletal muscle is thought to be the best fuel for healthy muscle during exercise [19], although intramuscular TAG is an important contributor to obesity, insulin resistance and type 2 diabetes. The content of skeletal muscle lipids is known as a marker of insulin resistance, while these neutral triglycerides are not thought to be harmful [20,21]. Intramuscular lipid content was increased in obese subjects with T2DM while glycogen stores were simultaneously reduced [22], suggesting a pathway to modulate oxidative substrate selection and induce muscle insulin resistance. Surprisingly, recent findings demonstrate that modulation of oxidative substrate selection to increase muscle glucose utilization also results in muscle insulin resistance [23]. Insulin resistance did not increase proportionally to fat mass or lipid accumulation in obese and nonobese subjects with T2DM [24]. Thus, the balance between storage and utilization of TAG is critical for understanding the role of skeletal muscle lipid accumulation in insulin resistance. Mechanistically, reduced skeletal muscle mitochondrial activity results in ectopic lipid accumulation and reduced glucose tolerance in elderly adults, independent of obesity [25]. Skeletal muscle fibers differentiate into specific fiber types with distinct metabolic properties determined by their oxidative substrates. Mitochondrial quality control is mostly determined by mitochondrial biogenesis, fusion and fission and mitochondrial autophagy (*i.e.* mitophagy) [26,27]. PGC-1α is a master nuclear coactivator to mediate skeletal muscle mitochondrial biogenesis and enhance lipid-supported mitochondrial respiration [28,29]. Conversely, nuclear corepressors NCoR1 and RIP140 have been documented to antagonize the transcription activation of PGC-1α and inhibit muscle mitochondrial biogenesis and oxidative metabolism [30,31]. In addition, previous studies have investigated the importance of autophagy in metabolic regulation. Skeletal muscle autophagy is essential for muscle mass, mitochondrial function and insulin sensitivity [32]. Induction of autophagy decreased lipid accumulation and increased oxidative phosphorylation in hepatic cells. Rapamycin treatment induced autophagy and decreased hepatic TAG and glycogen content, as well as corrected hepatic lipid accumulation [33]. Endothelial autophagy is critically important for limiting lipid accumulation within the vessel wall. Induction of autophagy prevented the age-dependent decline in autophagic flux and treated atherosclerosis [34]. These findings had linked autophagy to ectopic lipid accumulation and the related diseases.

Ecological studies have shown that increased B vitamin consumption is strongly correlated with the prevalence of obesity and diabetes [35]. Although previous studies emphasized the beneficial or detrimental effects of NAM in treatment of obesity and type 2 diabetes, the understanding of how NAM modulates lipid metabolism has not been directly addressed *in vivo*. To examine this question, we sought to determine whether long-term treatment with NAM (100 mg/kg/day, 8 weeks) would affect insulin sensitivity and skeletal muscle lipid metabolism. We found that NAM induced insulin resistance and skeletal muscle lipotoxicity in nonobese mice. Compared to diet-induced obesity and insulin resistance, NAM specifically increased lipid accumulation in skeletal muscle rather than in adipose tissue and liver.

2. Materials and methods

2.1. Mice and drugs treatment

Male C57/BL6 mice were obtained from Sino-British Sippr/BK Lab Animal (Shanghai, China) and Jackson Laboratories. The animals were housed in plastic cages in a room kept at a room temperature of 23±2°C and a light–dark cycle, and mice had *ad libitum* access to water and food. Mice were fed regular chow (18% fat, 58% carbohydrate and 24% protein by calories; Harlan Teklad) or 8 weeks of high-fat diet (HFD; 55% fat, 24% carbohydrate and 21% protein by calories; Harlan Teklad). For chronic treatment with NAM and RSV, the mice received intraperitoneal injection at the dose of 100 mg/kg body weight/day for 8 weeks. The same volume of vehicle (10% DMSO in saline) was injected to the control mice. Fat and lean body mass was assessed by magnetic resonance spectroscopy [magnetic resonance imaging (MRI)]. All experiments were performed in accordance with the guidelines established by China Ministry of Health for the Care and Use of Laboratory Animals. Experimental procedures were approved by the Experimental Animal Care and Use Committee at East China Normal University (ECNU 2006-05) and the local research institution. Every effort was made to optimize comfort and to minimize the use of animals.

2.2. Glucose and insulin tolerance tests

Three days after the last drug injection, the mice were deprived of food for 16 h and then subjected to glucose tolerance tests (GTT) and insulin tolerance tests (ITT). GTT were performed as previously described [36]. Mice were injected intraperitoneally with glucose (1 g/kg), and blood was collected by tail bleed at 0, 15, 30, 45, 60, 90 and 120 min for plasma glucose measurements. For ITT, mice were injected intraperitoneally with insulin (0.75 U/kg), and blood was collected for plasma glucose measurements.

2.3. Blood analysis and tissue lipid content

Three days after the last drug injection, the mice were sacrificed to collect blood and tissues. Fasting serum glucose, triglyceride (TG) and free fatty acids (FFA) were determined by enzymatic colorimetric assays according to the manufacturer's instruction. Serum insulin was measured by ELISA (R&D Systems, USA). Tissue triglyceride was extracted as described previously [37] and measured by an enzymatic assay (Sigma-Aldrich). Diacylglycerol (DAG) and ceramides were extracted and subjected to liquid chromatography–tandem mass spectrometry analysis as described previously [37]. [NAD+]/[NADH] ratio was measured as described by So-young Jang [5]. Tissues were homogenized in the assay buffer and centrifuged for lactate oxidase assay and pyruvate oxidase assay; the ratio of pyruvate/lactate was used to calculate [NAD+]/[NADH] ratio.

2.4. Glycogen content assays

Approximately 100 mg of tissues was homogenized in 10:1 volume/weight dilution, and glycogen content was measured as described previously [38]. Briefly, homogenates were centrifuged at 5000*g* for 5 min at 4°C. The supernatants or glycogen standards were spotted onto GF/A filters. Filters were washed in ice-cold ethanol for 15 min followed by two washes in 70% ethanol at room temperature. After drying overnight, filters were placed in glass tubes and incubated with 1 ml amyloglucosidase (0.04% in 0.05 M sodium acetate) for 90 min at 37°C. Glucose present in the reaction was analyzed using a commercial kit according to the manufacturer's instructions.

2.5. Fatty acid metabolism

As described previously [39], muscle strips were equilibrated in 2 ml of pregassed Krebs–Henseleit buffer [4% bovine serum albumin (BSA), 30°C] with 1 mM palmitate and 5 mM glucose in a gentle shaking bath for 30 min. Muscle strips continued to be incubated for additional 60 min with the addition of 0.5 μCi/ml [1-^{14}C]palmitate. Exogenous palmitate oxidation and esterification were monitored by the production of $^{14}CO_2$ and incorporation of [1-^{14}C] palmitate into intramuscular lipids, respectively.

2.6. Mitochondrial functions

For mitochondrial isolation, fresh gastrocnemius muscle was rinsed with phosphate-buffered saline and put into ice-cold isolation buffer [0.075 M sucrose, 0.225 M sorbitol, 1 mM EGTA, 0.1% fatty acid (FA)-free BSA and 10 mM Tris–HCl, pH 4]. Tissues were sheared carefully to mince, rinsed to get rid of residual blood and then homogenized in 1 ml isolation buffer per 100 mg tissue. The homogenate was centrifuged at 1000*g* for 5 min at 4°C; the resulting supernatant was decanted and saved. The pellet was washed once with isolation buffer. The supernatant was combined and centrifuged at 9000*g* for 10 min at 4°C. The mitochondrial pellet was washed and centrifuged twice at 15,000*g* for 2 min at 4°C with isolation buffer. Mitochondrial