Selenium acts as an insulin-like molecule for the down-regulation of diabetic symptoms via endoplasmic reticulum stress and insulin signalling proteins in diabetes-induced non-obese diabetic mice

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To investigate whether selenium (Sel) treatment would impact on the onset of diabetes, we examined serum biochemical components including glucose and insulin, endoplasmic reticulum (ER) stress and insulin signalling proteins, hepatic C/EBP-homologous protein (CHOP) expression and DNA fragmentation in diabetic and non-diabetic conditions of non-obese diabetic (NOD) mice. We conclude that (i) Sel treatment induced insulin-like effects in lowering serum glucose level in Sel-treated NOD mice, (ii) Sel-treated mice had significantly decreased serum biochemical components associated with liver damage and lipid metabolism, (iii) Sel treatment led to the activation of the ER stress signal through the phosphorylation of JNK and eIF2 protein and insulin signal mechanisms through the phosphorylation of Akt and PI3 kinase, and (iv) Sel-treated mice were significantly relieved apoptosis of liver tissues indicated by DNA fragmentation assay in the diabetic NOD group. These results suggest that Sel compounds not only serve as insulin-like molecules for the downregulation of glucose level and the incidence of liver damage, but may also have the potential for the development of new drugs for the relief of diabetes by activating the ER stress and insulin signalling pathways.

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1. Introduction

Selenium (Sel), long known as a ubiquitous trace element in nature, has been proven to be essential for animal and human health (Wilber 1980). This element has been also demonstrated to be required for normal growth and reproduction during spermatogenesis (Smith and Picciano 1986). Furthermore, its deficiency was demonstrated to induce several diseases associated with oxidative damage, an endemic fatal cardiomyopathy in Keshan, China (Yang

Keywords. Selenium; ER stress; insulin signal; NOD; lipid metabolism; apoptosis

Abbreviations used: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST aspartate aminotransferase; CHO, cholesterol, Tg, triglyceride, HDL, high-density lipoprotein; CHOP, C/EBP-homologous protein; ER, endoplasmic reticulum; Glut-4, glucose transporter-4; HRP, horseradish peroxidase; IRS, insulin receptor substrate; LDH, lactate dehydrogenase; LDL, low-density lipoprotein; NOD, non-obese diabetic; SECIS, selenocysteine insertion sequence; Sel, selenium; Sel, selenocysteine

et al 1984) as well as muscular dystrophy in patients subjected to long-term unsupplemented parenteral nutrition (Van Rij *et al* 1979). It is also well known that Sel deficiency can be partially replaced with vitamin E (Schwarz and Folz 1957; Reddy *et al* 1987).

Sel, an important dietary antioxidant, is now recognized as an essential component of the active site of a number of selenoenzymes (Behn *et al* 1995; Stadtman 1996), and has insulin-like effects both *in vitro* and *in vivo*. Incubation of rat adipocytes with Sel stimulated glucose transport activity, due to the translocation of two types of glucose transporters, cAMP phosphodiesterase activity and ribosomal S6 protein phosphorylation (Ezaki 1990). Furthermore, Sel regulated the activity of various enzymes involved in the processes of glycolysis and gluconeogenesis in rats with streptozotocin-induced diabetes, but the regulation of these enzyme activities was not induced by a change in insulin level (McNeill *et al* 1991; Ghosh *et al* 1994; Beaker *et al* 1996; Battell *et al* 1998; Ghose *et al* 2001). Ayaz *et al* (2002 and 2004) reported that Sel treatment may prevent and alleviate the symptoms of disease in diabetes animal models involving heart, kidney and platelet defects. Also, Sel treatment has been reported to show insulin-like actions during glucose metabolism due to the stimulation of tyrosine kinase in the insulin signalling pathway (Hei *et al* 1998; McKenzie *et al* 2002). However, few studies have been conducted to investigate whether Sel treatment of NOD mice, as a classical diabetes model, significantly alleviates the symptoms of diabetes including decreased hepatic DNA apoptosis via the change in the signalling proteins involved in the ER stress and insulin signalling pathways.

As demonstrated by our data, significant changes were observed in the signalling proteins of the ER stress and insulin pathways, and apoptosis in the liver of Sel-treated mice in response to Sel. The data presented here provide strong evidence that Sel would be a powerful candidate for the prevention or alleviation of diabetes-related disease.

2. Materials and methods

2.1 *Care and use of NOD mice*

The animals were handled in an accredited Korea FDA animal facility in accordance with the AAALAC International Animal Care policies (Accredited Unit—Korea Food and Drug Administration: Unit Number-000996). All mice were housed in cages under a strict light cycle (light on at 06:00 h and off at 18:00 h), given a standard irradiated chow diet (Purina Mills Inc.) *ad libitum* and maintained in a specified pathogen-free state. The 25–27-week-old NOD mice used in this study were purchased from Jackson Laboratories. These mice were divided into 2 groups according to their plasma glucose levels; a diabetes (>250 mg/dl for 2 weeks) and a non-diabetes (<250 mg/dl for 2 weeks) group.

2.2 *Treatment with Sel and detection of glucose level*

Sodium selenite (Na_2SeO_3) obtained from Sigma (S5261, USA) was dissolved in distilled water to give a final concentration of 0.2 μmol/μl. Diabetic and non-diabetic animals were randomly divided into two subgroups per group. The first group of diabetic and non-diabetic animals received a comparable volume of daily water via intraperitoneal injections (untreated diabetes and non-diabetes group), while the second group received 5 μmol/kg body weight/day of sodium selenite via intraperitoneal injections for 3 weeks (sodium selenite-treated mice in both the diabetic and non-diabetic NOD groups). The serum glucose level from mice was detected after 24 h fasting using the sensitive strip of the blood glucose monitoring system (I-sens Co., Korea).

2.3 *Serum biochemical analysis*

After the final administration of Sel, all mice were kept fasting for 24 h and blood was collected from the abdominal vein of the mice. Serum was obtained by centrifugation of blood incubated for 30 min at room temperature. Serum biochemical components were assayed by the Automatic Serum Analyzer (HITACHI 747, Japan). All assays were conducted in duplicate on fresh serum using standard enzymatic methods.

2.4 *Western blot*

Proteins prepared from the tissue of diabetic and non-diabetic mice were separated for 3 h using electrophoresis in a 4–20% SDS-PAGE gel, and then transferred to nitrocellulose membranes for 2 h at 40 v. Each membrane was separately incubated overnight at 4°C with the primary, anti-ATF6α (SantaCruz), anti-IRE1β (SantaCruz), anti-JNK (SantaCruz), anti-p-JNK (SantaCruz), anti-eIF2α (Cell Signalling), anti-p-eIF2α (Cell Signalling), anti-GADD15 (CHOP) (SantaCruz), anti-Akt (SantaCruz), anti-p-Akt (SantaCruz), anti-PI3 kinase (Cell Signalling), anti-p-PI3 kinase (Cell Signalling) and anti-actin antibodies (Sigma, A5316). The membranes were washed with washing buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4 and 0.05% Tween 20), and incubated at room temperature for a further 2 h with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Zymed) in a 1: 1,000 dilution. Membrane blots were developed using a chemiluminescence reagent plus kit (ECL, Pharmacia).

2.5 *Quantification of insulin by ELISA*

The level of insulin in sera from diabetic and non-diabetic mice was detected using the ultra-sensitive assay procedure and reagents in the Mercodia Rat Insulin ELISA kit (Mercodia; Cat. 10-1137-01, Sweden). Sera and standards were incubated in a plate shaker at 100–150 rpm at room temperature for 2 h on antibody-coated plates. Wells were then washed six times using an automatic plate washer (Hoefer; PV100, USA). HRP conjugate was added to all the plates, which were then incubated in a shaker for 30 min at room temperature. The reaction was terminated by the addition of 50 μl of stop solution (0.5 M H_2SO_4). The plates were read at 450 nm using the Molecular Devices V_{max} Plate reader (Sunnyvale, CA, USA).

2.6 *Immunohistochemistry*

Immunohistochemical analysis was performed as previously described (Hwang *et al* 2001). Briefly, for the detection of CHOP using light microscopy, tissue was fixed in 5% formalin for 12 h, embedded in paraffin, and 4 μm thick sections were prepared. These sections were de-paraffinized with xylene, rehydrated and pretreated for 30 min at room temperature with PBS-blocking buffer, containing 10% goat serum. Next, the sections were incubated with mouse anti-CHOP antibody, at a dilution of 1:1,000 in PBS-blocking buffer. The antigen–antibody complexes were visualized with biotinylated secondary antibody (goat anti-rabbit)-conjugated HRP streptavidin (Zymed, Histostain-Plus Kit), at a dilution of 1:1,500 in PBS-blocking buffer. CHO proteins were detected using stable diaminobenzidine (DAB) (ResGen, Invitrogen Corp.) and the Imazing Densitometer (Model GS-690, Bio-Rad).

2.7 *DNA fragmentation assay*

DNA fragmentation assay was performed using the methods suggested by Gervais *et al* (1999). In brief, the frozen tissues (100 mg) were chopped with a pair of scissors and suspended in 100 μl of lysis buffer containing 0.6% SDS and 10 mM EDTA (pH 7.5) and NaCl was added to give a final concentration of 1 M. After overnight incubation at 4°C, the resultant supernatant containing fragmented DNA was separated by centrifugation at 15,000 rpm. The resultant supernatant was then treated with 10 μg/ml RNAase at 37°C for 40 min and phenol/chloroform solution added to remove protein. The pellets were then incubated with isopropanol and washed with 70% alcohol. Immediately after drying, the fragmented DNA was loaded in a buffer containing 30% glycerol, 0.25% BPB and 0.25% XC, and determined by electrophoresis on 1.2% agarose gel and a ladder-like appearance visualized with EtBr.

2.8 *Statistical analysis*

Tests for significance between Sel-treated and non-treated mice were performed using a one-way ANOVA test of variance (SPSS for Windows, Release 10.10, Standard Version, Chicago, IL). Also, tests for significance between the diabetic NOD and non-diabetic NOD groups were performed using a post-hoc test of variance (SPSS for Windows, Release 10.10, Standard Version, Chicago, IL) and significance levels were given in the text. All the values are reported as the mean±standard deviation (sd). A P value <0.05 was considered significant.

3. Results

3.1 *Sel effects on body weight, serum glucose and insulin, and serum biochemical profiles*

In order to investigate whether Sel treatment would affect the onset of diabetes, we measured serum glucose and insulin levels, and other serum biochemical components in both the diabetic and non-diabetic groups during the course of Sel treatment. In the non-diabetic NOD group, the body weight and levels of glucose and insulin in the Sel-treated mice were unaffected compared with those in mice that had received no treatment. In the diabetic NOD group, however, the body weight of the Sel-treated mice was significantly higher in comparison with that of non-treated mice. The Sel-treated mice also showed a significant decrease in serum glucose levels compared with the untreated mice, while insulin remained at a constant level (table 1). These results suggest that Sel treatment has a positive effect on maintaining body weight and serum glucose during progression of diabetes in diabetes-induced NOD mice.

Since Sel was also found to have several insulin-like effects in the NOD mice, we investigated the level of serum cholesterol (CHO), triglyceride (TG), high-density lipoprotein (HDL) and low-density lipoprotein (LDL) to see whether Sel treatment could affect lipid metabolism in the NOD mice (figure 1). Serum biochemical components were markedly raised in the diabetic NOD group by 2–3-fold, compared with those in the non-diabetic NOD group. Furthermore, the Sel-treated mice had remarkably decreased levels of CHO, TG, HDL and LDL compared with untreated mice in the diabetic NOD group. However, there was no difference in the serum biochemical profiles between Sel-treated and untreated mice in the non-diabetic NOD group (figure 1). These results suggest that Sel treatment is highly effective in reducing circulating lipid levels in diabetes.

Table 1. Selenium treatment significantly decreased serum glucose but not insulin level in the diabetic NOD group

NOD mice	Body weight (g)	Blood glucose (mg/dl)	Blood insulin (pmol/ml)
Non-diabetic NOD			
Not treated	30.78±2.4	131±5.2	95±8.5
Sel-treated	30.29±2.5	126±4.5	98±7.8
Diabetic NOD			
Not treated	21.11±1.4*	310±28*	8.5±0.8*
Sel-treated	26.21±2.1*,**	160±15*,**	9.2±0.8*,**

Mice were injected intraperitoneally with sodium selenite (5 μmol/kg body weight/day) for 3 weeks. The control group received the vehicle only. Blood glucose and insulin levels were estimated as described in Materials and methods. Values are mean±SD, and the coefficients of variation of glucose and insulin in our serum samples were 5.2% and 2.4%, respectively. Non-diabetic NOD group: diabetes phenotype non-induced mice, Diabetic NOD group: diabetes phenotype-induced mice, Sel treated groups: mice treated with sodium selenite for three weeks. * P<0.05 is the significance level compared with the non-diabetic NOD group. ** P<0.05 is the significance level compared with the untreated mice.

To study whether Sel treatment could affect hepatic damage or necrosis in the NOD mice, the levels of serum alanine transaminase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were monitored in all the groups of NOD mice. As shown in figure 2, these biochemical parameters in mice in the diabetic NOD group were significantly different from those in the non-diabetic NOD group; the diabetic NOD group showed a higher level of these enzymes than the non-diabetic NOD group. In the diabetic NOD group, the levels of ALT, AST and ALP were significantly lowered in the Sel-treated mice, while there was no change in the non-diabetic NOD group (figure 2). These results suggest that Sel treatment can effectively alleviate liver damage caused by the progression of diabetes among diabetic NOD mice.

3.2 *Sel effects on IRE1 signalling pathway*

To test whether Sel treatment can stimulate IRE1 signalling in the ER stress pathway, the levels of IRE and JNK protein were monitored in liver tissues from all groups of mice using western blot with corresponding antibodies. The diabetic NOD group showed a basically higher expression of IRE protein than the non-diabetic NOD group. The expression of this protein significantly increased in Sel-treated mice in the diabetic NOD group compared with untreated mice, but remained unchanged in the non-diabetic NOD group. In addition, JNK protein was found to have a higher expression level in the non-diabetic NOD group compared with the diabetic group. In particular, the level of JNK protein in Sel-treated mice of the diabetic NOD group significantly decreased compared with that in untreated mice. The diabetic NOD group had a higher expression of p-JNK protein than the non-diabetic NOD group. Sel-treated mice in the diabetic NOD group also had significantly increased levels of p-JNK protein compared with untreated mice, while in the non-diabetic NOD group it remained unchanged (figure 3A). These results suggest that Sel treatment can effectively induce an IRE1 signal transduction pathway in the ER stress signalling system.

3.3 *Sel effects on the PERK signalling pathway*

To examine whether the eIF2α signal would be affected by Sel in the NOD mice, the phosphorylation level of eIF2α protein was measured in the liver of mice in the diabetic and non-diabetic NOD groups. For the eIF2α protein, in general, the diabetic NOD group showed a significantly lower level of eIF2α protein expression than the non-diabetic group. When both the groups were treated with Sel, the Sel-treated diabetic NOD group showed the low level of eIF2α protein compared with untreated mice. With regard to the p-eIF2α protein, however, the diabetic NOD group showed a higher expression of p-eIF2α protein than the non-diabetic group. As with eIF2α protein, only the diabetic NOD group responded to Sel treatment; the resulting level of p-eIF2α protein expression in the Sel-treated mice was considerably increased compared with the untreated mice (figure 3B). Our observations indicate that Sel treatment in the diabetes-induced group can influence eIF2α and p-eIF2α phosphorylation in the PERK signalling pathway.

3.4 *Sel effects on CHOP activation*

C/EBP homologous protein (CHOP, also known as GADD153) encodes a small nuclear protein from the C/EBP family, which was originally isolated from cultured adipocytes. Although the cells are inactive under normal conditions, the CHOP gene is markedly induced by a variety of cellular stresses such as nutrient deprivation and metabolic perturbations (Barone *et al* 1994; Nakagawa *et al* 2000). Therefore, to determine whether the signalling of ER stress by Sel treatment increases the level of CHOP in the liver of the Sel-treated mice, CHOP expression was detected by western blot analysis and immunostaining. The expression of CHOP in the total, microsomal and nuclear fractions of the liver in the non-diabetic NOD group was higher compared with that in the diabetic NOD group (figure 3C). In the non-diabetic NOD group, the level of CHOP in the total and nuclear fractions was significantly

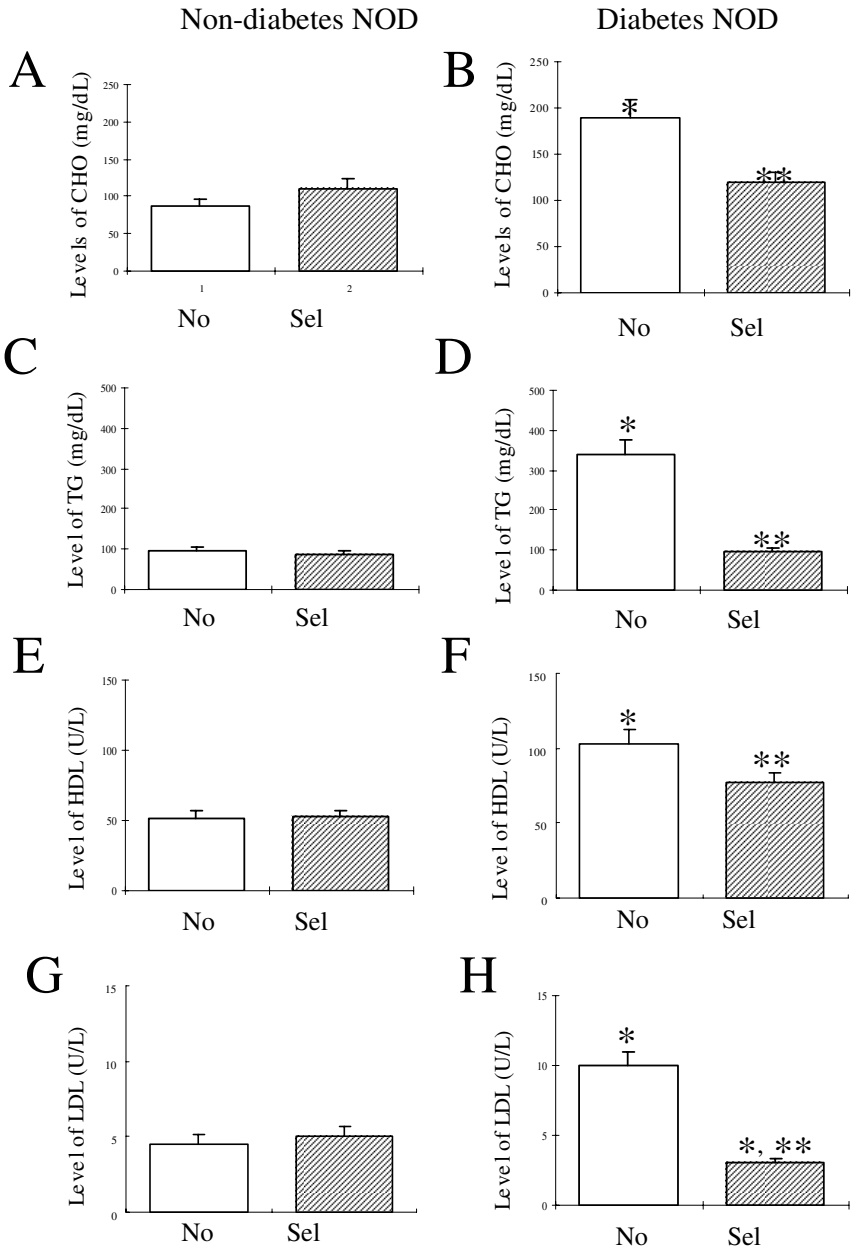

Figure 1. Selenium treatment is very effective in decreasing serum biochemical profiles related with lipid metabolism in 25–27-week-old non-diabetic and diabetic NOD mice. Blood was collected from the abdominal vein of the mice, and serum levels of cholesterol, triglyceride, HDL and LDL were analysed using a serum biochemical analyser. Three experiments were assayed in triplicate using serum biochemical analysis. Values are mean±SD. Non-diabetic NOD group: diabetes phenotype non-induced mice, Diabetic NOD group: diabetes phenotype-induced mice, Sel-treated group: mice treated with sodium selenite for three weeks. * $P<0.05$ is the significance level compared with the non-diabetic NOD group. ** $P<0.05$ is the significance level compared with the untreated mice.

increased by Sel treatment, but remained unchanged in the microsomal fraction. However, in the diabetic NOD group, total CHOP protein level and that in the nuclear fraction did not differ between Sel-treated and untreated mice. The level of CHOP protein in only the microsomal fraction significantly increased in the Sel-treated mice compared with that in untreated mice (figure 3C). CHOP immunoreactivity in the liver and pancreas was then analysed using a higher magnification of microscope. The intensity of CHOP immunoreactivity was found to be localized to the nuclear region of the liver in the Sel-treated mice of both the diabetic and non-diabetic NOD groups.

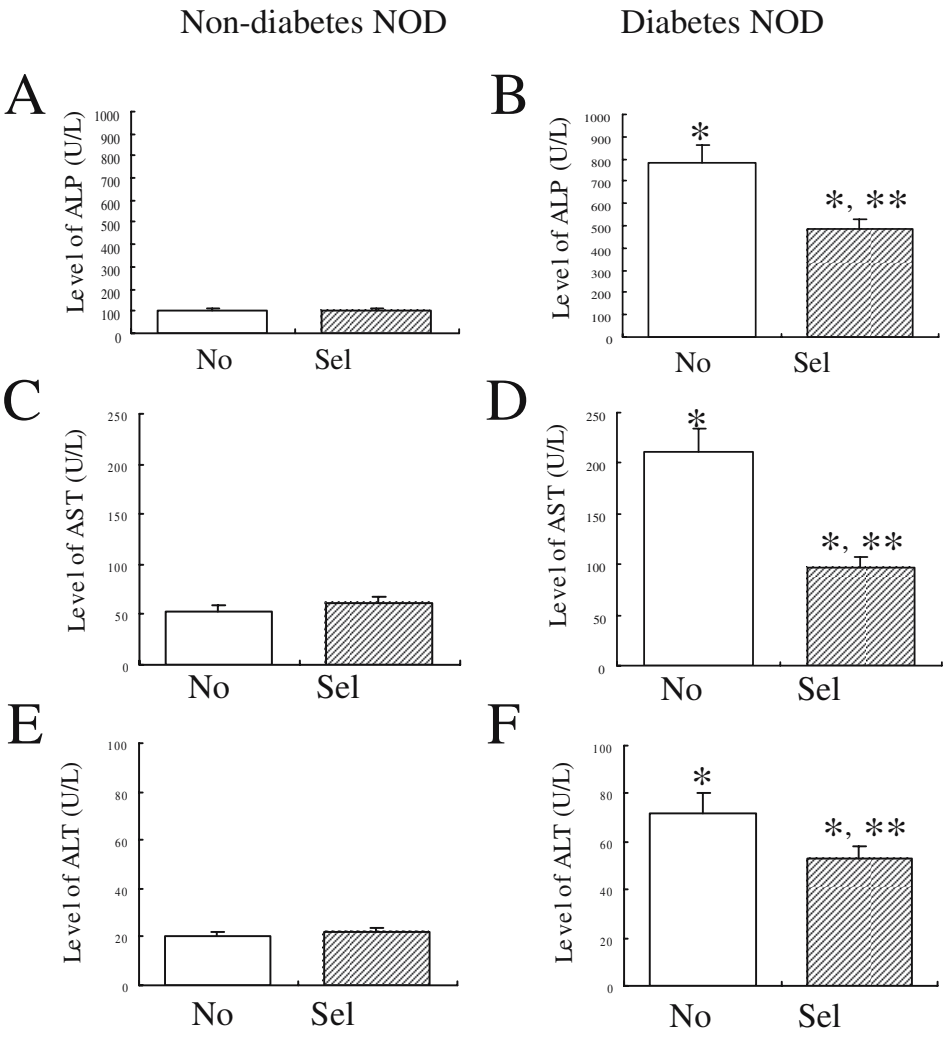

Figure 2. Selenium treatment alleviated liver damage during the progression of diabetes. Serum levels of AST, ALT and ALP in 25–27-week-old non-diabetic and diabetic NOD mice were analysed using a serum biochemical analyser. Three experiments were assayed in triplicate using serum biochemical analysis. Values are mean±SD. Non-diabetic NOD group: diabetes phenotype non-induced mice, Diabetic NOD group: diabetes phenotype-induced mice, Sel-treated mice: mice treated with sodium selenite for three weeks. * $P<0.05$ is the significance level compared with the non-diabetic NOD group. ** $P<0.05$ is the significance level compared with the untreated mice.

Furthermore, the diabetic NOD group showed a lower expression of CHOP than the non-diabetic group (figure 4), indicating that the induction of diabetes could significantly decrease the total level of CHOP in the liver and pancreas of the NOD mice. In addition, Sel treatment stimulated the translocation of CHOP from the microsome to the nuclear zone in the livers of both the diabetic and non-diabetic NOD groups.

3.5 *Sel effects on the insulin signalling pathway*

Insulin exerts profound metabolic and cellular growth effects when it binds to its receptor at the cell membrane. Its receptor, in turn, exhibits a change in intrinsic kinase activity. In the insulin signalling pathway, the response of the insulin receptor is directly transmitted through IRS-1, PI3-kinase and Akt-kinase in a cascade process of insulin action (Kahn *et al* 2006). In order to study the effects of Sel treatment on proteins associated with the insulin signalling pathway, the phosphorylation level of PI3 and Akt proteins was determined in the diabetic and non-diabetic groups using western blot analysis. The expression level of Akt and p-Akt proteins significantly increased in Sel-treated mice in the diabetic NOD group, but remained unchanged in the non-diabetic group. In particular, the expression level of p-Akt protein in Sel-treated diabetic mice was higher than that in untreated diabetic mice (figure 5A). In the case of PI3

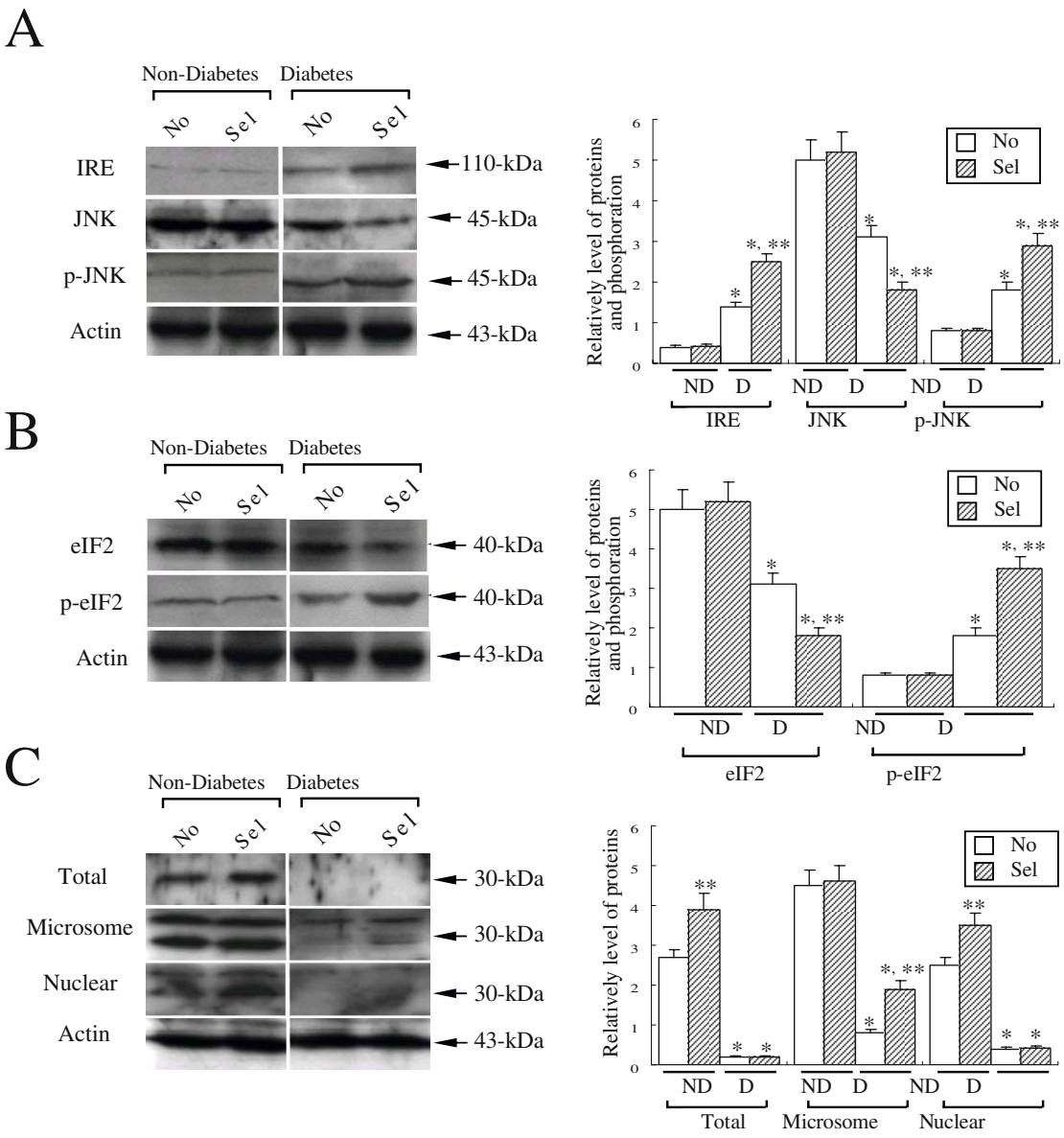

Figure 3. The ER stress signalling pathway was significantly changed by Sel treatment in the diabetic NOD group. (**A**) Protein expression levels of IRE, JNK and p-JNK. (**B**) Protein expression level of eIF2α and p-eIF2α. (**C**) Expression level of CHO protein. Microsomal and nuclear fractions were prepared from liver tissues of Sel-treated and untreated NOD mice, as described in Materials and methods. Fifty micrograms of protein per sample were immunoblotted with antibodies for each protein. Three samples were assayed in triplicate using western blotting. Values are the mean±SD. Non-diabetic NOD group: diabetes phenotype non-induced mice, Diabetic NOD group: diabetes phenotype-induced mice, Sel-treated mice: mice treated with sodium selenite for three weeks. * P<0.05 is the significance level compared with the non-diabetic NOD group. ** P<0.05 is the significance level compared with the untreated mice.

protein, Sel treatment significantly decreased the level of PI3 protein; the level of PI3 protein increased only in the diabetic NOD group. However, in the non-diabetic NOD group both protein levels did not change in response to Sel treatment (figure 5B). These results suggest that Sel treatment can effectively activate the insulin signalling pathway through the activation of Akt and PI3 phosphorylation.

3.6 *Sel effects on DNA fragmentation*

To test whether Sel affects apoptosis in the liver of NOD mice, the DNA fragmentation pattern was examined in both the NOD groups. As shown in figure 6, diabetic NOD mice treated with Sel showed a significant decrease in DNA fragmentation compared with untreated mice, whereas there

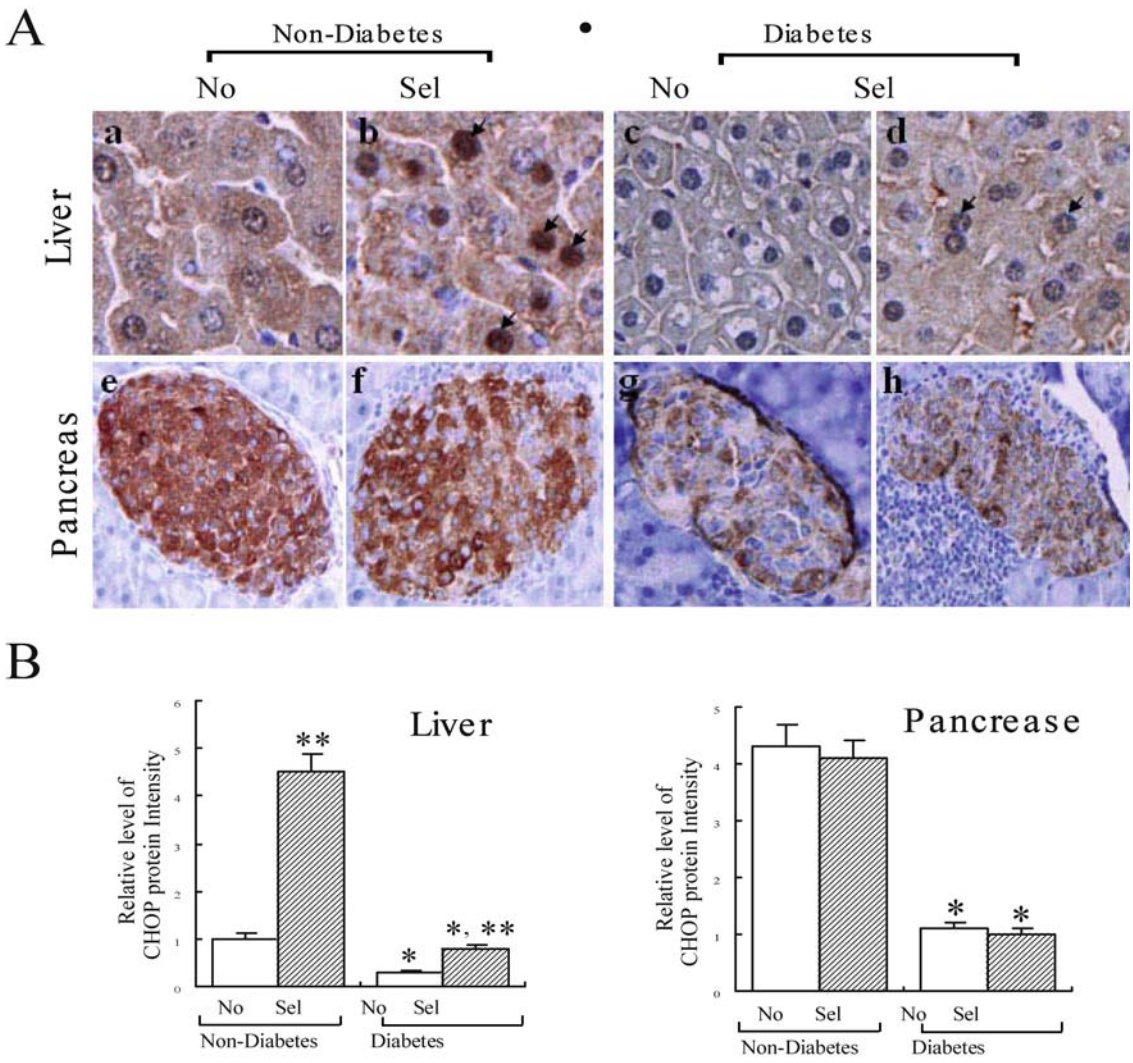

Figure 4. The CHOP was translocated into the nuclear region in the liver tissues of the NOD mice. CHOP expression in the liver and pancreas was detected with anti-CHOP primary antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG as described in Materials and methods. The intensity of the CHOP was calculated using an Imazing Densitometer. Values are the mean±SD. Non-diabetic NOD group: diabetes phenotype non-induced mice, Diabetic NOD group: diabetes phenotype-induced mice, Sel-treated mice: mice treated with sodium selenite for three weeks. * $P<0.05$ is the significance level compared with the non-diabetic NOD group. ** $P<0.05$ is the significance level compared with the untreated mice.

was no difference in its laddering pattern in the non-diabetic NOD group regardless of Sel treatment. These observations suggest that Sel treatment appears to significantly suppress the progression of apoptosis in the liver of diabetic NOD mice.

4. Discussion

NOD mice were discovered in Japan during the late 1970s (Makino *et al* 1980). They were inbred and distributed worldwide, and then used to establish numerous colonies. The frequency and age of onset of insulin-dependent diabetes mellitus (IDDM) differs widely among these colonies (Pozzilli *et al* 1993) due to multiple environmental factors. Diabetes usually appears at between 4 and 6 months of age, and is much more frequently seen in females than in males. In addition to diabetes, NOD mice also present with thyroiditis (Bernard *et al* 1992) and sialitis, and with autoimmune haemolytic anaemia later in life (Baxter and Mandel 1991). Until now, numerous studies have suggested that NOD mice are a good model for the study of diabetes and related diseases. Therefore, in this study, NOD mice were selected as a model of diabetes to study the effects of Sel. In addition, in an effort to minimize the genetic

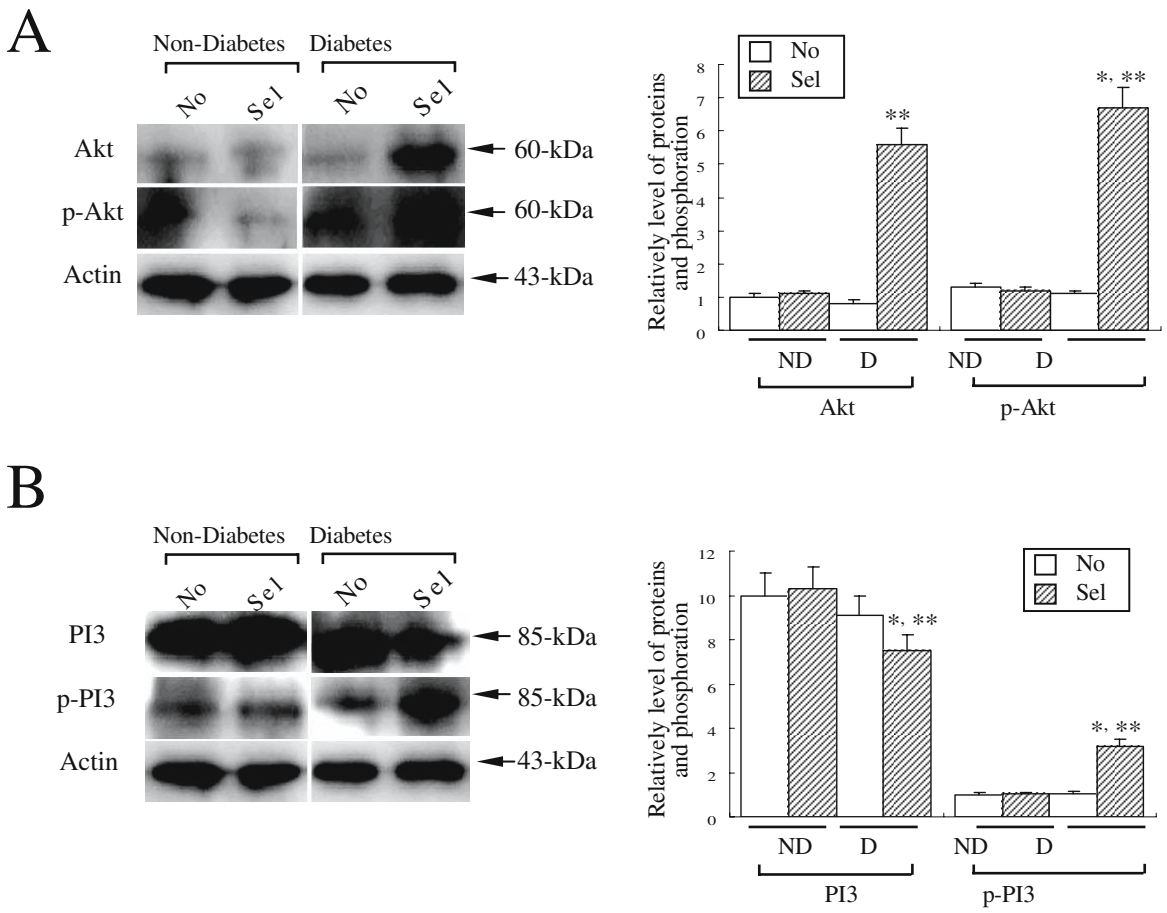

Figure 5. The insulin signalling pathway was significantly changed by Sel treatment in the diabetic NOD group. (**A**) Protein expression level of AKT and p-AKT. (**B**) Protein expression level of PI3 and p-PI3. Fifty micrograms of protein per sample were immunoblotted with antibodies for Akt, p-Akt, PI3, p-PI3 and β-actin. Three samples were assayed in triplicate using western blotting. Values are the mean±SD. Non-diabetic NOD group: diabetes phenotype non-induced mice, Diabetic NOD group: diabetes phenotype-induced mice, Sel-treated mice: mice treated with sodium selenite for three weeks. * $P<0.05$ is the significance level compared with the non-diabetic NOD group. ** $P<0.05$ is the significance level compared with the untreated mice.

variability of diabetic phenotype expression due to multiple factors, we divided all NOD mice into two categories based on the serum glucose level; NOD mice at 25–27 weeks of age with a serum glucose level >250 mg/dl were categorized as the diabetic NOD group and those with serum glucose levels below this as the non-diabetic NOD group.

Sel exists naturally in both organic (e.g. selenomethionine, selenocysteine) and inorganic forms (e.g. selenite, selenate, selenide) (Birringer *et al* 2002; Schweizer *et al* 2004). However, these compounds need to be catabolised into an inorganic precursor before being inserted into proteins, as the rare amino acid selenocysteine (Sec) is essential for the catalytic function of selenoenzymes (Carlson *et al* 2004). The presence of Sel instead of sulphur increases the activity of the respective enzymes by several orders of magnitude, as Sel is more nucleophilic and selenols are ionized at a physiological pH (Stadtman 1996; Zhong and Holmegren 2000). Sec, the 21st proteinogenic amino acid, was not initially recognized in the classical genetic code, as it is encoded by the UGA "stop" codon. For Sec to be inserted at UGA codons in the translation process, a specific RNA stem loop structure is required, which resides in eukaryotes in the 3'-untranslated region of the mRNA, the so-called selenocysteine insertion sequence (SECIS) (Berry *et al* 1991). Sel was also found to be an essential part of mammalian enzymes such as glutathione peroxidase, and the thyroid hormones deiodinase and thioredoxin reductase. There have been several reviews on the role of Sel, with emphasis on general health (Kohrle 2000; Kohrle *et al* 2000; Rayman 2000), Sel biochemistry (Birringer *et al* 2002) and selenoprotein biosynthesis (Hatfield and Gladyshev 2002). Inorganic forms of Sel such as selenite are better able to react

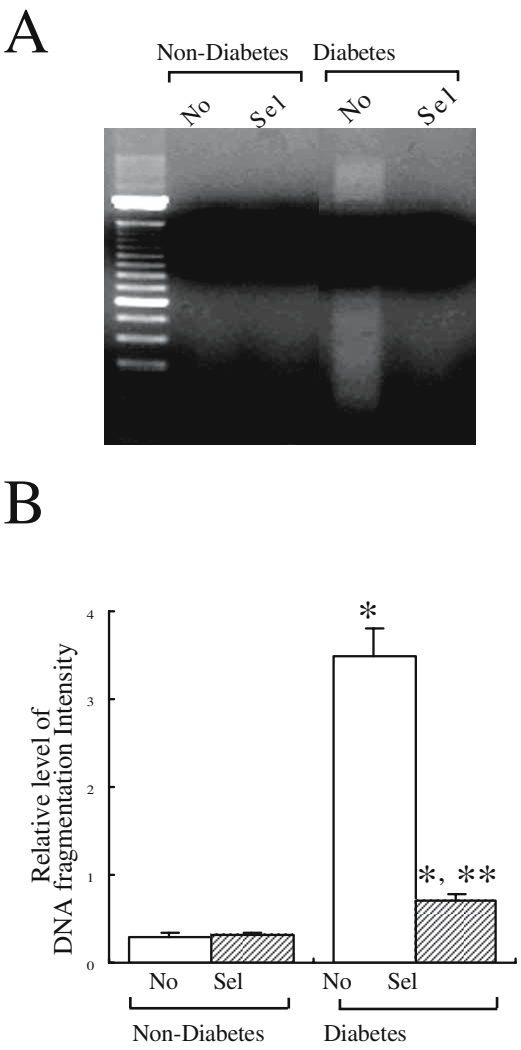

Figure 6. Progression of apoptosis in liver tissues from NOD mice was suppressed by Sel treatment. Fragmented DNA as an apoptosis indicator was purified from liver tissue and separated on 1.2% agarose gel as described in §2 . Three experiments were assayed in triplicate. Values are the mean±SD. Non-diabetic NOD group: diabetes phenotype non-induced mice, Diabetic NOD group: diabetes phenotype-induced mice, Sel-treated mice: mice treated with sodium selenite for three weeks. * P<0.05 is the significance level compared with the non-diabetic NOD group. ** P<0.05 is the significance level compared with the untreated mice.

at pharmacological concentrations with intracellular thiol groups than the organic compounds (e.g. selenomethione, Stadtman 1996). However, dietary supplementation with inorganic forms such as selenite could result in relatively poor absorption from the intestinal tract and subsequently poorer reserves of Sel in the body. Organic Sel, which also has high antioxidant properties primarily in the form of selenomethionine, can result in a higher bioavailability of Sel in the body compared with inorganic Sel (Enjalbert *et al* 1999).

In this study, 5 μmol/kg body weight/day of sodium selenite was injected into NOD mice to study the effect of Sel on diabetic symptoms. The dose of sodium selenite used in our study is very similar to that reported to be an effective dose to improve the mechanical and electrical activity of the heart in the diabetic rat by single-dose intraperitoneal injection for 4 weeks (Ayaz *et al* 2004). However, the dose of selenite given to the animals was such that it may have some toxic effects if given for prolonged periods. We were not able to measure plasma Sel levels and thus it was not possible to determine accurately the potential toxicity of the dose used. In a previously published study, the serum selenium concentration in humans spanned the following ranges: 400–30,000 μg/l associated with acute toxicity, 500–1,400 μg/l associated with chronic toxicity, and <1,400 μg/l free of toxicity; the category is determined by the signs and symptoms in the patient (Nuttall 2006).

Serum CHO and TG levels are markedly influenced by nutrition, endocrine and liver function. Raised levels are directly associated with metabolic diseases such as diabetes mellitus, chronic malfunction of the liver and hyperlipdaemia. In CHO metabolism, LDL plays a key role in transporting CHO to the tissues, whereas HDL acts as a scavenger of CHO in various tissues. Therefore, a decrease in some serum components such as TG, CHO and LDL cholesterol indicate that Sel treatment may alleviate the symptoms of diabetes in diabetic NOD mice. Furthermore, some serum biochemical enzymes such as AST, ALT, lactate dehydrogenase (LDH) and ALP are present in high concentrations in various organs such as the liver, muscle, brain and placenta. Raised levels of these enzymes in the blood indicates liver necrosis or disease (McCarthy *et al* 1980; Murray *et al* 1990). Increased serum LDH activity has been reported in animals deficient in antioxidant defence mechanisms, partly because of enhanced liver damage by lipid peroxidation (Reddy *et al* 1987). As seen in earlier studies, we observed a significant decrease in serum AST, ALT and ALP in the Sel-treated mice compared with untreated mice.

It has been shown that obesity is associated with the activation of cellular stress signalling and the inflammatory pathway (Hirosumi *et al* 2002; Ozcan *et al* 2004; Yuan *et al* 2001). In response to this cellular stress, the ER, a membranous network that functions in the synthesis and processing of secretory and membrane proteins, has been shown to be a key indicator (Berridge 2002). Some stress conditions disrupt ER homeostasis, which leads to the accumulation of unfolded or misfolded proteins in the ER lumen (Mori 2000; Harding *et al* 2002). It has been also reported that ER stress is particularly activated by glucose or nutrient deprivation, viral infections, lipids and increased synthesis of secretory proteins, as well as the expression of mutant or misfolded protein (Ma and Hendershot 2001; Kaufman *et al* 2002). In our study, we investigated the expression patterns of several

molecular indicators of ER stress in the liver of NOD mice both with and without Sel treatment. In the various ER stress pathways, ATF6 signalling was not induced by Sel treatment in either the diabetic or non-diabetic groups (data not show). However, Sel treatment significantly stimulated signalling through the IRE1-JNK protein pathway in the diabetic group, but not in the non-diabetic group. PERK-eIF2 signalling was also activated by Sel treatment in the diabetic NOD group. Thus, these results suggest the ER stress induced by diabetes is effectively counteracted by Sel treatment through the JNK and eIF2 signalling pathways.

In addition, the CHO protein, a key molecule of the ER stress signal in the final stage of the pathway, encodes a small nuclear protein from the C/EBP family, which was originally isolated from the culture of adipocytes. The CHOP gene is markedly activated by a variety of cellular stresses, including nutrient deprivation and metabolic perturbations (Barone *et al* 1994). When we examined the effect of Sel treatment on the induction and translocation of CHO protein by western blot and immunostaining assay, the onset of diabetes significantly reduced the expression of CHO protein in the total, and microsomal and nuclear fractions of liver tissues in the NOD mice. Immunostaining of the CHOP protein showed that Sel treatment markedly stimulated the translocation of the CHOP protein from the cytosol to the nuclear fraction in the non-diabetic group.

When insulin binds to its receptor at the surface of the cell membrane, the insulin signal is transmitted through the insulin receptor substrate (IRS)-1. Phosphorylation of IRS-1 has been linked to signal transduction from the insulin receptor to PI3-kinase. This leads to glucose transporter-4 (Glut-4) translocation and the subsequent stimulation of glucose uptake. Preliminary studies have shown that the enzyme PI3-kinase is correlated with glucose being absorbed by the entire body. Akt has also been proposed as a key step linking the activation of PI3-kinase to glucose uptake (Kahn *et al* 2006). Furthermore, this signalling pathway was disrupted by the ER stress signal in induced obesity through the activation of IRE, JNK and IRS-1 proteins (Ozcan *et al* 2004). In this study, we investigated the effects of Sel treatment on the interaction between ER stress signalling and insulin signalling. According to our data, Sel treatment significantly activated the insulin signalling pathway through the induction of Akt expression and phosphorylation only in the diabetic NOD group.

Finally, we investigated the effect of Sel treatment on apoptosis in the liver of the NOD mice. The amount of fragmented DNA increased slightly in diabetic NOD mice compared with non-diabetic mice. In particular, Sel treatment significantly suppressed the fragmentation of genomic DNA laddering in liver tissues of the diabetic group. However, few studies have been carried out to investigate the effect of Sel on the fragmentation pattern of liver genomic DNA in the diabetic NOD group. Our results suggest that Sel treatment can relieve the symptoms of diabetes through suppression of ER stress and insulin receptor signalling in diabetic NOD mice.

Taken together, our results show that Sel lowered serum glucose and biochemical profiles associated with liver damage and lipid metabolism in diabetic NOD mice, indicting that Sel can relieve or prevent the incidence of complicated diabetes. It is postulated that Sel has a crucial role in the regulation of diabetes via modulation of the ER stress signal through the activation of IRE, p-JNK and p-eIF2 proteins and insulin signalling through the phosphorylation of Akt and PI3 kinase in the diabetic NOD group. It seems that Sel compounds may not only serve as insulin-like molecules for downregulation of the glucose level, but also provide potential new drugs for the relief of diabetes by modulating the ER stress and insulin signalling mechanisms. Intensive work is still needed to define the role of Sel in preventing diabetes mellitus in NOD mice.

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