Protective effect of hypoxylonol C and 4,5,4′,5′-tetrahydroxy-1,1′-binaphthyl isolated from Annulohypoxylon annulatum against streptozotocin-induced damage in INS-1 cells

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ARTICLE INFO

Keywords:
Annulohypoxylon annulatum
Pancreatic β-cells
Apoptosis
Streptozotocin

ABSTRACT

We evaluated the protective effects of hypoxylonol C and 4,5,4′,5′-tetrahydroxy-1,1′-binaphthyl (BNT) isolated from Annulohypoxylon annulatum on pancreatic β-cell apoptosis, using the β-cell toxin streptozotocin (STZ). Hypoxylonol C and BNT restored the STZ-induced decrease in INS-1 cell viability in a dose-dependent manner. In addition, treatment of INS-1 cells with 50 μM STZ resulted in an increase in apoptotic cell death, which was observed as annexin V fluorescence intensity. Apoptotic cell death was decreased by co-treatment with 100 μM hypoxylonol C and 100 μM BNT. Similarly, STZ caused a marked increase in the expression of cleaved caspase-8, caspase-3, Bax, and poly (ADP-ribose) polymerase (PARP), as well as a decrease in the expression of B-cell lymphoma 2 (Bcl-2), which was reversed by co-treatment with 100 μM hypoxylonol C and 100 μM BNT. These findings suggest that hypoxylonol C and BNT play an important role in protecting pancreatic β-cells against apoptotic damage.

1. Introduction

The number of people with diabetes mellitus has been increasing in the last few decades, and it is now recognized as a global public health problem [1,2]. The loss and dysfunction of pancreatic β-cells is the key feature of diabetes [3,4]. Loss of pancreatic β-cell function leads to insulin deficiency, which then results in hyperglycemia [5].

β-cell death results from the action of pro-inflammatory cytokines, oxidative stress, and glucolipotoxicity [6]. Streptozotocin (STZ), an antibiotic derived from Streptomyces achromogenes, has been widely used to induce experimental diabetes in mouse and rat models, and may induce β-cell destruction and apoptosis via the generation of reactive oxygen species (ROS) [6,7]. β-cell toxicity is related to the glucose moiety in its chemical structure, which facilitates the permeation of STZ into β-cells and leads to DNA alkylation damage [3]. This induces the activation of poly (ADP-ribose) polymerase (PARP). Understanding these mechanisms is therefore necessary for the development of drugs to prevent the onset and progression of diabetes.

Natural product extracts and their compounds are more attractive than synthetic drugs as drug candidates, as synthetic drugs treat the primary symptoms of a specific disease as understood by scientific pathology, whereas natural products usually act by healing and repairing body functions that are affected by the primary symptoms. This encourages the development of many natural compounds that have biological potency [8–10].

In the treatment and prevention of diabetes, several natural product extracts and their compounds possess known beneficial properties related to pancreatic β-cell function, such as insulin secretion, anti-apoptosis activity, and cell proliferation. Panax ginseng root enhanced insulin secretion and prevented pancreatic β-cell apoptosis induced by cytokine expression in the INS-1 rat insulinoma cell line [11]. Cyclocarya paliurus tea leaves inhibited STZ-induced pancreatic β-cell...
apoptosis [12], and P. ginseng berries improved glucose tolerance and increased pancreatic β-cell proliferation in STZ-treated diabetic mice [13]. Sesamin isolated from sesame meal showed protective effects on STZ-treated NIT-1 pancreatic β-cells via a decrease in oxidative stress and NO synthesis [14]. Similarly, curcumin isolated from Curcuma longa not only enhanced insulin secretion, but also prevented STZ-induced pancreatic β-cell damage [15]. Genistein, isolated from Glycine max, and quercetin, found in a variety of plants, not only enhanced insulin secretion, but also prevented pancreatic β-cell apoptosis [11,16]. Studies of novel agents showing beneficial effects on the improvement of pancreatic β-cell function are important for the discovery of cost-effective agents. *Annulohypoxylon annulatum*, a ball-shaped wood-rotting fungus that contains unique benzofluoranthenecompounds [17], which protect against cisplatin-induced renal proximal tubular damage [18], and inhibits tumor angiogenesis [19,20]. However, the anti-diabetic effects of *A. annulatum* mediated by the improvement of pancreatic β-cell function have not yet been elucidated. Here, we investigated the protective effects of *A. annulatum* and its compounds on STZ-induced pancreatic β-cell dysfunction and their underlying molecular mechanisms in INS-1 cells.

2. Experimental

2.1. Preparation of three compounds from *A. annulatum*

*A. annulatum* was collected in Yeongok-myeon, Gangneung city, Korea, in March 2018 and a voucher specimen was deposited (MCO-NP-I-0176) at the Library of Natural Products Research Institute, Korea Institute of Science and Technology. The stroma sample (130 g) was extracted using methanol (1.5L) for 12h at room temperature and filtered. The crude extract (approximately 16g) was suspended in water and then successively partitioned with hexane (Hex), ethyl acetate (EA), n-butanol (BuOH), and water, yielding 364mg, 6.5g, 4.8g, and 3.9 g of residue, respectively. The EA-soluble fraction was analyzed via reversed-phase high-performance liquid chromatography (HPLC; Phenomenex C18 column, 250 × 21.2 mm ID) and eluted using water (mobile phase A) and acetonitrile (B), both containing 0.1% formic acid, at a flow rate of 20 mL/min, using the following gradient: 50% B over 5 min, 50–100% B over 50 min, and 100% B over 5 min, using a 254-nm ultraviolet (UV) detector to yield three sub-fractions (A-C). Further purification of each sub-fraction (A, B, and C) was conducted via semi-preparative HPLC (Phenomenex C6-Phenyl column, 250 × 10 mm ID) eluting with 25% aqueous MeOH and using a refractive index (RI) detector, at a flow rate of 4 mL/min to yield pure hypoxylonol C (Fig. 1 (1), 314 mg), hypoxylonol F (Fig. 1 (2), 218 mg), and 4,5,4′,5′-tetrahydroxy-1,1′-binaphthyl (BNT, Fig. 1 (3), 389 mg). The chemical structures of these three isolated compounds were determined by comparing with previously reported reference compounds (Fig. 1) [19].

2.2. Cell culture

Rat insulinoma INS-1 cells were obtained from Biohermes (Shanghai, China). The cells were maintained in RPMI-1640 medium (Cellgro, Manassas, VA, USA) supplemented with 1% penicillin/

streptomycin (Invitrogen Co., Grand Island, NY, USA), 10% fetal bovine serum (FBS), 11 mM α-glucose, 2 mM L-glutamine, 10 mM HEPES, 0.05 mM 2-mercaptoethanol, and 1 mM sodium pyruvate at 37 °C in a humidified atmosphere with 5% CO₂.

2.3. Analysis of protective effect against STZ-induced damage in INS-1 cells

STZ was purchased from Sigma-Aldrich (St. Louis, MO, USA). Cell viability was evaluated using a cell viability assay kit (EZ-Cytox) purchased from the Daeil Lab Service Co. (Seoul, Korea). Briefly, INS-1 cells were seeded in 96-well culture plates (1 × 10⁴ cells/well) for 24h and treated with the potential drug candidates and/or 50 µM STZ for 24h. Cells were incubated with Ez-Cytox reagent (10 µL/well) for 2h. The absorbance of the medium, which is proportional to the number of viable cells, was then measured at a wavelength of 450 nm using a PowerWave XS microplate reader (Bio-Tek Instruments, Winooski, VT, USA).

2.4. Determination of intracellular ROS

The level of intracellular ROS was measured using a cell-permeant 2′,7′-dichlorofluorescein diacetate (H₂DCFDA) (Sigma, St. Louis, MO, USA). Briefly, INS-1 cells were seeded in black 96-well culture plates (1 × 10⁴ cells/well) for 24h and treated with the potential drug candidates and/or 50 µM STZ for 24h. Cells were stained with 10 µM H₂DCFDA at room temperature for 30min in the dark followed by washing with PBS. The fluorescence intensity of DCF was measured with an excitation wavelength at 495 nm and emission wavelength at 517 nm, using a SPARK 10M microplate reader (Tecan, Männedorf, Switzerland) and represented by fold-increases. Furthermore, fluorescent images were acquired using a IX50 fluorescent microscope equipped with a CCD camera (Olympus, Tokyo, Japan).

2.5. Image-based cytometric assay

The percentage of apoptosis, live, necrotic cells was measured using an image-based cytometric assay kit, according to the manufacturer’s instructions. Briefly, INS-1 cells were seeded in 6-well culture plates (4 × 10⁵ cells/well) for 24h and treated with the potential drug candidates and/or 50 µM STZ for 24h. Cells were harvested and suspended in binding buffer (Life Technologies, Carlsbad, CA, USA) with annexin V-Alexa Fluor 488 and PI (Invitrogen, Temecula, CA, USA). After incubation at room temperature for 30 min in the dark, the percentage of apoptosis, live, necrotic cells were calculated using a Tali image-based cytometer (Invitrogen, Temecula, CA, USA).

2.6. Western blot analysis

Western blot analysis was performed to evaluate the relative expression levels of the proteins regulating apoptosis. Briefly, INS-1 cells were seeded in 6-well culture plates (4 × 10⁵ cells/well) for 24h and treated with the potential drug candidates and/or 50 µM STZ for 24h. Cells were harvested and lysed using radioimmunoprecipitation assay buffer (Cell Signaling, Danvers, MA, USA) supplemented with 1 mM phenylmethylsulfonyl fluoride. Lysate protein content was determined using a Pierce™ BCA protein assay kit (Thermo Scientific, Carlsbad, CA, USA).
USA). Equal amounts of protein (20 μg/lane) were loaded, resolved via 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% skim milk, and incubated for 1h with primary antibodies against cleaved caspase-8, cleaved caspase-3, B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax), PARP, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Cell Signaling, Boston, MA, USA) at room temperature.

Bound antibodies were incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibodies (Cell Signaling, Boston, MA, USA) for 1h at room temperature. Proteins were detected using western blotting detection reagents (ECL Advance, GE Healthcare, UK) and visualized using a chemiluminescence system (FUSION Solo, PEQLAB Biotechnologie GmbH, Erlangen, Germany). The relative optical density of each immunoreactive band was quantified using ImageJ software (Version 1.51J; National Institutes of Health, Bethesda, MD, USA). Quantitative expression data was normalized to the loading control GAPDH expression and presented as fold-increases compared to the untreated cells.

2.7. Statistical analysis

Statistical significance was determined via an analysis of variance (ANOVA) with Bonferroni correction applied for multiple comparisons. p < 0.05 was considered statistically significant. The analysis was performed using SPSS ver. 19.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Effect of A. annulatum and its fractions on STZ-induced damage in INS-1 cells

The crude A. annulatum extract and its fractions (hexane (Hex), ethyl acetate (EA), n-butanol (BuOH), and water) were prepared as described in Supporting information. Hypoxylonol C (Fig. 1 (1)), hypoxylonol F (Fig. 1 (2)), and 4,5,4′,5′-tetrahydroxy-1,1′-binaphthyl (BNT, Fig. 1 (3)) were isolated and determined by comparing with previously reported reference compounds (Fig. 2) [19].

To determine the effect of A. annulatum and its fractions on STZ-induced damage in INS-1 cells, we performed cytotoxicity assays. As shown in Fig. 3, the reduction in INS-1 cell viability induced by 50 μM STZ was counteracted by co-treatment with A. annulatum and its fractions in a concentration-dependent manner. Although cytotoxic effect was observed after treatment with 100 μg/mL MeOH extract (Fig. 2A), INS-1 cell viability reduced by STZ increased to 90.4 ± 2.7% after co-treatment with 50 μg/mL MeOH extract (Fig. 2F). Similarly, cytotoxic effects were also observed after treatment with 50 and 100 μg/mL EA (Fig. 2C), and 100 μg/mL BuOH (Fig. 2D) fractions. However, INS-1 cell viability reduced by STZ increased to 91.1 ± 2.5%, 95.8 ± 2.4%, 96.5 ± 3.3%, and 96.2 ± 4.1% after co-treatment with 100 μg/mL Hex, 25 μg/mL EA, 50 μg/mL BuOH, and 100 μg/mL water fractions, respectively (Fig. 2G–J).
3.2. Effect of hypoxylonol C, hypoxylonol F, and BNT isolated from A. annulatum on STZ-induced damage in INS-1 cells

To determine the effect of hypoxylonol C, hypoxylonol F, and BNT isolated from A. annulatum on STZ-induced damage in INS-1 cells, we performed cytotoxicity assays. As shown in Fig. 3, the reduction in INS-1 cell viability induced by 50μM STZ treatment was counteracted by co-treatment with hypoxylonol C and BNT in a concentration-dependent manner. The viability of INS-1 cells after STZ-induced damage increased to 90.0 ± 2.5% after co-treatment with 100μM hypoxylonol C (Fig. 3D) without inducing cytotoxicity (Fig. 3A). However, INS-1 cell viability was not recovered by treatment with 2.5–100μM hypoxylonol F (Fig. 3E). INS-1 cell viability after STZ-induced damage increased to 89.1 ± 1.6% after co-treatment with 100μM BNT (Fig. 4F) without inducing cytotoxicity (Fig. 3C).

3.3. Effect of hypoxylonol C and BNT on STZ-induced oxidative stress and apoptosis in INS-1 cells

Co-treatment with 100μM hypoxylonol C and 100μM BNT improved the characteristic changes in cell morphology related to apoptosis, including the cellular shrinkage, blebbing, and swelling observed in STZ-treated cells (Fig. 4A). To detect STZ-induced oxidative stress and explore the effect of hypoxylonol C and BNT on oxidative stress, cells were treated with 50μM STZ in the presence or absence of 100μM hypoxylonol C and 100μM BNT, and stained with H$_2$DCFDA. As shown in fluorescent images (Fig. 4B), after STZ treatment, intracellular ROS were increased, whereas 100μM hypoxylonol C and 100μM BNT almost completely reduced this increase. Consistent with this result, our quantitative results showed that STZ-induced significant increase in intracellular ROS level (5.12-fold increase) was markedly reduced by 100μM hypoxylonol C and 100μM BNT (3.52- and 2.13-fold increases, respectively) (Fig. 4E).

In addition, to explore the effect of hypoxylonol C and BNT on STZ-induced cell death, cells were treated with 50μM STZ in the presence or absence of 100μM hypoxylonol C and 100μM BNT and stained with annexin V conjugated with Alexa Fluor 488 and PI. The percentage of live cells (unstained cells; blue circles) was significantly decreased to 51.66 ± 0.04% by treatment with 50μM STZ, whereas it was increased to 71.66 ± 0.03% and 76.00 ± 0.04% by treatment with 100μM hypoxylonol C and 100μM BNT, respectively. The percentage of apoptotic cells (annexin V-positive cells; green circles) was significantly increased to 42.66 ± 0.57% by treatment with 50μM STZ, whereas it was decreased to 21.66 ± 1.57% and 19.33 ± 0.57% by treatment with 100μM hypoxylonol C and 100μM BNT, respectively. The cells treated with STZ in the presence or absence of 100μM hypoxylonol C and 100μM BNT showed a low and similar percentage of necrotic cells (Fig. 4C and 4D). The results revealed that the exposure of INS-1 cells to STZ significantly increased the apoptotic cell death after nuclear condensation, while treatment with hypoxylonol C and BNT reduced the percentage of apoptotic cell death.

3.4. Effect of hypoxylonol C and BNT on cleaved caspase-8, caspase-3, Bax, Bcl-2, PARP, expression in INS-1 cells with STZ-induced damage

We investigated whether hypoxylonol C and BNT exert preventive effects on STZ-induced cleavage of caspase-8, caspase-3, Bax, Bcl-2, PARP, and the expression of Bax and Bcl-2 in INS-1 cells. Treatment with 50μM STZ markedly increased the cleavage of caspase-8, caspase-3, and PARP. However, these changes were reversed by co-treatment with 100μM hypoxylonol C and 100μM BNT (Fig. 5A–D, 5H). Moreover,
treatment with STZ also increased in the expression of Bax and decreased in the expression of Bcl-2 protein expression. However, the increased ratio of Bax to Bcl-2 protein expression were reversed by co-treatment with 100μM hypoxylonol C and 100μMBNT (Fig. 5A, 5E-G).

4. Discussion

We investigated the effect of hypoxylonol C and BNT isolated from A. annulatum on the prevention of apoptotic damage induced by STZ treatment in pancreatic β-cells. The dysfunction of pancreatic β-cells plays a key role in the pathogenesis of both type 1 and type 2 diabetes [21,22]. In type 1 diabetes, pancreatic β-cells are destroyed by immunological factors known as cytokines [23]. In type 2 diabetes, these cells are destroyed by persistent hyperglycemia, high lipid levels, expression of inflammatory mediators, or oxidative stress [24]. This factors induce pancreatic β-cell apoptosis, leading to the inhibition of insulin secretion in animal models of diabetes mellitus [7,25]. Thus, maintaining pancreatic β-cell function is an important therapeutic strategy for the prevention and treatment of diabetes.

Treatment with STZ experimentally induces pancreatic β-cell apoptosis via DNA strand breaks and the production of ROS in cells [3]. The protective effects of the MeOH extract of A. annulatum and its fractions against STZ-induced damage were evaluated in INS-1 cells as STZ was previously reported to induce damage at 30mM for 1 h in INS-1 cells [26], and 20 mM or 12mM for 24 h in NIT-1 insulinoma cells [14,27]. In this study, a much lower concentration of STZ (50μM) was used to induce damage in INS-1 cells, while treatment with the MeOH extract of A. annulatum and its fractions (Hex, EA, BuOH, and water fractions) exerted protective effects. Hypoxylonol C and BNT isolated from A. annulatum showed protective effects in INS-1 cells, which were

Fig. 4. Effect of hypoxylonol C and BNT on streptozotocin-induced oxidative stress and apoptosis in INS-1 cells. Visualization of (A) cell morphology, (B) DCF-positive cells (green color: intracellular ROS accumulation), Scale bar, 100μm, (C) apoptotic (green circles), live (blue circles), necrotic cells (red circles) (40× magnification). (D) Bar graph showing percentage of apoptotic, live, necrotic cells. (E) Bar graph showing the fold increase in the intracellular ROS accumulation.

*p < 0.05 compared to streptozotocin-treated cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
accompanied by the improvement of characteristic changes in cell morphology related to apoptosis including cellular shrinkage, blebbing, or swelling. The representative fluorescence imaging revealed that DCF-positive cells were increased by STZ treatment, while treatment with hypoxylonol C and BNT reduced this effect. The results revealed that hypoxylonol C and BNT exert antioxidant effect against STZ-induced oxidative stress in INS-1 cells. In addition, the exposure of INS-1 cells to STZ significantly increased the apoptotic cell death after nuclear condensation, while treatment with hypoxylonol C and BNT reduced the percentage of apoptotic cell death.

In addition, exposure of INS-1 cells to STZ significantly increased the number of apoptotic cells stained with annexin V after nuclear condensation, while treatment with hypoxylonol C and BNT reduced the percentage of apoptotic cells. Furthermore, in the present study, we investigated the molecular mechanisms underlying the protective effects of 100μM hypoxylonol C and 100μM BNT in STZ-treated INS-1 cells. Studies on STZ-induced apoptosis in pancreatic β-cells have reported that STZ may enter pancreatic β-cells and lead to DNA strand breaks, as well as induce the activation of PARP, which is known to initiate the apoptotic pathway [7,28,29]. In the present study, we also demonstrated that treatment with STZ increased the expression of PARP, which is known to initiate the apoptotic pathway [7,28,29]. In the present study, we also demonstrated that treatment with STZ increased the expression of PARP, which is known to initiate the apoptotic pathway [7,28,29]. In the present study, we also demonstrated that treatment with STZ increased the expression of PARP, which is known to initiate the apoptotic pathway [7,28,29]. Our study revealed that caspase-8 was activated by STZ treatment, while treatment with hypoxylonol C and BNT reversed this effect. The decreasing the ratio of the expression of pro-apoptotic to anti-apoptotic Bcl-2 family members is also characteristics of apoptosis in pancreatic β-cell [5,32]. Here, we also demonstrated that treatment with STZ increased the expression of Bax and decreased the expression of Bcl-2, while treatment with hypoxylonol C and BNT reversed this. This indicated that STZ-induced the increase in the ratio of Bax to Bcl-2 was diminished by hypoxylonol C and BNT. Increased ratio of Bax to Bcl-2 up-regulates caspase-3 which is the effector caspase in both the extrinsic and intrinsic apoptotic pathways in STZ-treated pancreatic β-cells [12,29,30,33]. In line with a previous study, STZ treatment increased the cleavage of caspase-3, while treatment with hypoxylonol C and BNT reversed this effect. Together, these results indicate that hypoxylonol C and BNT significantly inhibited STZ-induced apoptotic pancreatic β-cell death.

5. Conclusions

Our findings confirmed that hypoxylonol C and BNT isolated from A. annulatum protect pancreatic β-cells from STZ-induced apoptotic damage via inhibiting oxidative stress and preventing the activation of apoptotic signal cascades. These results encourage further studies involving novel agents which have beneficial effects on the improvement of diabetic β-cell function.
Declaration of Competing Interest

The authors declared that there is no conflict of interest.

Acknowledgements

This work was supported by the Korea Institute of Science and Technology (KIST) institutional program (Project No. 2205610), and by the Basic Science Research Program of the National Research Foundation of Korea (NRF), funded by the Ministry of Science, ICT & Future Planning (2017R1C1B5015841, 2019R1F1A1059173).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioro.2019.103053.

References