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Chitooligosaccharides protect pancreatic β-cells from hydrogen peroxide-induced deterioration

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ABSTRACT

Chitooligosaccharides (COS) of four different molecular weight ranges (below 1 kDa, 1–3 kDa, 3–5 kDa and 5–10 kDa) were investigated for their ability to protect β-cells from damage induced by hydrogen peroxide. The viability of HIT-T15 pancreatic β-cells was found by MTT assay to be significantly reduced after 4 h of treatment with 150 μM H_2O_2. Chitooligosaccharides (500 μg/ml) with molecular weights of 3–5 kDa raised cell viability to 58.46% following H_2O_2 addition. The radical scavenging effect of chitooligosaccharides was examined by dichlorofluorescein assay. In addition to free radical scavenging, the effect of chitooligosaccharides on oxidative-stress related enzymes and genes was tested. In agreement with the expression levels of related genes, results of cell viability and cellular radical scavenging assays indicate that chitooligosaccharides protect β-cells from oxidative-stress-induced cellular damage, presumably due to radical scavenging activity.

1. Introduction

It is well known that most of the diabetic complications and impaired cell function in type 2 diabetes are mediated by hyperglycemia (Ceriello, 2005; Williamson et al., 1993). Increasing levels of reducing sugars in the blood under hyperglycemic conditions trigger sets of reactions resulting in formation of reactive oxygen species (ROS), which promote oxidative-stress-induced tissue damage (Poitout & Robertson, 2002; Robertson & Harmon, 2006). Glucose as a primary energy source and regulator of cell function especially induces such reactions. In type 2 diabetes, although patients can retain healthy pancreatic β-cells for many years after disease onset, chronic exposure to high glucose will impair β-cell function in later stages. Impaired β-cell function leads to cellular damage in type 2 diabetic patients (Ihara et al., 1999; Robertson, Harmon, Tran, Tanaka, & Takahashi, 2003). Research into glucose-dependent reactions in pancreatic cells indicates that glucose can harm β-cell function by producing ROS through insulin secretion and glycation pathways (Robertson, 2004). In addition, pancreatic β-cells are already known to be more susceptible to damage from oxidative-stress compared to other tissues, due to the low expression of anti-oxidative enzymes (Lenzen, Drinkgern, & Tiedge, 1996). Therefore, due to glucose-related oxidative stress, β-cells can lose their ability to synthesize insulin and enter an apoptotic stage upon exposure to hyperglycemic conditions (Donath, Gross, Cerasi, & Kaiser, 1999; Lenzen et al., 1996). As a result, this type of oxidative stress is liable for deterioration of pancreatic β-cells in the later stages of type 2 diabetes. It is also shown that β-cells can be protected from tissue damage or apoptosis by treatment with chemical anti-oxidants such as NAC and cysteine (Tanaka, Gleason, Tran, Harmon, & Robertson, 1999).

Chitooligosaccharides (COS) are hydrolyzed derivatives of chitosan, which is a soluble form of chitin, a cellulose-like polymer present in the exoskeleton of crustaceans, cuticle of insects and cell wall of some microorganisms (Pantaleone, Yalpani, & Scollar, 1992). Compared to chitin, COS have low viscosities, relatively small molecular sizes and are soluble in neutral aqueous solutions. With a high absorption rate in *in vivo* systems, COS are expected to be more efficient than either chitosan or chitin. Moreover, it has been reported that COS possess anti-tumoral, anti-fungal, anti-microbial, anti-viral, fat lowering and free radical scavenging activities according to their molecular weight ranges (Kim & Rajapakse, 2005). Liu, Liu, Han, & Sun (2007) showed that low molecular weight chitooligosaccharides exhibit anti-diabetic effects in pancreatic β-cells by enhancing proliferation *in vitro* and by improving insulin secretion in diabetic rats. In this study, we extended previous research by investigating the protective effects conferred by different molecular weight COS on pancreatic β-cells. We showed that COS protected β-cells from H_2O_2-oxidative stress and impaired insulin secretion due to high glucose.

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2. Experimental

2.1. Reagents

Chitooligosaccharides were kindly donated by Kitto Life Co. (Seoul, Korea). Ham's F12K medium, antibiotics and fetal bovine serum (FBS) were purchased from Gibco BRL (Gaithersburg, MD, USA). Primers and other RT-PCR chemicals were obtained from Bioneer Inc. (Alameda, CA, USA). Primary and secondary antibodies for Western blotting were acquired from Santa Cruz Biotechnology (Heidelberg, Germany). All other chemicals and reagents were obtained from Sigma–Aldrich Inc. (St. Louis, MO, USA).

2.2. Cell culture

The hamster (*Mesocricetus auratus*) pancreatic β-cell line, HIT-T15, was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). HIT-T15 cells were grown in Ham's F12K medium containing 2 mM L-glutamine, 10% fetal bovine serum and 5 mM glucose, followed by equilibration in 5% CO_2 and 95% air at 37 °C. Cells were harvested before passage number 18 for experiments.

2.3. Cell viability assay

The viability levels of β-cells were determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Mosmann, 1983). Briefly, cells were grown in 96-well plates at a density of 1×10^4 cells/well. After overnight incubation, cells were treated with hydrogen peroxide in the absence or presence of different molecular weight COS solutions and were incubated for 4 h. The supernatants in the wells were exchanged with 100 μl of MTT solution (final concentration, 1 mg/ml) and incubated for another 4 h. The amount of newly formed formazan in cells was determined by the addition of DMSO (dimethyl sulfoxide) to wells and by measuring optical density (OD) at 540 nm with a microplate reader (GENios Tecan Austria GmbH, Austria). Relative cell viability was calculated as a percentage of the viability of untreated cells that were used as a control.

2.4. Cellular ROS determination by 2′,7′-dichlorofluorescein diacetate (DCFH-DA) assay

The oxidation-sensitive dye DCFH-DA was used to detect formation of intracellular ROS as previously described (Engelmann, Volk, Leyhausen, & Geurtsen, 2005). β-Cells growing in fluorescence microtiter 96-well plates were labeled with 20 μM DCFH-DA in HBSS (Hank's Buffered Salt Solution, Sigma–Aldrich Inc., USA) for 30 min under dark conditions. Cells were treated with different molecular weights of COS and incubated for 24 h. After cells were washed with PBS, 150 μM H_2O_2 was added and the intensity of the fluorescence signal emitted by DCF after oxidation by cellular ROS was detected in a time-dependent manner using a fluorescence microplate reader (GENios Tecan Austria GmbH, Salzburg, Austria). Fluorescence intensities were plotted in order to compare treatment, control and blank groups.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from HIT-T15 cells treated with H_2O_2 in the presence or absence of 3–5 kDa COS using Trizol reagent (Invitrogen Co., CA, USA), as reported in the manufacturer's manual. After RNA was quantified by spetrophotometry, 2 μg of total RNA was reverse-transcribed to cDNA in a master mix containing 1× RT buffer, 1 mM dNTPs, 500 ng oligo (dT), 140 U M-MLV reverse transcriptase and 40 U RNase inhibitor at 42 °C for 60 min and at 72 °C for 5 min using an automatic Whatman thermocycler (Biometra, UK). PCR was performed to amplify synthesized cDNAs in the presence of specific primers for 32 cycles at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min, with an initial cycle of 95 °C for 2 min. PCR reactions were carried out using specific primers for Caspase-3 (forward 5′-GGA CCT GTG GAC CTG AAA AA-3′ and reverse 5′-TGA TTT GAC CAT TTG CCT GA-3′), Caspase-9 (forward 5′-AAG ACC ATG GCT TTG AGG TG-3′ and reverse 5′-CAG GAA CCG CTC TTC TTG TC-3′), Bcl-2 (forward 5′-ATA CCT GGG CCA CAA GTG AG-3′ and reverse 5′-TGA TTT GAC CAT TTG CCT GA-3′), Bax (forward 5′-TGC AGA GGA TGA TTG CTG AC-3′ and reverse 5′-GAT CAG CTC GGG CAC TTT AG-3′) and GAPDH (forward 5′-GTC AAC GGA TTT GTC GTA TT-3′ and reverse 5′-AGT CTT CTG GGT GGC AGT GAT-3′). The resulting cDNAs were separated by electrophoresis on a 1% agarose gel for 30 min at 120 V, followed by visualization under UV light after ethidium bromide staining. Band intensities were quantitated with Multi Gauge Software (Fujifilm Life Science, Tokyo, Japan), and bands of specific genes were normalized using GAPDH as a reference.

2.6. Western blotting analysis

β-Cells were treated with 150 μM H_2O_2 for 6 h in the presence or absence of different molecular weight COS followed by lysis in RIPA buffer (Sigma, St. Louis, MO, USA). The cell lysate was used for immunoblotting analysis. Total protein of cell lysates was measured by the Lowry method, and the same amount of total protein was used for further procedures. Proteins were separated by denaturating SDS-PAGE on 4% stacking and 10% separating gels containing 25 mM Tris, 192 mM glycine and 0.1% SDS. Separated proteins were electrotransferred to a nitrocellulose membrane, which was blocked in 5% skim milk powder in TBS containing 0.1% Tween 20 (TBS-T buffer). After washing 2 times with TBS-T buffer, the membrane was incubated with primary insulin antibody (1:500) (Santa Cruz Biotechnology Inc., CA, USA) and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody (1:5000) (Santa Cruz Biotechnology Inc., CA, USA) for 2 h. The resulting protein bands were visualized by chemiluminescence assay (Amersham Bioscience) using a LAS3000 image analyzer (Fujifilm Life Science, Tokyo, Japan). The intensities of the bands were quantitated with Multi Gauge Software.

2.7. Statistical analysis

Statistical significance of experiments were determined and expressed as mean of three independent experiments ± standard deviation.

3. Results and discussion

3.1. Maintaining cell viability

COS have been reported as biologically active polymers with no cytotoxicity that possess anti-cancer, anti-viral, anti-bacterial and anti-oxidative activities (Kim & Rajapakse, 2005). Liu et al. (2007) showed that COS enhanced β-cell proliferation and lowered plasma glucose in streptozotocin-treated diabetic rats. Recent studies have already shown that COS exert differing free radical scavenging effects in living cells depending on molecular weight (Je, Park, & Kim, 2004). However, no study has been performed on whether COS can protect pancreatic β-cells against oxidative stress or reverse impaired insulin secretion.

In the present study, we found that COS moderately protected HIT-T15 β-cells from H_2O_2-induced cell death in HIT-T15 cells. Hydrogen peroxide was chosen as the stimulus for oxidative-stress instead of β-cell-specific toxins like alloxan and streptozotocin because hydrogen peroxide is known to act in most oxidative-stress

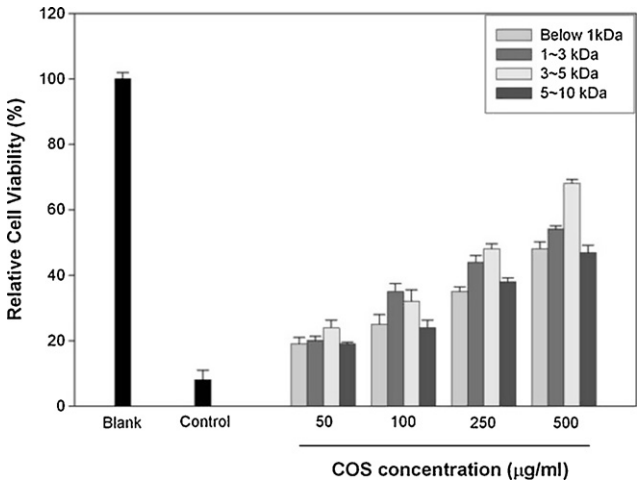

Fig. 1. HIT-T15 cell viability after H_2O_2 treatment with or without COS. Cells were treated with 150 μM H_2O_2 for 4 h in the absence (control) or presence of COS of varying molecular weight and concentration. Results are the percentage of viable cells compared to untreated (blank) cells. Values are means ± SD ($n = 3$) (Control: +H_2O_2–COS; Blank: –H_2O_2–COS).

processes (Morita, Kaminaka, Masumura, & Tanaka, 1999; Wilson, Patton, McCord, Mullins, & Mossman, 1984). Moreover, results suggest that β-cells were protected from death apparently due to the free radical scavenging activity of COS.

In recent decades, studies have suggested that β-cells are induced to undergo apoptosis during type 2 diabetes by a cascade of reactions stimulated by the immune system, DNA damage, hormonal disorders and oxidative stress (Porte, 1991). Oxidative stress under diabetic conditions is most likely due to hyperglycemia, which causes higher levels of glycation, reduced anti-oxidative activity and elevated glucose metabolism that eventually produces ROS (Robertson & Harmon, 2006). Therefore, chronic hyperglycemia causes harmful oxidative stress to insulin-secreting β-cells through induced glycation during type 2 diabetes. Increasing evidence both *in vivo* and *in vitro* has supported the notion that such cell deterioration is due to chronic hyperglycemia and oxidative stress (Tanaka et al., 1999).

In order to determine whether COS protect β-cells against H_2O_2-induced damage, MTT assay was performed (Fig. 1). The viability

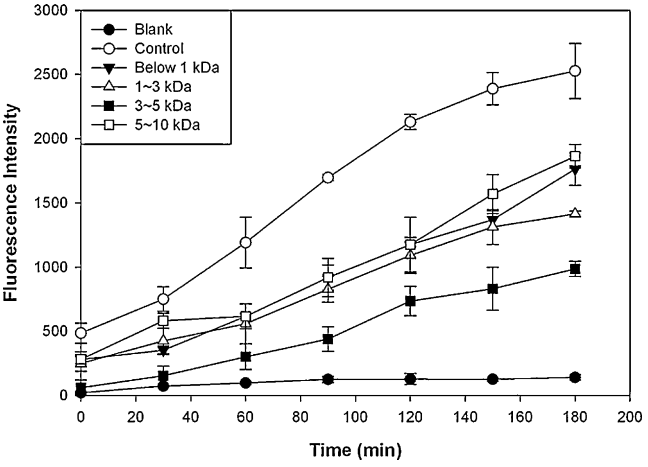

Fig. 2. Effect of COS on the intracellular generation of ROS in HIT-T15 cells. Cells were labeled with DCFH-DA and treated with COS of varying molecular weight at a concentration of 500 μg/ml for 24 h (except blank and control). Oxidation of DCFH to DCF was induced by 250 μM H_2O_2 (except blank). Fluorescence signal intensity of DCF was measured at 30 min intervals for 3 h. Values are means ± SD ($n = 3$) (Control: +H_2O_2–COS; Blank: –H_2O_2–COS).

of HIT-T15 cells was reduced to 16.79% after exposure to 150 μM H_2O_2 for 6 h. COS of varying molecular weight were applied to β-cells at different concentrations. It was found that COS 3–5 kDa in molecular weight at a concentration of 500 μg/ml raised cellular viability to 58.46% of untreated cells. At this concentration, all COS protected β-cells against H_2O_2-induced cell death. However, COS 3–5 kDa in molecular weight provided the best protection among the four different molecular weight categories.

Exposure to 150 μM H_2O_2 caused a significant decrease in the viability of HIT-T15 cells while COS treatment from 50 μg/ml to 500 μg/ml was effective in elevating β-cell viability. Five-hundred μg/ml of COS was the most effective treatment for preserving cell viability and thus was designated as the highest accepted concentration for a non-toxic biologically active material. As mentioned in Je et al. (2004), the radical scavenging activities of COS differ through their molecular weights. Accordingly, different molecular weight COS were used for cell viability assays and were applied in different concentrations from 50 μg/ml to 500 μg/ml. Among the four different molecular weight ranges, COS between 3 and 5 kDa provided the highest protection by raising HIT-T15 cell viability to 58.46% of that of untreated viable cells. Cell viability assay clearly suggested that COS, especially those 3–5 kDa, protect β-cells against oxidative-stress-induced cell death in a dose-dependent manner.

3.2. Free radical scavenging and anti-apoptotic activities

Next, to evaluate the free radical scavenging effects of different molecular weight COS on H_2O_2-induced β-cells, a DCFH-DA assay was conducted on HIT-T15 cells (Fig. 2). Oxidation of DCF was followed by emitted fluorescence from cells at 30 min intervals. All molecular weight ranges exhibited free radical scavenging effects after incubation for 30 min at 500 μg/ml. COS 3–5 kDa in molecular weight significantly reduced free radical-mediated oxidation in HIT-T15 cells compared to other molecular weight COS.

Free radical scavenging is a common and basic cellular anti-oxidant defense system, and free radical scavengers are accepted as known anti-oxidant compounds (Sun, 1990). To assess whether COS can exhibit the same free radical scavenging activity in HIT-T15 cells as they do in other living cells, DCFH-DA assay was performed. Oxidation of DCF at 30 min intervals for 3 h was conducted in the presence of 150 μM H_2O_2 and 500 μg/ml of different molecular weight COS as in the cell viability assay. COS 3–5 kDa in molecular weight impaired DCF oxidation better than other COS, and corroborating results were seen in the cell viability assay. According to assay results, the free radical scavenging activity of COS clearly acts to inhibit oxidation in HIT-T15 β-cells, which can be considered protection against ROS-mediated oxidative stress. The results of the cell viability and DCFH-DA assays corroborated each other, suggesting that COS 3–5 kDa in molecular weight were the most active in protecting β-cells from oxidation among the four different molecular weight COS. Thus, further experiments were needed to evaluate the mechanism behind the protection of β-cells against oxidative-stress conferred by COS.

Therefore, mRNA expression levels of apoptosis-related genes were examined to assess this molecular mechanism (Fig. 3). H_2O_2 addition increased apoptotic Bax mRNA expression while significantly decreasing anti-apoptotic Bcl-2 mRNA expression. Expression of caspases 3 and 9 was also increased after H_2O_2 addition, but to a lesser degree than those of Bax and Bcl-2. Moreover, prior to H_2O_2 addition, 3–5 kDa COS significantly suppressed apoptotic gene expression in HIT-T15 cells while simultaneously increasing anti-apoptotic gene expression.

Apoptosis is triggered by a variety of stimuli, such as cell surface receptors and expression of stress-induced genes. Among them, caspases comprise a class of cystein proteases, many members of

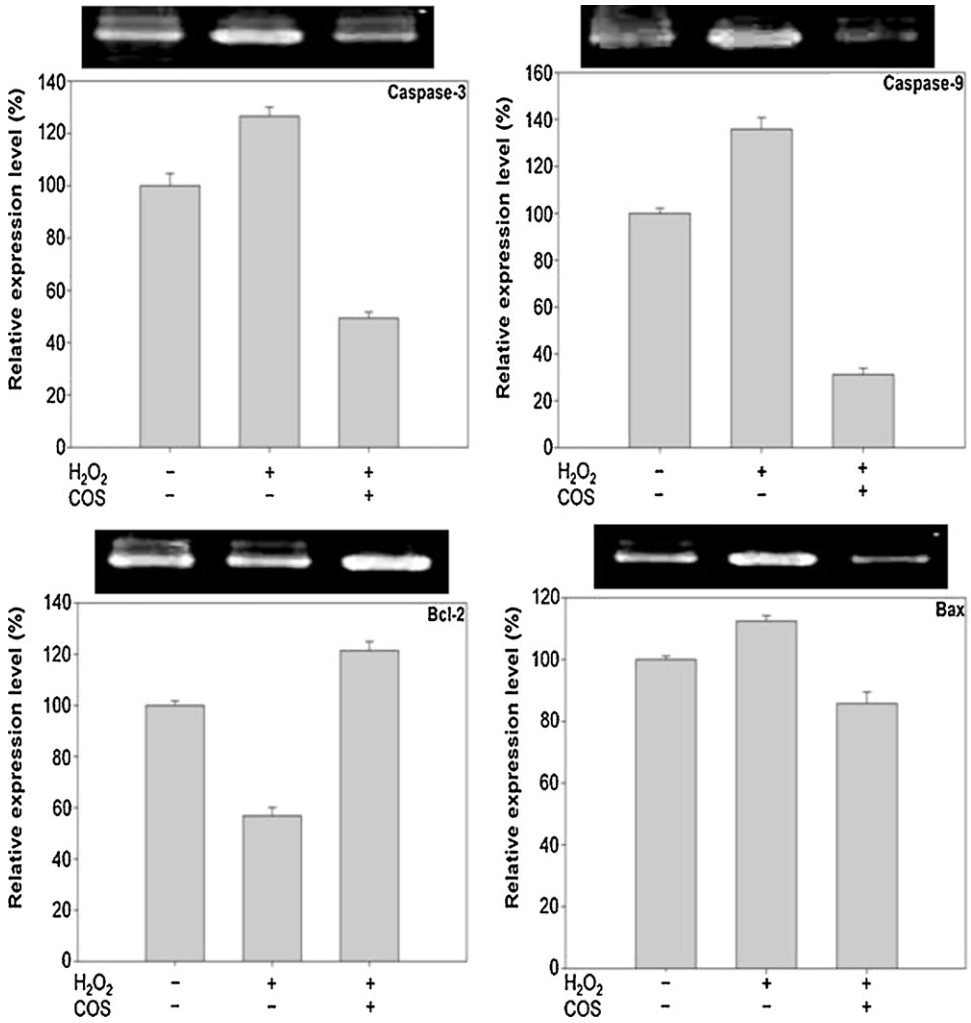

Fig. 3. Effect of COS on the stress and apoptosis-related gene expression levels. Cells were treated with 150 μM H_2O_2 for 4 h in the presence or absence of 3–5 kDa COS at a concentration of 500 μg/ml before isolation of total mRNA. Changes in the expression levels of caspases 3 and 9, bcl-2 and bax were measured by RT-PCR. Expression levels of mRNA are exhibited as the percentage of COS-untreated. (For band normalization, GAPDH is used as an internal control.) Values are means ± SD ($n = 3$).

which are involved in apoptosis. Caspases cleave and activate other caspases, which leads to the induction of enzymes that degrade cellular targets and cause cell death (Cohen, 1997). Bcl-2 and bax prevent activation of this caspase cascade as well as apoptosis by blocking pore formation and the release of caspase activators (Adams & Cory, 1998). In light of this knowledge, the expression levels of apoptotic caspases 3 and 9 as well as anti-apoptotic bcl-2 and bax were examined in HIT-T15 cells under oxidative stress in the

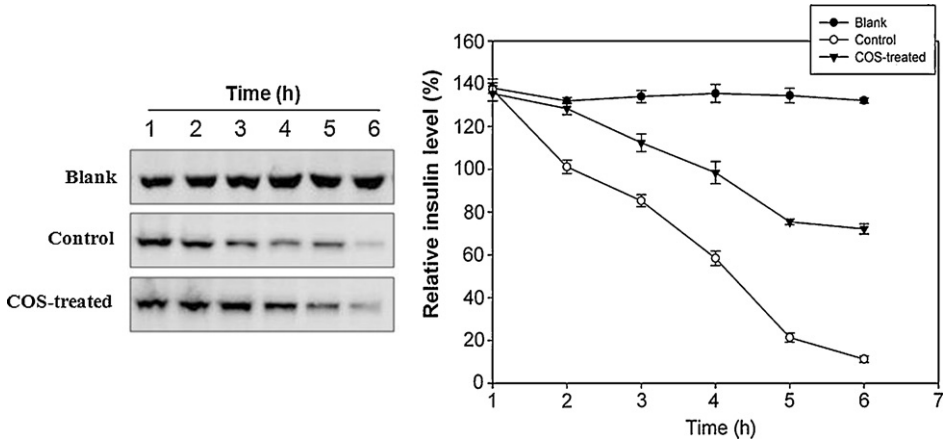

Fig. 4. Effect of COS on the insulin secretion from HIT-T15 cells. Cells were treated with 50 μM H_2O_2 and 20 mM glucose for 6 h in the presence or absence of 3–5 kDa COS at a concentration of 500 μg/ml. Cell culture medium was collected after each hour to evaluate the insulin concentration by immunoblotting. The amount of insulin was expressed as of the percentage of untreated cells (Insulin amount of cells maintained in 5 mM glucose medium was used as control for normalization). Values are means ± SD ($n = 3$) (Control: +H_2O_2–COS; Blank: –H_2O_2–COS).

presence or absence of 3–5 kDa COS. After 3 h incubation with H_2O_2, expression of caspases 3 and 9 was significantly suppressed. However, bax expression was not noticeably affected by COS treatment. On the other hand, bcl-2 expression was enhanced by COS addition after exposure to oxidative stress. Among all stress-induced genes, the expression of bax was least affected by COS, whereas caspase 3 expression was highly suppressed. Therefore, induction of stress-related genes and suppression of apoptotic enzymes suggest that COS protect β-cells from oxidative stress and apoptotic cell death.

Oxidative stress is a well-known contributor to the deterioration of β-cells. Therefore, anti-oxidants have been well researched for their ability to reduce β-cell damage and diabetic complications. N-acetylcysteine (NAC) and aminoguanidine have been observed to protect against glucose toxicity in HIT-T15 cells as well as diabetic progression in Zucker diabetic rats (Tanaka et al., 1999). COS have been well established as anti-oxidant free radical scavengers that enhance β-cell proliferation (Lui et al., 2007). Therefore, the results of this study suggest that COS contain free radical scavenging activity that protects β-cells against oxidative stress and therefore apoptotic effects.

3.3. Reduction of insulin secretion

To check insulin secretion, HIT-T15 cells were treated with 20 mM glucose and 50 μM H_2O_2 in the presence or absence of 3–5 kDa COS. The amount of insulin secreted to the cell culture medium was evaluated by Western blotting analysis at 1 h intervals for 6 h (Fig. 4). Glucose treatment significantly elevated insulin secretion during the first hour. However, H_2O_2-induced oxidative-stress impaired insulin secretion after 1 h. COS treatment from the second hour protected cell function and kept insulin secretion steady. Therefore, the reduction of insulin secretion is due to the anti-oxidative activity of COS. In accordance with prior results, insulin secretion was reduced from the second hour following COS treatment, which protected β-cells from glucose-induced stress.

4. Conclusion

In all assays performed, COS 3–5 kDa conferred the highest protection among all COS tested. Importantly, it remains unknown why 3–5 kDa was the most effective molecular weight for COS activity. However, this result is confirmed by previous studies (Lee, Park, Kim, Ahn, & Je, 2009; Ngo, Lee, Kim, & Kim, 2009) that examined the ability of COS to relieve oxidative stress. This suggests that COS possess anti-oxidative effects that protect β-cells from both H_2O_2 and glucose-induced damage.

This study provides evidence that COS protect against H_2O_2-induced cell damage in β-cells. However, the underlying mechanism must be studied more. Our results suggest that the protective effect of COS presumably originates from its anti-oxidative effect that inhibits apoptosis. Therefore, COS are potent natural compounds that have potential as nutraceutical products in support of diabetic patients.

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