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Protective effects of a nicotinamide derivative, isonicotinamide, against streptozotocin-induced β-cell damage and diabetes in mice

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ABSTRACT

Objective: Nicotinamide rescues β-cell damage and diabetes in rodents, but a large-scale clinical trial failed to show the benefit of nicotinamide in the prevention of type 1 diabetes. Recent studies have shown that Sirt1 deacetylase, a putative protector of β-cells, is inhibited by nicotinamide. We investigated the effects of isonicotinamide, which is a derivative of nicotinamide and does not inhibit Sirt1, on streptozotocin (STZ)-induced diabetes in mice.

Research design and methods: Male C57BL/6 mice were administered with three different doses of STZ (65, 75, and 100 mg/kg BW) alone or in combination with subsequent high-fat feeding. The mice were treated with isonicotinamide (250 mg/kg BW/day) or phosphate-buffered saline for 10 days. The effects of isonicotinamide on STZ-induced diabetes were assessed by blood glucose levels, glucose tolerance test, and immunohistochemistry.

Results: Isonicotinamide effectively prevented hyperglycemia induced by higher doses of STZ (75 and 100 mg/kg BW) alone and low-dose STZ (65 mg/kg BW) followed by 6-week high-fat diet in mice. The protective effects of isonicotinamide were associated with decreased apoptosis of β-cells and reductions in both insulin content and insulin-positive area in the pancreas of STZ-administered mice. In addition, isonicotinamide inhibited STZ-induced apoptosis in cultured isolated islets.

Conclusions: These data clearly demonstrate that isonicotinamide exerts anti-diabetogenic effects by preventing β-cell damage after STZ administration. These findings warrant further investigations on the protective effects of isonicotinamide and related compounds against β-cell damage in diabetes.

1. Introduction

Diabetes develops when pancreatic β-cells no longer function properly or have been destroyed. Irreversible damage and death of β-cells play a key role in the pathogenesis of type 1 and type 2 diabetes, both of which are characterized by the progressive loss of functional β-cell mass. Development of preventive and/or therapeutic strategies to protect β-cells from damage and death in diabetes has been an issue of intense investigation for decades, but still remains a major challenge in the field.

The protective effects of nicotinamide have been studied in patients with, and rodent models of, type 1 diabetes. Previous studies have shown that treatment with nicotinamide prevents or ameliorates streptozotocin (STZ)-induced diabetes [1] and progression of diabetes in non-obese diabetic mice [2,3]. Small-scale clinical trials demonstrated the beneficial effects of nicotinamide on metabolic outcome in newly diagnosed type 1 diabetes patients [3–6] and the prevention of type 1 diabetes in individuals at high risk [7,8]. A previous study has shown that pretreatment of islet grafts with nicotinamide prevents early graft failure after islet transplantation in mice [9]. A large-scale randomized clinical trial, however, failed to show the benefit of nicotinamide in the prevention of diabetes in islet cell antibody-positive first degree relatives of patients with type 1 diabetes [10].

The inhibition of poly(ADP-ribose) polymerase (PARP) activity by nicotinamide is considered as an important contributor to the

Abbreviations: STZ, streptozotocin; PARP, poly(ADP-ribose) polymerase; NAD^+, nicotinamide adenine dinucleotide; TUNEL, TdT-mediated dUTP Nick-End Labeling; HD-STZ, high-dose streptozotocin; MD-STZ, medium-dose streptozotocin; LD-STZ, low-dose streptozotocin.

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protective effects of nicotinamide against β-cell damage [11,12]. PARP activation, which is triggered by DNA damage, consumes nicotinamide adenine denucleotide (NAD^+), leading to NAD^+ depletion. This in turn causes death of β-cells. PARP-1 knockout mice are resistant to STZ-induced diabetes [13,14]. On the other hand, nicotinamide also serves as a precursor of NAD^+, as well as an inhibitor of PARP. NAD^+ is biosynthesized from nicotinamide by the nicotinamide phosphoribosyl transferase (also known as visfatin and pre-β cell colony enhancing factor) pathway [15].

Nicotinamide has been identified as an end-product inhibitor of Sirt1, a NAD^+-dependent deacetylase and the closest mammalian homologue of the putative yeast longevity gene, Sir2 [16]. A recent study has shown that activation of Sirt1 protects β-cells from cytokine-induced damage [17]. β-Cell-specific Sirt1 overexpressing transgenic mice exhibit enhanced insulin secretion and improved glucose tolerance [18], while insulin secretion is blunted in Sirt1 knockout mice [19]. These findings suggest that Sirt1 inhibition by nicotinamide might hamper the beneficial effects of nicotinamide in diabetes, and contribute to the failure to show the benefit in the large-scale clinical trial. One can speculate, therefore, that derivatives of nicotinamide, which do not interfere with Sirt1 activity, could be a new class of reasonable drug candidate to protect β-cells in diabetes. Isonicotinamide is such a derivative of nicotinamide, as it does not inhibit Sirt1 activity but antagonizes the inhibition of Sirt1 by nicotinamide [20]. These recent findings motivated us to study the effects of isonicotinamide on diabetes induced by different doses of STZ in combination with and without high-fat diet in mice.

2. Materials and methods

2.1. Animals

Male C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The Institutional Animal Care Committee approved the study protocol. The animal care facility is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. At 7 weeks of age mice were administered with streptozotocin (STZ) dissolved in citrate buffer (pH = 4.5) (65, 75, or 100 mg/kg BW, IP, Sigma, St. Louis, MO) twice with an interval of 2 days. Treatment with isonicotinamide (250 mg/kg BW, IP, once daily, Sigma) or phosphate-buffered saline (PBS) was initiated at 1 h prior to the first STZ injection and continued for 10 days. Blood glucose levels were measured by a blood glucose meter, CONTOUR (Bayer, Pittsburgh, PA), under fed condition, unless otherwise indicated.

2.2. Glucose tolerance test

At 26 days after the last injection of low-dose STZ (65 mg/kg BW), the mice received a peritoneal injection of glucose (2 g/kg BW) after 4-h fasting. Blood glucose levels and plasma insulin concentrations were measured just before and at 15, 30, 60, 90, and 120 min after the glucose injection. Plasma insulin concentrations were measured by ELISA kit (ALPCO, Salem, NH).

2.3. High-fat diet

The mice administered with low-dose STZ (65 mg/kg BW) were fed high-fat diet (D12451, Research Diet, New Brunswick, NJ) for 6 weeks starting at 28 days after the last injection of STZ.

2.4. Immunohistochemical analysis

Pancreas was collected under anesthesia with pentobarbital sodium (50 mg/kg BW) from low-dose (65 mg/kg BW), medium-dose (75 mg/kg BW), and high-dose (100 mg/kg BW) STZ-administered mice at 10 weeks, 28, and 7 days after the last STZ injection, respectively. Pancreata were fixed in 4% paraformaldehyde and embedded as paraffin blocks. Paraffin sections were cut (5-μm thickness, 100-μm interval) and processed for immunohistochemistry as previously described [21]. The sections were stained with anti-insulin (Dako, Carpinteria, CA) and anti-glucagon (Millipore, Billerica, MA) antibodies. Insulin-positive, glucagon-positive and islet areas were evaluated from 4 non-continuous pancreatic sections, each of which contains more than 30 islets, using a Nikon Eclipse TE2000-U microscope (Nikon Instruments, Melville, NY) and camera, and measured using Image J software (NIH, Bethesda, MD). The percentages of insulin- or glucagon-positive area per islet and per section area were calculated [21].

2.5. Insulin content

Whole pancreata were removed under anesthesia at 7 days after the last STZ injection or from naïve mice at 8 weeks of age. Insulin in the pancreata was extracted as previously described [22], and measured by ELISA kit.

2.6. Culture of isolated islets

Islets were isolated from male C57/BL6 mice by collagenase digestion followed by centrifugation over a Histopaque (Sigma) gradient, as previously described [23]. Isolated islets were incubated overnight with RPMI1640 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in atmosphere of 5% CO_2 in humidified air. Then, the islets were treated with and without STZ (0.8 mM) in the presence and absence of isonicotinamide (0.3 mM). Isonicotinamide was added to the culture at 2 h prior to STZ treatment. 50 islets per well were incubated in 24-well plates for 8 h after the addition of STZ, and then fixed in paraformaldehyde. The fixed islets were frozen in Tissue-Tek O.C.T. compound (SAKURA Fineteck, Torrance, CA) and apoptosis was evaluated by TdT-mediated dUTP Nick-End Labeling (TUNEL) staining in frozen section using a kit (Promega, Madison, MI). To assess islet cell death, the islets were incubated in 96-well plates for 24 h after the addition of STZ and fluorescence intensity of Sytox (Invitrogen, Carlsbad, CA) staining was quantified using Image J.

2.7. Statistical analysis+

Statistical analysis was performed by Student's *t*-test or one-way ANOVA followed by Bonferroni post hoc analysis. The effect of isonicotinamide on the development of diabetes was also analyzed by χ^2 test. A value of $p < 0.05$ was considered statistically significant. Data are expressed as means ± SEM.

3. Results

3.1. Isonicotinamide treatment prevented high- and medium-dose streptozotocin-induced diabetes in mice

After two injections of high-dose streptozotocin (HD-STZ, 100 mg/kg BW), all of the mice developed diabetes (blood glucose > 300 mg/dL) when treated with PBS. Isonicotinamide treatment significantly reduced the incidence of HD-STZ-induced diabetes based on the statistical analysis by χ^2 test ($p < 0.05$ vs. PBS) (Four out of eight mice became diabetic after HD-STZ injections when treated with isonicotinamide.) (Fig. 1A). At 7 days after the last injection of HD-STZ, robust hyperglycemia following 4-h fasting was observed in PBS-treatment mice, which was markedly ameliorated by isonicotinamide treatment (Fig. 1B).

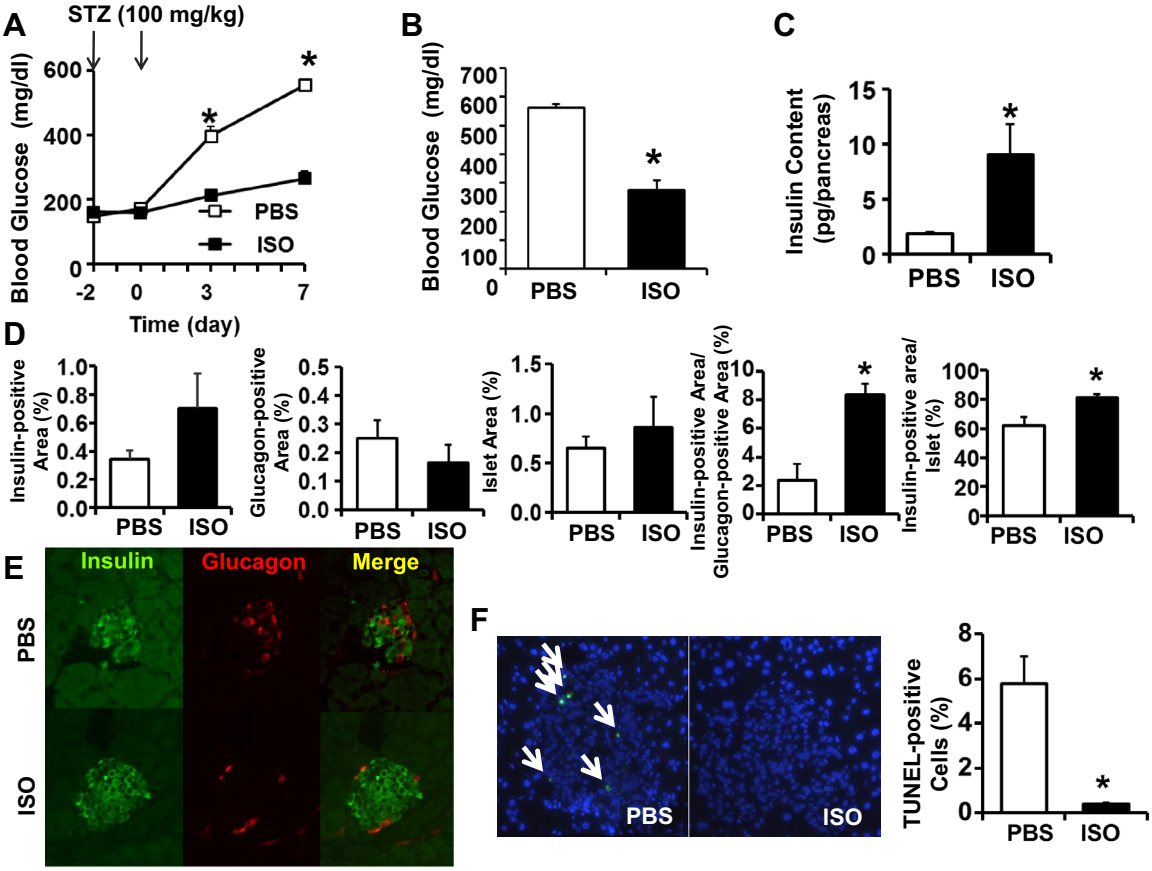

Fig. 1. Isonicotinamide inhibited the development of overt diabetes induced by high-dose STZ in mice. Mice were administered with high-dose STZ (100 mg/kg BW) and treated with isonicotinamide (ISO, 250 mg/kg BW) ($n = 8$) or PBS ($n = 8$) for 10 days. Isonicotinamide significantly protected the mice from high-dose STZ-induced overt diabetes, as reflected by blood glucose levels under fed (A) and fasted (B) conditions. Isonicotinamide prevented STZ-induced decrease in insulin content at 7 days after the last injection of STZ (C). The ratio of insulin-positive area to glucagon-positive area, and insulin-positive area were significantly greater in isonicotinamide (ISO)-treated mice than PBS-treated mice (D and E). At 1 day after the last STZ injection, the percentage of TUNEL-positive nuclei in total DAPI-positive nuclei was profoundly decreased in the mouse islets by isonicotinamide compared with PBS (F). $^{*}p < 0.05$ vs. PBS.

Induction of overt diabetes by HD-STZ was associated with reduced insulin content in the pancreas at 7 days after the last HD-STZ injection. Isonicotinamide significantly increased insulin content in HD-STZ-administered mice (Fig. 1D and E). When treated with isonicotinamide, insulin content in HD-STZ-administered mice was restored to the level comparable to that observed in naïve mice (Insulin content [pg/pancreas]: Naïve [$n = 8$]: 4985 ± 1685 [mean ± SEM]; HD-STZ + ISO [$n = 4$]: 5622 ± 1764; HD-STZ + PBS [$n = 4$]: 1153 ± 95.12). HD-STZ administration increased apoptosis in the islets of PBS-treated mice, as judged by TUNEL staining. Isonicotinamide decreased apoptosis in the islets in HD-STZ-administered mice (Fig. 1F). There were trends of increases in insulin-positive area and islet area, and decrease in glucagon-positive area in the pancreas of isonicotinamide-treated mice, as compared with PBS. There were, however, no significant differences. On the other hand, the ratio of insulin-positive area to glucagon-positive area, and insulin-positive area per islet were significantly greater in isonicotinamide-treated mice than in PBS-treated mice after HD-STZ administration (Fig. 1D and E). Body weight was also significantly greater in isonicotinamide-treated mice than in PBS-treated mice at 7 days after HD-STZ injections (Supplementary Fig. 1A), presumably secondary to the amelioration of diabetes by isonicotinamide.

Similarly, isonicotinamide prevented medium-dose streptozotocin (MD-STZ, 75 mg/kg BW)-induced increase in blood glucose levels, as compared with PBS (Fig. 2A). At 28 days after MD-STZ injections, insulin-positive area and islet area in the pancreas, and the ratio of insulin-positive area to glucagon-positive area were significantly greater in isonicotinamide-treated mice than in PBS-treated mice (Fig. 2B and C). There were trends of decreased glucagon-positive area and increased insulin-positive area per islet in isonicotinamide-treated mice relative to PBS, but no significant differences were found. Body weight did not differ between the two groups at 28 days after MD-STZ injections (Supplementary Fig. 1B), possibly because mild hyperglycemia did not significantly affect body weight. We did not observe any adverse effects of treatment with isonicotinamide (250 mg/kg BW) for up to 10 days in naïve or STZ-administered mice, including body weight gain and food intake (data not shown).

3.2. Isonicotinamide treatment inhibited the development of diabetes by low-dose STZ followed by high-fat diet in mice

Low-dose streptozotocin injections (LD-STZ, 65 mg/kg BW) did not significantly increase blood glucose levels in either PBS- or isonicotinamide-treated mice up to 28 days after LD-STZ injections (Fig. 3A and data not shown). At 26 days after the last LD-STZ injection, glucose tolerance test revealed that PBS-treated mice were more glucose-intolerant than isonicotinamide-treated mice (Fig. 3B). Prior LD-STZ administration abrogated increase in plasma insulin concentration during glucose tolerance test in PBS-treated mice, which was restored by isonicotinamide.

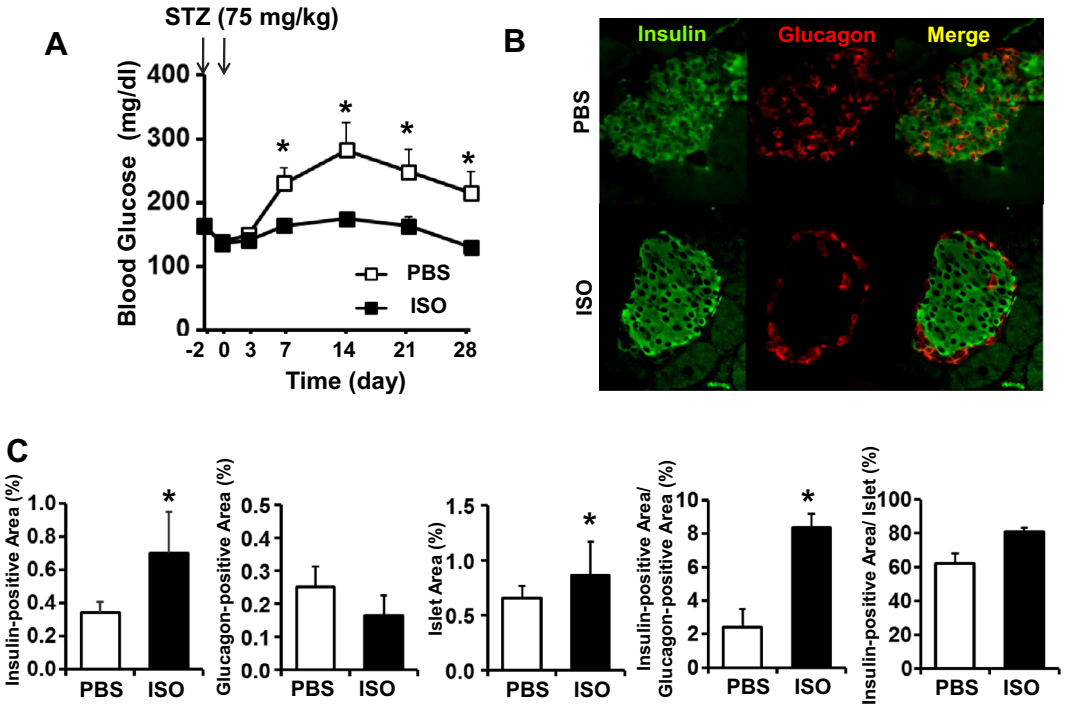

Fig. 2. Protective effects of isonicotinamide against medium-dose STZ-induced diabetes. Mice were administered with medium-dose STZ (75 mg/kg BW) and treated with isonicotinamide (ISO, 250 mg/kg BW) ($n = 6$) or PBS ($n = 6$) for 10 days. Medium-dose STZ administration increased in blood glucose levels in PBS-treated mice, which was prevented by isonicotinamide. Insulin-positive area and islet area in the pancreas, and the ratio of insulin-positive area to glucagon-positive area were significantly greater in isonicotinamide (ISO)-treated mice than in PBS-treated mice (B and C). $^{*}p < 0.05$ vs. PBS.

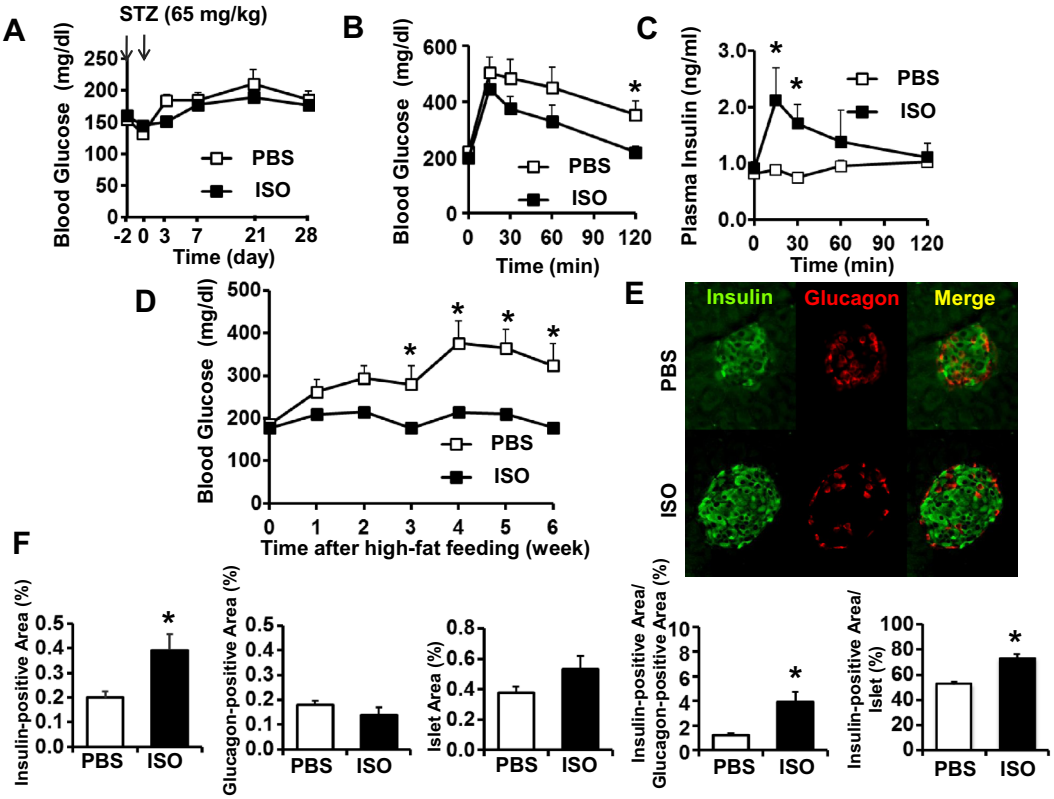

Fig. 3. Protective effects of isonicotinamide in low-dose STZ-administered mice. Mice were administered with low-dose STZ (60 mg/kg BW) and treated with isonicotinamide (ISO, 250 mg/kg BW) ($n = 8$) or PBS ($n = 8$) for 10 days (A). Blood glucose levels did not significantly increase up to 28 days after the last injection of low-dose STZ regardless of the treatment (A and data not shown). On day 26 post-STZ injections glucose tolerance test showed that plasma insulin concentration did not increase after glucose injection (2 g/kg BW) in PBS-treated mice, while blood glucose levels were greater in PBS-treated mice than isonicotinamide-treated mice (B). In contrast, isonicotinamide-treated mice responded to glucose load with over 2-fold increase in plasma insulin concentration (C). At 4 weeks after the low-dose STZ administration, high-fat diet was initiated and continued for 6 weeks. High-fat diet induced hyperglycemia in PBS-, but not isonicotinamide-, treated mice (D). Insulin-positive area was greater in isonicotinamide-treated mice than PBS-treated mice after 6-week high-fat diet (E and F). $^{*}p < 0.05$ vs. PBS.

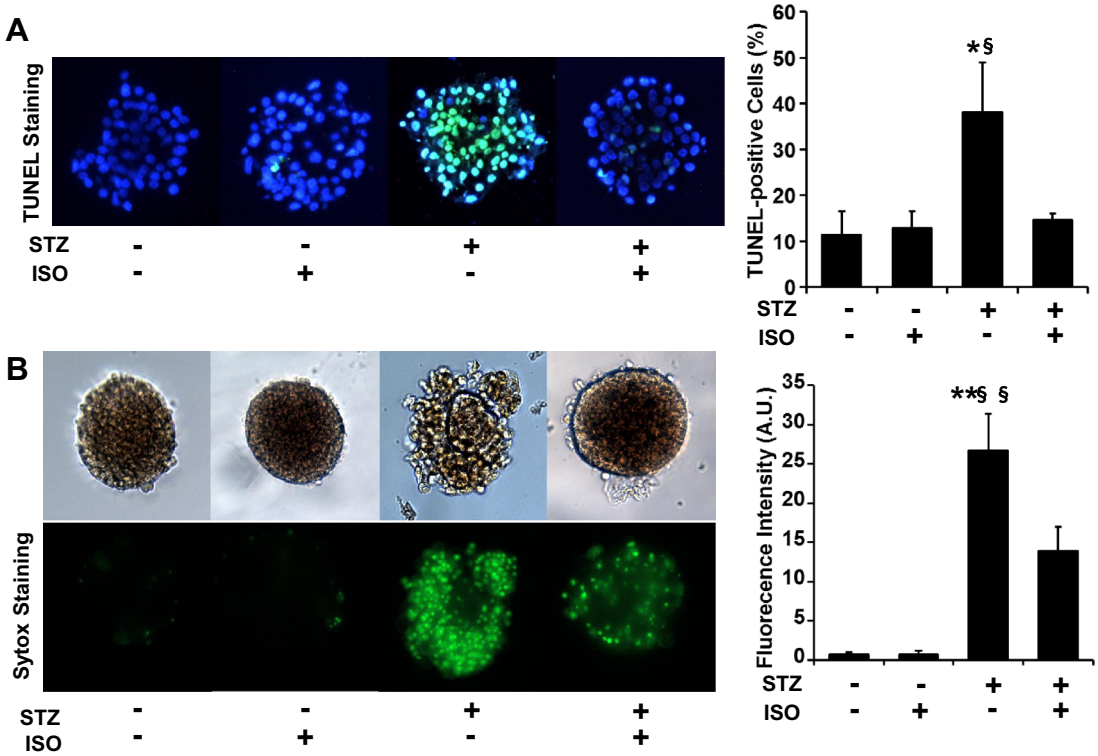

Fig. 4. Isonicotinamide inhibited STZ-induced cell death in cultured mouse islets. Isolated islets were incubated with and without STZ (0.8 mM) in the presence and absence of isonicotinamide (ISO, 0.3 mM). Isonicotinamide inhibited STZ-induced apoptosis and death of cultured islet cells, as quantified by TUNEL (A) and Sytox staining (B), respectively. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. STZ (−); $^{\S}p < 0.05$. $^{\S\S}p < 0.01$ vs. STZ (+) ISO (+).

Starting at 28 days after LD-STZ injections (viz., 21 days after the last isonicotinamide injection), the mice were fed high-fat diet for 6 weeks. High-fat diet induced hyperglycemia in PBS-, but not isonicotinamide-, treated mice (Fig. 3D).

Consistently, insulin-positive area in the pancreas, the ratio of insulin-positive area to glucagon-positive area, and insulin-positive area per islet were significantly greater in isonicotinamide-treated mice than in PBS-treated animals after 6-week high-fat feeding (Fig. 3E and F). Islet area in the pancreas tended to be greater in isonicotinamide-treated mice relative to PBS, but there was no significant difference. Glucagon-positive area did not differ between isonicotinamide- and PBS-treated mice. There was no difference in food intake between the two groups (Food intake during high-fat diet [g/day]: Isonicotinamide: 2.1 ± 0.2; PBS: 1.9 ± 0.2). Body weight was, however, greater in isonicotinamide-treated mice than in PBS-treated mice after 6-week high-fat diet (Supplementary Fig. 1C), presumably reflecting hyperglycemia-associated body weight loss in PBS-treated mice.

3.3. Isonicotinamide protected against STZ-induced apoptosis in isolated islets

To further investigate the protective effects of isonicotinamide against STZ-induced β-cell damage, we treated cultured isolated islets with or without STZ (0.8 mM) in the presence and absence of isonicotinamide (0.3 mM). Consistent with previous studies [24], STZ induced apoptosis and death of islet cells. Isonicotinamide was effective to inhibit STZ-induced apoptosis and cell death in cultured islets, as judged by TUNEL and Sytox staining, respectively (Fig. 4).

4. Discussion

Here, we demonstrate that isonicotinamide effectively prevented diabetes induced by three different doses of STZ alone or in combination with high-fat diet. The protective effects of isonicotinamide against STZ-induced diabetes were associated with prevention of β-cell damage, as reflected by reduction in apoptosis in the islets and restoration of insulin content in the pancreas. Moreover, isonicotinamide inhibited STZ-induced apoptosis and cell death in cultured isolated islets (Fig. 4). These findings indicate that isonicotinamide can directly protect β-cells from STZ-induced damage.

STZ dose-dependently induced hyperglycemia when mice were treated with PBS. LD-STZ failed to cause overt hyperglycemia regardless of treatment with isonicotinamide or PBS (Fig. 3A). Of note, isonicotinamide preserved β-cell function in LD-STZ-administered mice, as indicated by glucose tolerance test (Fig. 3B). The LD-STZ-administered mice were treated with isonicotinamide or PBS for 10 days, and 3 weeks later high-fat feeding was initiated and continued for 6 weeks. Isonicotinamide prevented high-fat diet-induced hyperglycemia along with greater β-cell mass (Fig. 3D–F). These findings indicate that LD-STZ elicited irreversible or persistent inhibitory effects in β-cells, which was blocked by isonicotinamide. Capacity of adaptive expansion of β-cell mass in response to high-fat diet was lost by LD-STZ, but isonicotinamide restored it.

In contrast to the protective effects in β-cells, high-dose nicotinamide induces insulin resistance in individuals at high risk of developing type 1 diabetes [5], although the molecular mechanism is unknown. The insulin-desensitizing effect has been thought as a potential limitation of nicotinamide treatment in diabetes [5,25,26]. It should be noted that activation of Sirt1 improves insulin sensitivity in rodent models of obesity-induced diabetes [27,28], as well as promotes insulin secretion and β-cell survival in cell culture [17]. Together, it is conceivable that the inhibition of Sirt1 might underlie the insulin-desensitizing effect of nicotinamide. Recent studies have demonstrated that insulin resistance is an independent risk for the progression of type 1 diabetes [29,30]. In aggregate, one can speculate that the inhibition of Sirt1

by nicotinamide might be a contributor to the failure in the large-scale clinical trial by impairing insulin sensitivity and/or inhibiting the protective effects of Sirt1 in β-cells. It is reasonable to speculate that anti-diabetogenic actions of nicotinamide-related compounds might deserve reappraisal in case these limitations could be overcome.

A previous study has shown that endogenous levels of nicotinamide limit Sir2 activity in yeast cells [20]. The inhibition of Sir2 by nicotinamide is antagonized by isonicotinamide, leading to activation of Sir2 *in vitro* and in intact cells [20]. Isonicotinamide has been recently used as a Sirt1 activator in mouse mesenchymal stem cells [31] and human osteosarcoma cells [32]. A recent study has show that isonicotinamide represses the transcription of phosphoenolpyruvate carboxykinase (PEPCK), the rate-limiting enzyme of gluconeogenesis, by activating Sirt1 in cultured hepatocytes, while nicotinamide increases mRNA expression of PEPCK [33]. In early studies, isonicotinamide (250 mg/kg BW) attenuated iron-induced renal damage in rats although the mechanisms were unknown [34], and the safety of long-term administration of isonicotinamide was studied in mice [35]. A lifelong administration of isonicotinamide as 1% solution continuously in drinking water did not affect the lifespan or tumorigenesis as compared with untreated control mice (the average intake of isonicotinamide was approximately 100 mg/day) [35]. Collectively, our results suggest that isonicotinamide and its related compounds, which preserves or activates Sirt1, may represent a novel group of small molecules as a drug candidate to prevent and/or reverse diabetes by protecting β-cells from damage and death. This possibility is arguably worthy of further investigation.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.11.024.

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