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ORIGINAL ARTICLE

Schizandra arisanensis extract attenuates cytokine-mediated cytotoxicity in insulin-secreting cells

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Abstract

AIM: To explore the bioactivity of an ethanolic extract of *Schizandra arisanensis* (SA-Et) and isolated constituents against interleukin-1β and interferon-γ-mediated β cell death and abolition of insulin secretion.

METHODS: By employing BRIN-BD11 cells, the effects of SA-Et administration on cytokine-mediated cell death and abolition of insulin secretion were evaluated by a viability assay, cell cycle analysis, and insulin assay. The associated gene and protein expressions were also measured. In addition, the bioactivities of several peak compounds collected from the SA-Et were tested against cytokine-mediated β cell death.

RESULTS: Our results revealed that SA-Et dose-dependently ameliorated cytokine-mediated β cell death and apoptosis. Instead of suppressing inducible nitric oxide synthase/nitric oxide cascade or p38MAPK activity, suppression of stress-activated protein kinase/c-Jun NH2-terminal kinase activity appeared to be the target for SA-Et against the cytokine mix. In addition, SA-Et provided some insulinotropic effects which re-activated the abolished insulin exocytosis in cytokine-treated BRIN-BD11 cells. Finally, schiarisanrin A and B isolated from the SA-Et showed a dose-dependent protective effect against cytokine-mediated β cell death.

CONCLUSION: This is the first report on SA-Et ameliorating cytokine-mediated β cell death and dysfunction *via* anti-apoptotic and insulinotropic actions.

Key words: *Schizandra arisanensis*; C_{19} homolignans; Type 1 diabetes; Insulin-secreting cells; Interleukin-1β; Interferon-γ

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INTRODUCTION

The onset of type 1 diabetes occurs when approximately 60%-90% of insulin secreting cells are lost and/or are dysfunctional due to β-cell directed autoimmunity[1].

During the progression of autoimmunity, heavy infiltrations of mononuclear cells lead to substantial damage to β cells by generating locally high concentrations of pro-inflammatory cytokines, perforin, and FasL-Fas interactions within the micro-environment of islets[2,3]. As a result, deleterious outcomes include excessive proinsulin secretion, the abolition of glucose-induced insulin secretion, and ultimately β cell death[4].

Currently, scientists have developed a wide range of approaches to prevent or intervene in type 1 diabetes by stimulating β-cell proliferation, attenuating the cytotoxic effects of inflammatory agents, or modulating autoimmune response, and so on[1]. While many clinical trials are ongoing, the application of herbal medicine remains to be fully explored.

In cell culture systems, exposing β cells to an interleukin (IL)-1β and interferon (IFN)-γ mixture can mimic the consequences of an immune attack in type 1 diabetes, which affects pro-insulin secretion[5] and induces β cell death[6]. By employing such a platform, potential β cell protective herbal material can be identified. *Schizandra arisanensis* (SA-Et) is one of the schizandraceous plants from Taiwan. This plant also contains various C_{18} dibenzocyclooctadiene lignans and C_{19} homolignans[7,8]. The indications for this herb in traditional Chinese medicine include diabetes, hepatitis, immunomodulation, and cancer[7,9-11]. Therefore, in the present study, we investigated the potential β cell protective bioactivity of the ethanol extract of SA-Et, including its isolated constituents against cytokine-mediated β cell cytotoxicity and dysfunction.

MATERIALS AND METHODS

Plant material and reagents

Stems of SA-Et were collected and authenticated by Dr. Kuo YH in October 2005 in Chiayi County, Taiwan. A voucher specimen (No. NRICM20051003) was deposited at the National Research Institute of Chinese Medicine. The following were used as positive controls in the various biological assays performed; recombinant cytokines (IL-1β and IFN-γ; PeproTech, NJ, United States); epigallocatachin gallate (EGCG; Fluka, MO, United States); nitro-L-arginine methyl ester (L-NAME), glucose, KCl and $CaCl_2$ (Sigma-Aldrich Corp., MO, United States). These reagents were obtained at the highest purity available (> 97%) from the suppliers indicated.

Preparation of the ethanol extract of SA-Et

Dried stems of SA-Et were ground and then extracted with 95% (v/v) EtOH at 45 ℃ three times for 48 h each time. Under reduced pressure, the combined ethanol extracts were concentrated to an ethanolic extract residue of SA-Et. DMSO was employed as the dissolving reagent, and aliquots were prepared and stored at -20 ℃ before the biological assays were carried out.

High-performance liquid chromatographic analysis of the SA-Et

A filtered volume (100 μL) of the SA-Et solution (1.0 mg/mL in methanol) was prepared and injected into a reverse-phase high-performance liquid chromatography (HPLC) system. The HPLC analysis was performed using a Waters™ HPLC (cont 600 Pump, 996 photodiode array detector, 600 controller, and 717 plus autosampler; Milford, Massachusetts, United States), using a reverse-phase RP-18 column (4.6 mm × 250 mm *i.d.*). The solvent system used was a gradient of water and CH_3CN. The gradient system was as follows: 0-60 min, 45%-60% CH_3CN, and 60-70 min, 60%-75% CH_3CN; with elution performed at a solvent flow rate of 1 mL/min. The detection chromatogram was recorded at 215 nm.

Isolation of C_{19} homolignan and C_{18} lignan compounds

Peak compounds including schiarisanrin A (peak 5), schiarisanrin B (peak 1), schiarisanrin C (peak 4), schiarisanrin E (peak 3), and macelignan (peak 6) were obtained from the SA-Et followed by the procedures described below. Isolation of taiwanschirin A (peak 2) was based on a previous method[12].

Five grams of SA-Et was partitioned with water and *n*-hexane, to give the *n*-hexane residue (SA-Et-H). Further separations of SA-Et-H (100 mg) were performed on a Waters™ HPLC, using a preparative Cosmosil 5C_{18} AR-Ⅱ column (250 mm × 10 mm *i.d.*; Nacalai Tesque, Kyoto, Japan), employing a gradient system [water (A) and CH_3CN (B), mobile phase was as follows: 0-2 min, 45%-50% (B); 2-20 min, 50%-55% (B), 20-55 min, 55%-60% (B), 55-60 min, 60%-70% (B), and 60-70 min, 70%-100% (B)], at a flow rate of 3 mL/min, under 215 nm UV, to yield schiarisanrin A (4.6 mg), schiarisanrin B (4.1 mg), schiarisanrin C (5.6 mg), schiarisanrin E (6.3 mg), macelignan (11.5 mg), and schizanrin A (3.6 mg), which were identified by comparison with authentic samples and reported spectroscopic data (UR, infrared, and nuclear magnetic resonance) in the literature[8,12]. Their purity was over 75% as analyzed by HPLC.

Cytotoxicity test

BRIN-BD11 cells were routinely cultured in RPMI-1640 containing 10% (v/v) fetal bovine serum and 2 g/L glucose[13]. For the cytotoxicity test, BRIN-BD11 cells were seeded into 24-well plates at the density of 5×10^4 cells/well. After overnight attachment, cells were incubated with cytokines according to the figure legends. The viability at the end of treatment was measured by a neutral red assay as described previously[14]. The absorbance under control conditions was set to 100% viability.

Cell cycle analysis

At the end of treatment, cells were trypsinized and collected by centrifugation. Following a phosphate-buffered saline (PBS) wash, cell pellets were resuspended in ethanol overnight at -20 ℃. Fixed cells were then washed with PBS before being resuspended in 1% (v/v) Triton X-100 and incubated for 15 min. The cells were then resuspended in propidium iodide (PI) staining solution, consisting of 20 μg/mL PI, 50 μg/mL RNase A, and 0.0001% (v/v) Triton X-100 in PBS, and incubated at 4 ℃ for 30 min

Table 1 Sequence of primer sets for conventional reverse transcription-polymerase chain reaction

β-actin	
Forward	5′-CGTAAAGACCTCTATGCCAA-3′
Reverse	5′-AGCCATGCCAAATGTGTCAT-3′
Glucokinase	
Forward	5′-AAGGGAACTACATCGTAGGA-3′
Reverse	5′-CATTGGCGGTCTTCATAGTA-3′
Insulin	
Forward	5′-TGCCCAGGCTTTTGTCAAACAGCACCTT-3′
Reverse	5′-CTCCAGTGCCAAGGTCTGAA-3′
iNOS	
Forward	5′-TTTTCACGACACCCTTCACC-3′
Reverse	5′-GACCTGATGTTGCCACTGTTAG-3′

iNOS: inducible nitric oxide synthase.

with protection from light. Finally, the cells were passed through a mesh prior to cell cycle analysis by employing FACSan (BD Bioscience, San Jose, California, United States).

Western blot analysis

This procedure followed a previous methodology[15]. In brief, cells were washed with ice-cold PBS and scraped into ice-cold lysis buffer. Cell debris was removed by centrifugation. Equal amounts of protein (40 g) were subjected to separation on sodium dodecylsulfate 10% polyacrylamide gels. Following transfer to nitrocellulose membranes, blots were blocked with 5% (w/v) non-fat milk in Tris-buffered saline containing 0.1% (v/v) Tween 20 for 1 h, and incubated with primary antibodies at 4 ℃ overnight prior to incubation for 1 h at room temperature with the secondary antibody. Finally, results were visualized after development of films with the aid of an enhanced chemiluminescence kit (Amersham Biosciences, Uppsala, Sweden).

Measurement of gene expression

Total RNA was extracted using the TRI-reagent according to the manufacturer's instructions. Total RNA (1 μg) was reverse-transcribed to generate templates. Complementary (c) DNA at 50 ng was employed for the polymerase chain reaction (PCR). The sequences of primers are listed in Table 1. The annealing temperatures for amplification of β-actin (57 ℃), glucokinase (57 ℃), insulin (52 ℃), and inducible nitric oxide synthase (iNOS) (55 ℃) were employed to generate respective sequences of 349 bp, 130 bp, 187 bp, and 441 bp. Once the reaction was complete, PCR products were separated by gel electrophoresis, visualized, photographed using a digital camera, and quantified with Genetools 3.06 (Syngene, Cambridge, United Kingdom). When iNOS mRNA was determined by real-time reverse transcription-PCR, 1 μg of total RNA was reverse transcribed into cDNA using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Maryland, United States) before being applied to the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Warrington, United Kingdom). Each reaction was carried out based on the description of the TaqMan® Gene Expression Assay kit with pre-designed probes for iNOS (Rn00561646_m1) and β-actin (Rn00667869_m1). Data were ct values (i.e., cycle number where logarithmic PCR plots cross a calculated threshold line). For comparative quantification, Δct values were firstly calculated using the formula: Δct = (ct of iNOS) - (ct of β-actin). Then the ΔΔct value was determined using the following formula: ΔΔct = (Δct of each sample) - (average Δct of control samples). Fold increase in iNOS level under each condition was presented as $2^{-\Delta\Delta ct}$.

Acute insulin-secretion test

The acute insulin-secretion test was as previously described[14]. In brief, the culture medium was discarded and the cells were washed twice with 1 mL Krebs-Ringer bicarbonate (KRB) buffer. The cells were then pre-incubated in KRB for 40 min at 37 ℃. Pre-incubation buffer was then poured off followed by the addition of 1 mL of test medium per well at a constant rate. After 20 min, the test medium was collected at a constant rate and stored at -20 ℃ prior to insulin determination by a rat insulin enzyme-linked immunosorbent assay (ELISA) kit (Linco Research, St. Charles, Missouri, United States). The limit of sensitivity of the assay was 0.2 ng/mL (35 pmol/L) insulin using a 10 μL sample. The appropriate range of the insulin assay was 0.2 ng/mL to 10 ng/mL insulin (10 μL sample size). Samples with results greater than this range were diluted with sample buffer in order to fit the range of the standard curve.

Determination of nitrite production

Test samples at 100 μL were mixed with 1.32% sulfanilamide (50 μL) in 60% acetic acid and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride HCl (50 μL) in distilled water. After 10 min incubation, the absorbance was read on a spectrophotometer at 540 nm. Actual concentrations were calculated from an absorbance *vs* nitrite (μmol/L) standard curve.

Statistical analysis

The significance of various treatments was determined by the Student's unpaired *t*-test under non-parametric statistical conditions. Results are expressed as the mean ± SE. Differences were considered significant at $P < 0.05$.

RESULTS

As shown in Figure 1, HPLC analysis of the ethanolic extract of SA-Et was carried out, and seven major peaks were obtained and identified as schiarisanrin B, taiwanschirin A, schiarisanrin E, schiarisanrin C, schiarisanrin A, macelignan, and schizanrin A, respectively, by matching them to authentic samples. In addition, the model of cytokine-mediated β cell destruction was established using BRIN-BD11 cells. As shown in Figure 2A, a synergistic effect on cytotoxicity was only observed when IL-1β was mixed with IFN-γ. Maximum cell death occurred at 48 h when cells were treated with IL-1β (2 ng/mL) and IFN-γ (100 ng/mL). As shown in Figure 2B, the pres-

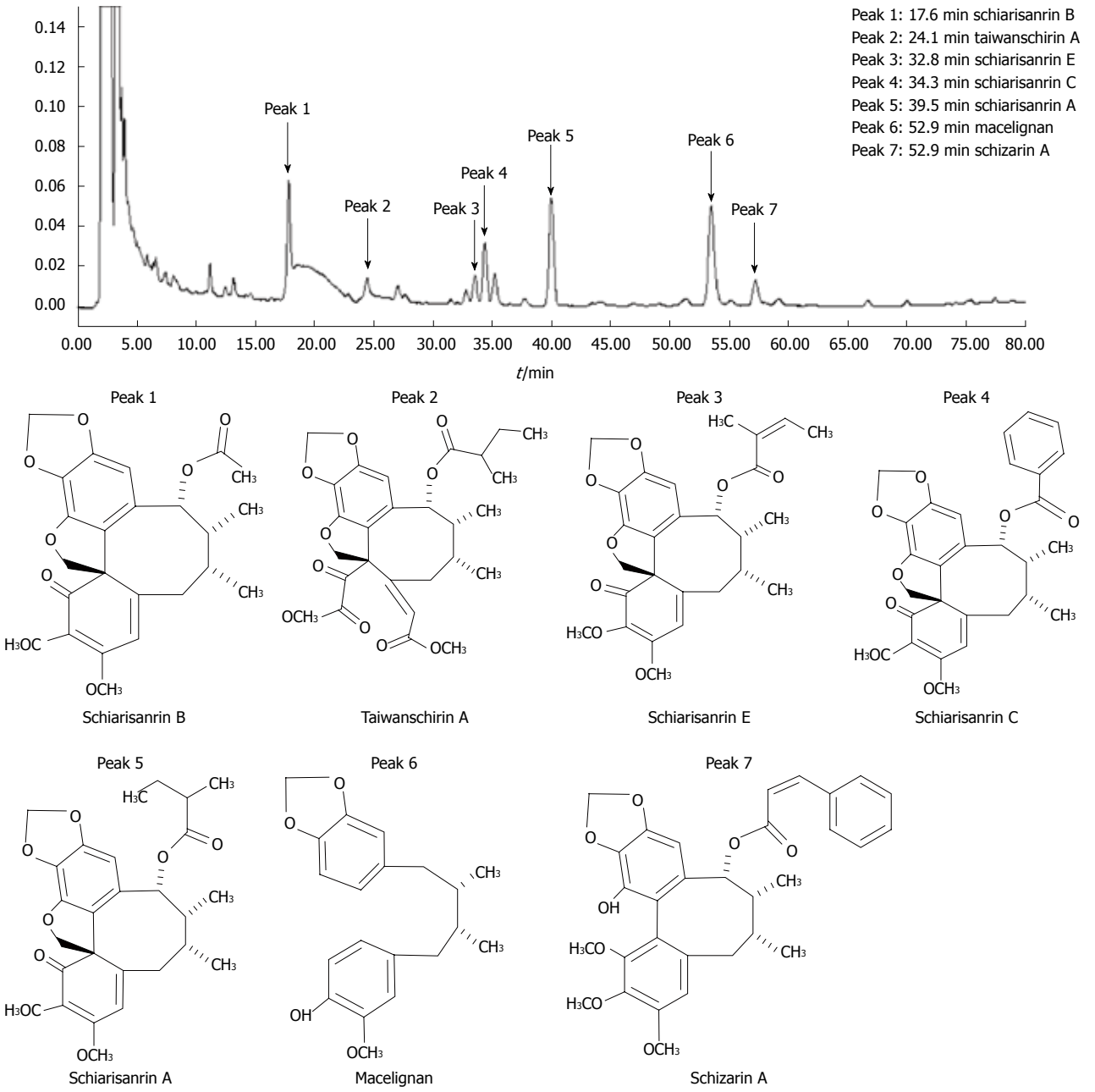

Figure 1 Chemical fingerprinting of the ethanolic extract of *Schizandra arisanensis*. The ethanolic extract of *Schizandra arisanensis* (SA-Et) (1.0 mg) was subjected to a high-performance liquid chromatography system to obtain chemical fingerprints. According to the retention times, the chemical identities and structures of seven major peaks are listed.

ence of IFN-γ alone resulted in significant G_1 arrest ($P < 0.05$). In contrast, the presence of IL-1β alone caused significant inhibition at S phase. However, when IFN-γ was combined with IL-1β, the accumulated G_1 arrested cells appeared to have progressed into the subG_1 phase ($P < 0.01$) at the end of cytokine treatment. By affecting the mechanisms of IL-1β + IFN-γ using various inhibitors, our results showed that IL-1β + IFN-γ-mediated cytotoxicity could be attenuated by SP600125 and SB203580. On the other hand, the presence of U0126 and L-NAME had no beneficial effects on cell viability. Moreover, the viability of cytokine-treated cells was worse in the presence of wortmannin (Figure 2C).

By employing our cell model, as shown in Figure 3A, the SA-Et at 20 μg/mL provided β cell protective activity as shown by an increase of approximately 1.3-fold ($P < 0.01$) in the viability in the cytokine treated condition. Consistently, induction of the subG_1 phase in the presence of IL-1β + IFN-γ was attenuated by the SA-Et (Figure 3B). Finally, full-length caspase-3 degradation in the presence of IL-1β + IFN-γ was observed and was parallel to the increase in cleaved caspase-3 (active form) of about 1.2-fold at 24 h post-treatment with IL-1β + IFN-γ (Figure 3C). However, while SA-Et alone reduced the cleaved form of caspase-3 by 30%, the protease-mediated caspase-3 activation by IL-1β + IFN-γ was also ameliorated by SA-Et.

Following determination of the protective effect of

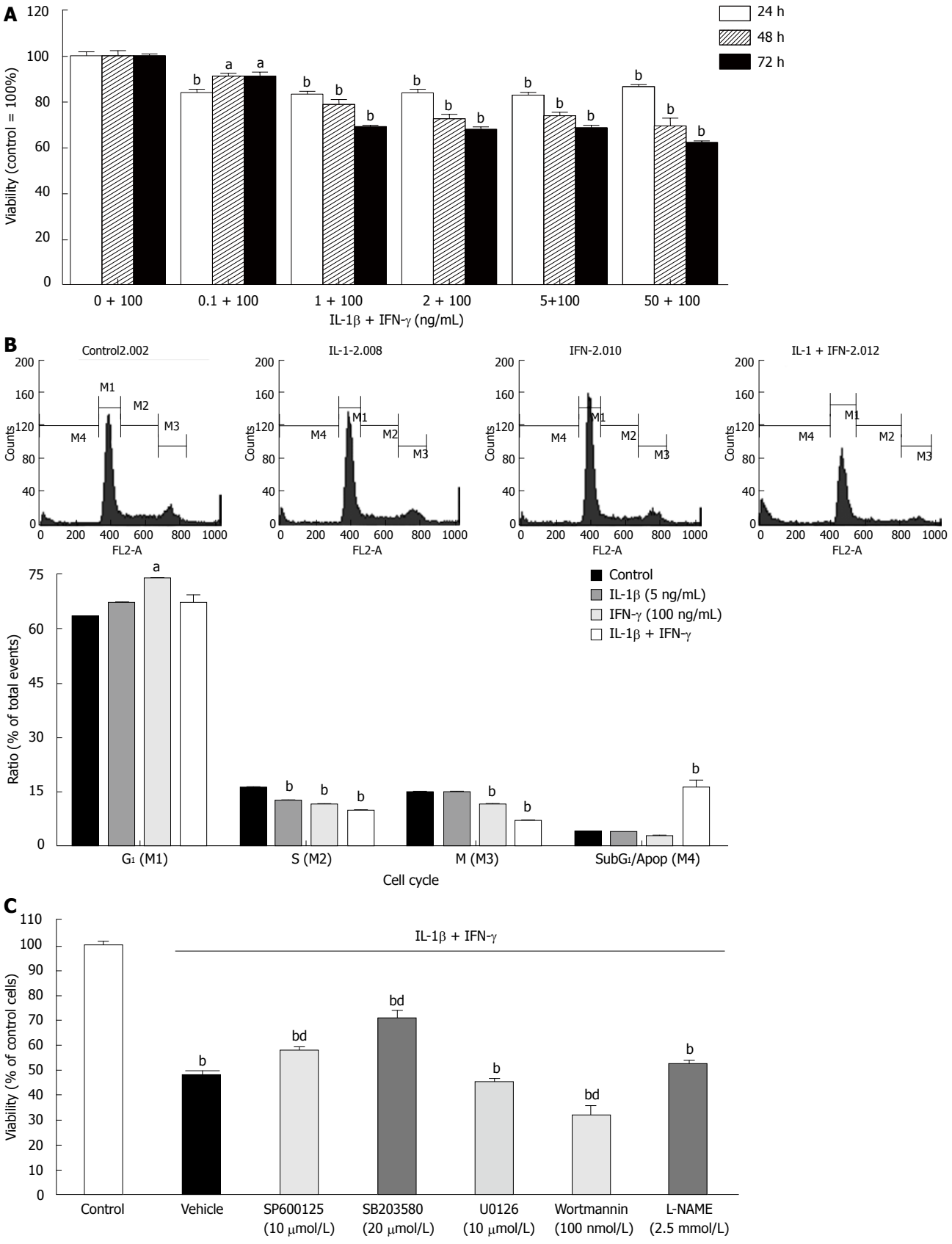

Figure 2 Interleukin-1β and interferon-γ-mediated BRIN-BD11 cell apoptosis is attenuated by p38MAPK and stress-activated protein kinase/c-Jun NH2-terminal kinase inhibitors. A: The viability of interferon (IFN)-γ (100 ng/mL)-treated cells in the presence of various concentrations of interleukin (IL)-1β for 24 h, 48 h and 72 h was measured. Data are presented as the mean ± SE, $n = 4$. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* the viability of untreated cells; B: Cell cycle analysis was carried out at 48 h post-treatment with IL-1β (5 ng/mL), IFN-γ (100 ng/mL), or a mixture of the two. Data are presented as the mean ± SE, $n = 4$. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* the control; C: The viability of cytokine-treated BRIN-BD11 cells in the presence of inhibitors was measured. Data are presented as the mean ± SE, $n = 4$. $^{b}P < 0.01$ *vs* the viability of untreated cells; $^{d}P < 0.01$ *vs* the viability of cells with the cytokine mix.

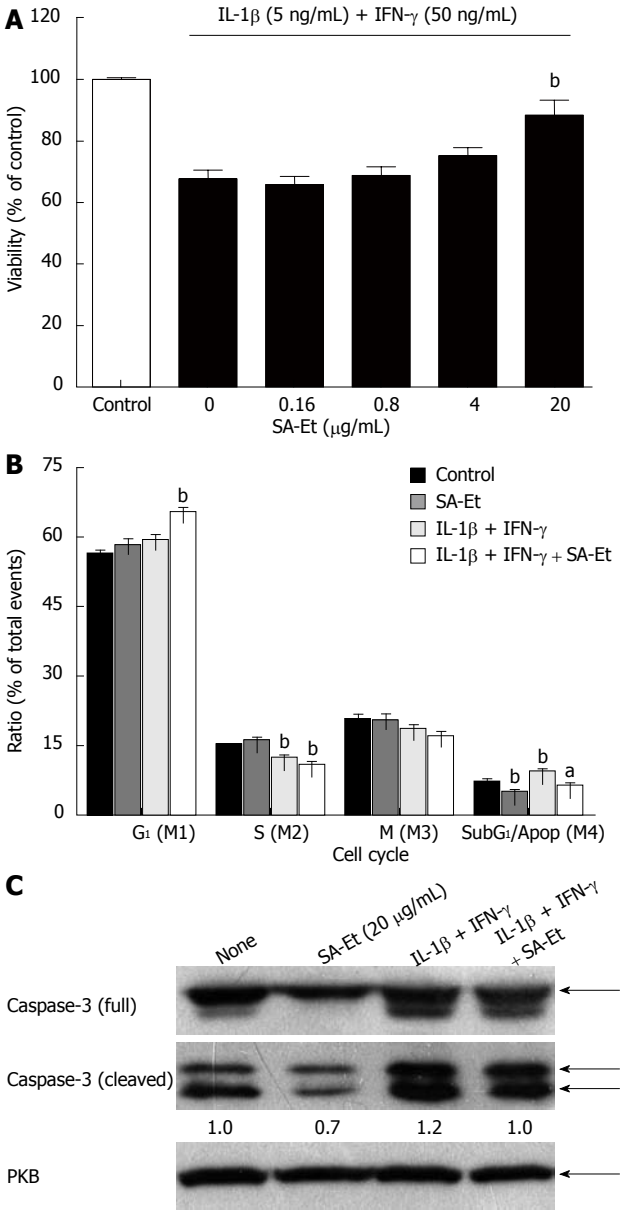

Figure 3 The anti-apoptotic effect of the ethanolic extract of *Schizandra arisanensis*. A: The viability of BRIN BD11 cells was determined after 48 h of cytokine treatment in the presence or absence of the ethanolic extract of *Schizandra arisanensis* (SA-Et); B: Cell cycle of BRIN BD11 cells was determined after 48 h of cytokine treatment in the presence or absence of the SA-Et. Data are presented as the mean ± SE, $n = 4$. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* the control; C: Both full and cleaved forms of caspase-3 protein were analyzed by Western blot after 24 h of cytokine treatment in the presence or absence of the SA-Et. A representative from three experiments is shown. IL-1β: Interleukin 1β; IFN-γ: Interferon-γ.

SA-Et, we further examined the impact of SA-Et on signal transduction of IL-1β + IFN-γ. As shown in Figure 4A, stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK), p38MAPK, and STAT-1α activation in cytokine-treated cells was evident, while ERK1/2 was not activated. In the presence of SA-Et, the phosphorylation of SAPK/JNK (at T183/Y182) and its substrate c-Jun (at S63) was attenuated by this treatment, while other pathways appeared to be unaffected by SA-Et.

In addition, cytokine treatment also promoted IκBα degradation which was unaffected by the addition of SA-Et (Figure 4B). Consistently, the presence of SA-Et (20 μg/mL) was unable to inhibit cytokine-mediated iNOS mRNA through cytokine induction (Figure 4C and D). Interestingly, SA-Et administration up to 20 μg/mL provided minor, but significant inhibition of cytokine-mediated NO formation. However, such inhibition did not reach the level of the known iNOS inhibitor, L-NAME, which provided > 50% of the inhibition of cytokine-mediated NO production (Figure 4E).

In terms of SA-Et treatment on β-cell function with or without cytokine challenge (Figure 5A), SA-Et treatment enhanced the insulinotropic effects of glucose and extracellular Ca^{2+}, and modestly but significantly affected the insulinotropic effect of extracellular K^{+}. As shown in Figure 5B, glucose responsiveness of BRIN-BD11 cells was unaffected by SA-Et alone. On the other hand, a detectable amount of secreted insulin under a basal or high concentration of glucose was not detected in surviving BRIN-BD11 cells following cytokine treatment. In contrast, we detected partial secretion of insulin from cytokine-treated BRIN-BD11 cells in the presence of SA-Et ($P < 0.01$).

When determining the insulin level in BRIN-BD11 cells under the indicated conditions, there was a modest reduction in insulin mRNA expression in the presence of SA-Et or the cytokine mix 48 h post-treatment (Figure 5C). In addition, insulin protein expression and the intracellular insulin content of BRIN-BD11 cells were consistent with each other (Figure 5D). Cellular insulin protein level appeared to be similar regardless of the addition of SA-Et. In contrast, cellular insulin contents in cytokine-treated cells were significantly elevated ($P < 0.001$). Interestingly, insulin content in cytokine-treated BRIN-BD11 cells with SA-Et treatment was significantly reduced ($P < 0.05$) compared with that in untreated cells.

Finally, we compared the biological activities of four major peaks as shown in Figure 6. Epigallocatechin gallate (EGCG) was employed as a reference drug which has been shown to protect against cytokine-mediated β cell death[16]. Our results indicated that both peak 1 (schiarisanrin B) and peak 5 (schiarisanrin A) provided β cell protective bioactivity similar to EGCG (20 μg/mL). The protective potency of schiarisanrin B reached its maximum at a concentration of 5 μg/mL, and higher concentrations led to a deleterious outcome. On the other hand, schiarisanrin A provided a dose-dependent protective effect starting at a concentration of 5 μg/mL ($P < 0.01$). Peak 4 (schiarisanrin C) led to cell death in a dose-dependent manner. Different from C_{19} homolignans, the C_{18} lignan, peak 6 (macelignan), played no role in cytokine-mediated cytotoxicity at any concentration.

DISCUSSION

Conventional therapy in patients with type 1 diabetes does not allow minute-to-minute control of blood glucose and does not prevent complications associated with the disease. Whole pancreas or islet transplantation facilitates glucose control on a real-time basis but the lack of

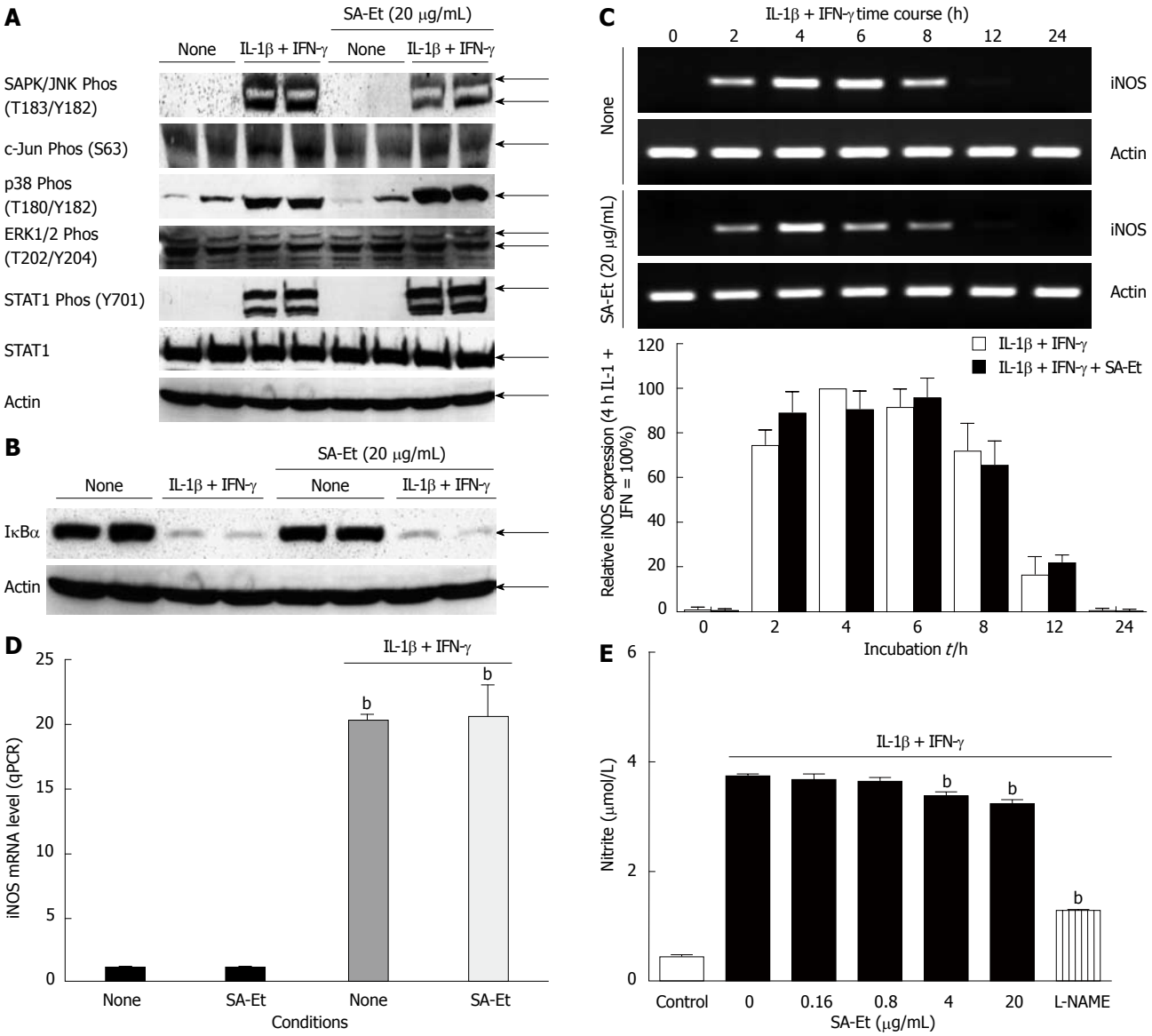

Figure 4 Characterizing the impact of ethanolic extract of *Schizandra arisanensis* on cytokine signal transduction. A: BRIN-BD11 cells stimulated with the cytokine mix in the presence or absence of ethanolic extract of *Schizandra arisanensis* (SA-Et) (20 μg/mL) were harvested after 30 min treatment for Western blot with the indicated antibodies. A representative Western blot from three experiments is shown; B: BRIN-BD11 cells stimulated with the cytokine mix in the presence or absence of SA-Et were harvested after 60 min treatment to measure IκBα protein level. A representative Western blot from three experiments is shown; C: Cytokine-induced inducible nitric oxide synthase (iNOS) mRNA levels in BRIN-BD11 cells during 24 h were determined by an reverse transcription-polymerase chain reaction. Relative *iNOS* gene expression was calculated by employing endogenous β-actin mRNA level as an internal control. The maximum *iNOS* gene expression at 4 h post-treatment with the cytokines alone was set to 100%. Data are presented as the mean ± SE, $n = 4$; D: Changes in cytokine-induced iNOS mRNA levels in BRIN-BD11 cells at 4 h in the presence or absence of the SA-Et (20 μg/mL) were also determined by real-time reverse transcription-polymerase chain reaction. β-actin was used as an internal control. The relative quantification of iNOS mRNA was presented as $2^{-\Delta\Delta ct}$. Data are presented as the mean ± SE, $n = 3$. $^{b}P < 0.01$ *vs* cells under control conditions; E: Nitric oxide production in cytokine-treated BRIN-BD11 cells in the presence or absence of the SA-Et (0-20 μg/mL) or nitro-L-arginine methyl ester (L-NAME) (0.5 mmol/L) was determined. Data are presented as the mean ± SE, $n = 4$. $^{b}P < 0.01$ *vs* cells treated with the cytokine mixture alone. IL-1β: Interleukin 1β; IFN-γ: Interferon-γ.

sufficient organs for transplantation is a major obstacle. Employing toxin-resistant cells for type 1 diabetes therapy is another developing approach in cell-based therapies. However, disassociation between toxin resistance and insulin-secretory functions may occur during the induction of toxin resistance[17]. Moreover, to our knowledge, no "master" toxin-resistant cells have been created so far[17-19]. As a result, supplementation with β cell protective phytochemicals to enhance the survival rate and secretory functions of cell-based therapeutics for curing type 1 diabetes is an interesting and flexible approach[20,21].

To pursue this objective, we created a simple platform by employing BRIN-BD11 cells. The use of a cytokine mix successfully promoted β cell death and abolished glucose-stimulated insulin secretion after 48 h of treatment. Through cell cycle analysis, it was apparent that only the combination of IL-1β and IFN-γ elicited apoptosis as judged by caspase 3 cleavage and a significant increment of the subG$_1$ population in BRIN-BD11 cells under such conditions. In the present investigation, the anti-apoptotic effect of SA-Et against cytokine-mediated β cell death was clearly demonstrated. Consistent with the restoration

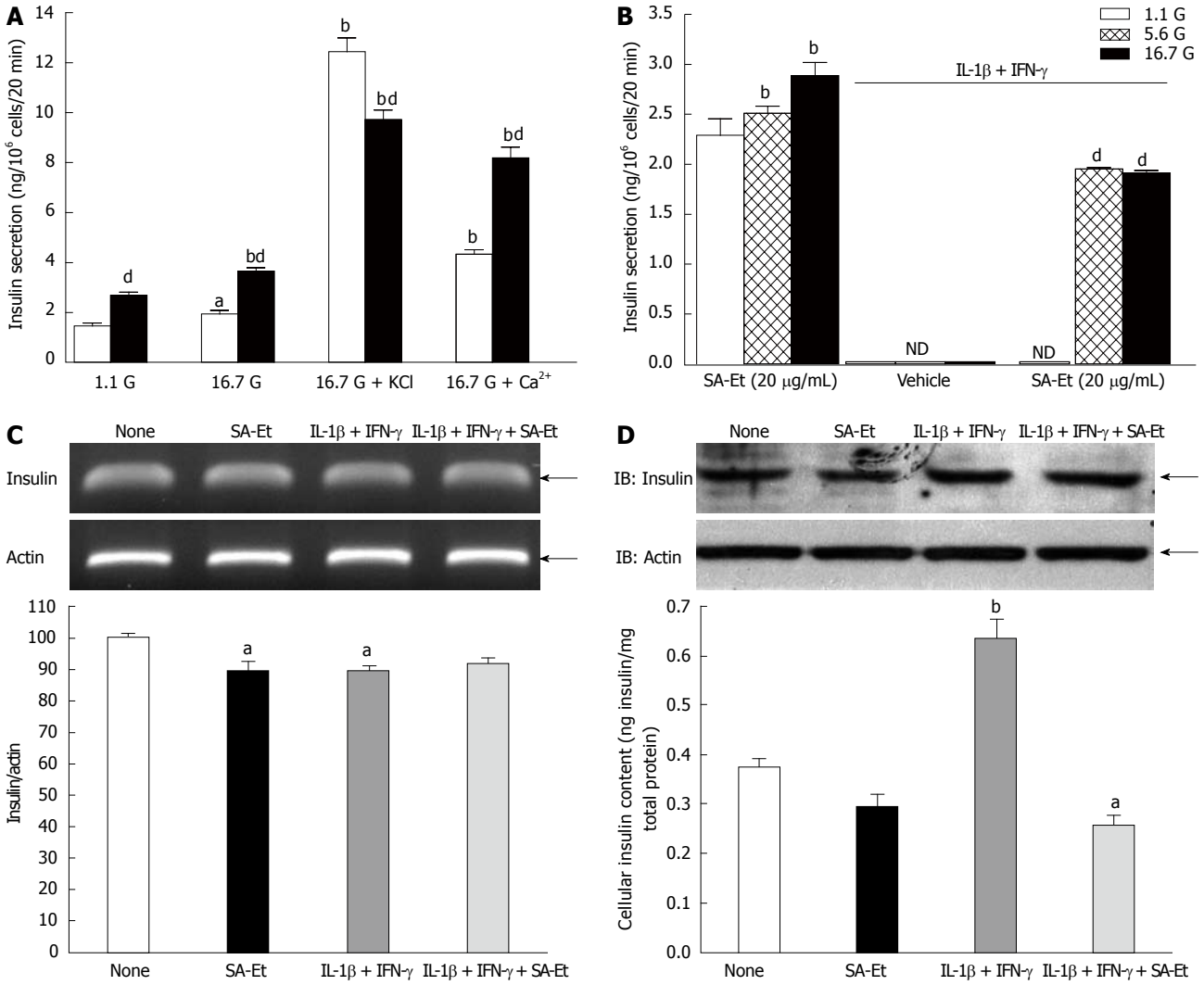

Figure 5 Ethanolic extract of *Schizandra arisanensis* treatment partially rescued the abolished insulin secretion in cytokine-treated BRIN-BD11 cells. A: Acute insulin release in response to glucose, KCl and Ca^{2+} in the presence or absence of the ethanolic extract of *Schizandra arisanensis* (SA-Et) was evaluated. Data are presented as the mean ± SE, $n = 4$. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* insulin released from cells treated with 1.1 mmol/L glucose; $^{d}P < 0.01$ *vs* control cells under the same conditions; B: Cultured BRIN-BD11 cells were treated with the cytokine mixture in the presence or absence of the SA-Et. At 48 h post-treatment, treated cells were evaluated for glucose responsiveness. Data are presented as the mean ± SE, $n = 4$. $^{b}P < 0.01$ *vs* insulin released from cells treated with 1.1 mmol/L glucose; $^{d}P < 0.01$ *vs* SA-Et-treated cells under the same glucose conditions. C: In addition, a portion of the treated cells was harvested to measure insulin mRNA level using reverse transcription-polymerase chain reaction. Relative insulin gene expression was calculated by employing endogenous β-actin mRNA level as an internal control. $^{a}P < 0.05$ *vs* control conditions (none); D: Protein level and cellular insulin content were independently measured by Western blot and enzyme-linked immunosorbent assay. Data are presented as the mean ± SE, $n = 4$. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control conditions (none). IL-1β: Interleukin 1β; IFN-γ: Interferon-γ; ND: Not determined.

of cell viability, the ratio of the subG$_1$ phase and protein level of cleaved caspase-3 in the cytokine-treated condition was blocked or reduced by SA-Et.

According to previous research, NFκB, p38MAPK, and SAPK/JNK pathways all play a role in cytokine-mediated cytotoxicity in β cells. Interestingly, unlike other cell lines, NFκB downstream iNOS/NO induction is not responsible for apoptosis of BRIN-BD11 cells. Instead, insulin secretion in response to glucose was significantly affected by NO production in BRIN-BD11 cells[22]. Our results are consistent with previous findings and further demonstrated that p38MAPK and JNK/SAPK play important roles in cytokine-mediated BRIN-BD11 cell death.

Using this cell model, we screened several anti-diabetic herbal extracts and discovered that SA-Et has protective bioactivity. According to the HPLC profile of the SA-Et, seven major peaks were identified: five C$_{19}$ homolignans (schiarisanrins) and two C$_{18}$ lignans (schizanrin A and macelignan). Through bioactivity screening, it was evident that some C$_{19}$ homolignans could account for the β cell protective effects of the SA-Et against the cytokine challenge. To our knowledge, this is the first report to reveal such bioactivity in C$_{19}$ homolignans. The results also implied that C$_{19}$ homolignans which possess an acetoxyl group at C-5 exhibited more-potent bioactivity than did those with the benzonic acid ester at C-5, regardless of the different C$_{19}$ homolignan skeletons as shown in Figure 1. Notably, schiarisanrin C exhibited dose-dependent negative protection activity, which was consistent with a previous report which found that schiarisanrin C exhibited cytotoxicity against other cell lines[8]. The structure-

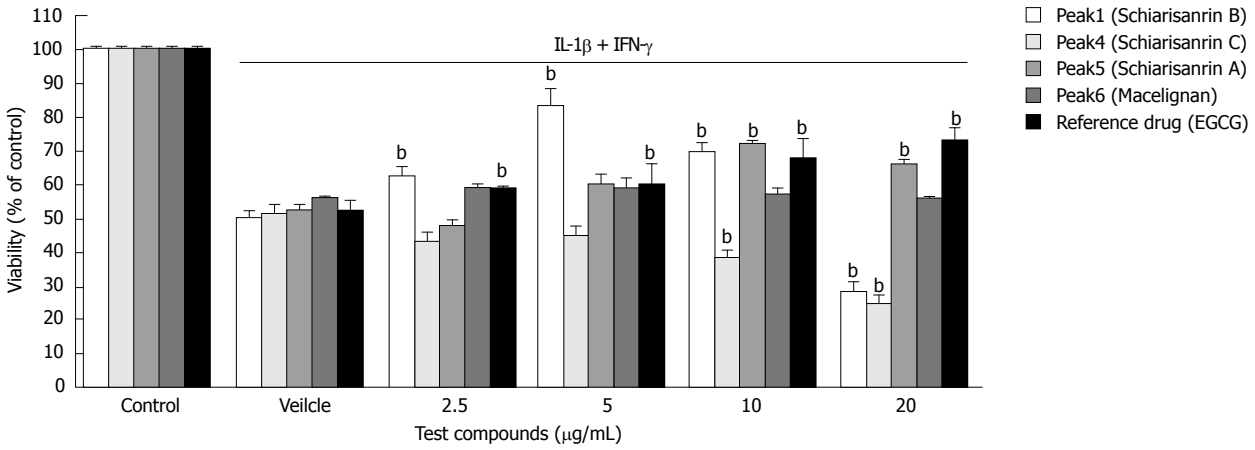

Figure 6 Identification of bio-active compounds isolated from *Schizandra arisanensis*. After major peak compounds were collected, BRIN-BD11 cells were treated with cytokine mix in the presence of each peak compounds for 48 h. The viability of cells was then measured by neutral red assay. Epigallocatechin gallate (EGCG) is used as reference drug. Data are presented as the mean ± SE, $n = 8$. $^{b}P < 0.01$ vs the viability of cells with no treatment.

activity relationship will likely be more obvious when more types of C_{19} homolignan derivatives are compared.

To further elucidate the potential drug target of SA-Et, we evaluated the effects of SA-Et on key players in each pathway involved in cytokine-mediated β cell death. The results indicated that SA-Et appeared to impact on JNK/SAPK kinase activities and downstream substrate, i.e., c-Jun, to facilitate its protective activity against the cytokine mix. Recent research discovered that the JNK/c-Jun cascade could transactivate the expression of Bcl-2 homology 3 (BH3)-only member death protein 5 (DP5)/harakiri (Hrk) leading to β cell apoptosis[23]; therefore, our results suggest that SA-Et treatment might intervene in such a cytokine pathway to facilitate β cell protection.

Interestingly, our results also demonstrated that SA-Et treatment had no inhibitory effect on cytokine-mediated NF-κB release judged by a similar pattern of cytokine-mediated IκB degradation and iNOS mRNA expression in the presence of SA-Et. NF-κB is an important player in abolishing glucose-stimulated insulin secretion (GSIS) by affecting the glucose-induced influx of Ca^{2+}[24,25]. In addition, IL-1β-mediated NO production also plays a negative role in insulin secretion in BRIN-BD11 cells[22]. Therefore, whether SA-Et can not only preserve the viability of cytokine-treated BRIN-BD11 cells, but also rescue the impaired insulin secretion of the cytokine-treated cells was our next question.

In terms of the insulinotropic action of SA-Et, our results indicate that SA-Et acutely enhanced both glucose- and calcium-stimulated insulin secretion, although K^+-stimulated insulin secretion appeared to be slightly reduced by SA-Et. As a result, we speculated that the insulinotropic effect of SA-Et might be associated with calcium-mediated insulin exocytosis, an overall proximal step in insulin secretion. In contrast, under cytokine treatment, insulin exocytosis rather than insulin mRNA expression appeared to be affected in BRIN-BD11 cells. This was based on the observations that insulin secreted by cytokine-treated BRIN-BD11 cells was undetectable using the insulin ELISA kit; however, the insulin mRNA and intracellular insulin content (protein level) of BRIN-BD11 cells remained intact. When SA-Et was present, the abolished insulin exocytosis by the cytokine mix might be counteracted; therefore, resulting in partial recovery of glucose-stimulated insulin secretion and relief of accumulated insulin content in cytokine treated BRIN-BD11 cells.

In conclusion, the current investigation revealed, for the first time, that C_{19} homolignans isolated from SA-Et possessed protective bioactivity against cytokine-induced β cell death. The cytokine-impaired insulin secretory function was also partially restored. However, iNOS/NO inhibition appeared to be an important factor if intact β cell insulin secretory function is to be preserved after cytokine challenge. In the future, continued research on potent β cell-protective phytochemicals should substantially contribute to the development of islet/stem cell transplantation or cell-based therapies for type 1 diabetes.

COMMENTS

Background

Conventional therapy (i.e., insulin injection) in patients with type 1 diabetes does not allow minute-to-minute control of blood glucose and does not prevent complications associated with the disease. However, the employment of whole pancreas or islet transplantation suffers from a lack of sufficient organs for transplantation. Therefore, the application of cell-based therapeutics for curing type 1 diabetes is an interesting and hot topic in the field.

Research frontiers

Employing toxin-resistant cells for type 1 diabetes therapy is a developing cell-based approach for curing type 1 diabetes. At least 5 toxin resistant β cell lines have been generated and investigated for defensive mechanisms. However, disassociation between toxin resistance and insulin-secretory functions may occur during the induction of toxin resistance. To our knowledge, no "master" toxin-resistant cells have been created so far. As a result, employing β-cell protective agent(s) including phytochemicals from traditional Chinese medicine to protect cell-based transplants has become an alternative and practical approach in the field.

Innovations and breakthroughs

By employing the glucose-responsive, insulin-secreting cell line, BRIN-BD11 cells, the authors successfully established a platform mimicking hormonal part of immune-attack in type 1 diabetes. In addition, the authors noted dissociation not only between cytokine-mediated nitric oxide and cell death, but also

between cell survival rate and secretory function. Moreover, the NFκB-induced inducible nitric oxide synthase-nitric oxide pathway is not necessarily a maser regulator for cell viability and function. *Schizandra arisanensis* (SA-Et) is one of the schizandraceous plants from Taiwan. The indications for this herb in traditional Chinese medicine include diabetes, hepatitis, immunomodulation and cancer. The current investigation is likely to be the first report on C_{19} homolignans from SA-Et which possess β-cell protective effects. Different to previous β cell protective phytochemicals, such as epigallocatechin gallate and silymarin, the action of C_{19} homolignans is not due to the blockage of NFκB-inducible nitric oxide synthase-nitric oxide.

Applications

By employing this glucose-responsive, insulin-secreting cell line to generate several platforms which represent key steps in autoimmune mediated β-cell destruction. The authors will be able to design a formulation which can be used to enhance the survival rate and maintain secretory functions in cell-based therapeutics to achieve needle-free control of type 1 diabetes in the future.

Terminology

Type 1 diabetes (T1D) is an autoimmune disease which occurs when approximately 60%-90% of insulin secreting cells are lost and/or are dysfunctional due to β-cell directed autoimmunity. During the progression of T1D, heavy infiltrations of mononuclear cells lead to substantial damage to β cells by generating locally high concentrations of pro-inflammatory cytokines, perforin, and FasL-Fas interactions within the micro-environment of islets. As a result, deleterious outcomes including excessive pro-insulin secretion, the abolition of glucose-induced insulin secretion, and ultimately β cell death are observed.

Peer review

The article is very interesting and shows novel findings in insulin secreting cells. The article is well written. Furthermore, it correctly cites the most important articles in the field.

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