Reducing sugars trigger oxidative modification and apoptosis in pancreatic β-cells by provoking oxidative stress through the glycation reaction

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Several reducing sugars brought about apoptosis in isolated rat pancreatic islet cells and in the pancreatic β-cell-derived cell line HIT. This apoptosis was characterized biochemically by internucleosomal DNA cleavage and morphologically by nuclear shrinkage, chromatin condensation and apoptotic body formation. *N*-Acetyl-L-cysteine, an antioxidant, and aminoguanidine, an inhibitor of the glycation reaction, inhibited this apoptosis. We also showed directly that proteins in β-cells were actually glycated by using an antibody which can specifically recognize proteins glycated by fructose, but not by glucose. Furthermore, fluorescence-activated cell sorting analysis using dichlorofluorescein diacetate showed that reducing sugars increased intracellular peroxide levels prior to the induction of apoptosis. Levels of carbonyl, an index of oxidative modification, and of malondialdehyde, a lipid peroxidation product, were also increased. Taken together, these results suggest that reducing sugars trigger oxidative modification and apoptosis in pancreatic β-cells by provoking oxidative stress mainly through the glycation reaction, which may explain the deterioration of β-cells under conditions of diabetes.

INTRODUCTION

Reactive oxygen species (ROS) have been implicated in a wide range of biological functions, but they can be both essential and highly toxic to cellular homoeostasis [1]. Under normal conditions, potentially toxic ROS are primarily generated by mitochondrial respiratory metabolism and are efficiently neutralized by cellular antioxidant defence mechanisms. However, several conditions are known to disturb the balance between the production of ROS and cellular defence, resulting in cellular destruction and dysfunction. An imbalance between pro- and anti-oxidant factors plays an important role in many disease processes, including diabetes mellitus. It has been reported that DNA damage and mutations are caused by ROS, and that apoptosis is also induced by ROS in several cell types [2–5].

Reducing sugars are known to produce ROS mainly through the glycation reaction [6–8]. D-Ribose and 2-deoxy-D-ribose, sugars with a large reducing capacity, have been known to induce cell death in mononuclear cells [9,10]. Under diabetic conditions, glucose is converted into fructose through the polyol pathway, leading to increased levels of the latter sugar [11–13]. Fructose has a stronger reducing capacity than glucose, and the glycation reaction is easily induced by fructose [14,15]. Consequently, fructose is believed to play an important role in the diabetes-induced deterioration in various organ systems [14,15].

Incubation of β-cells with a large amount of reducing sugar results in deterioration of β-cell function, e.g. inhibition of insulin secretion [16,17], but the precise mechanism by which this occurs is not known. The aim of the present study was to explore DNA damage in β-cell nuclei, morphological changes and oxidative modifications (e.g. lipid peroxidation) after exposure to reducing sugars, and to investigate the mechanisms causing β-cell deterioration. This is the first demonstration that reducing sugar triggers oxidative modification and apoptosis in pancreatic β-cells by provoking oxidative stress through the glycation reaction. This may explain the deterioration of β-cells that is seen under diabetic conditions.

EXPERIMENTAL

Materials

The hamster pancreatic β-cell-derived cell line HIT was obtained from the American Type Culture Collection. Ribose, ADP-ribose and type XI collagenase were purchased from Sigma. Fructose, sorbitol and aminoguanidine were from Wako Chemicals. Glucose and *N*-acetyl-L-cysteine (NAC) were from Nacalai Chemical Co. Ficoll was from Pharmacia Biotech. 2′,7′-Dichlorofluorescein diacetate (H_2DCF-DA) was from Molecular Probes. The protein kinase inhibitors H7 and H8 were from Seikagaku Kogyo Co. Other reagents were of the highest grade available.

Cell culture conditions

HIT cells were grown in RPMI 1640 medium (Nikken Biomedical Laboratories) supplemented with 10% (v/v) fetal calf serum, 100 units/ml penicillin and 0.1 mg/ml streptomycin sulphate in a humidified atmosphere of 5% CO_2 at 37 °C. All experiments were carried out in the presence of serum.

Isolation of rat pancreatic islets

Islets were isolated from Sprague–Dawley rats by collagenase

Abbreviations used: BSO, DL-buthionine sulphoximine; DAPI, 4′,6-diamidino-2-phenylindole hydrochloride; HBSS, Hanks' balanced salt solution; H_2DCF-DA, 2′,7′-dichlorofluorescein diacetate; NAC, *N*-acetyl-L-cysteine; NF-κB, nuclear factor κB; ROS, reactive oxygen species.

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digestion and use of a Ficoll gradient as reported previously [18]. With the rat under ether anaesthesia, the common bile duct was clamped at its entrance to the duodenum and cannulated under a dissecting microscope with a 30-gauge hypodermic needle. Approx. 12 ml of cold Hanks' balanced salt solution (HBSS) containing 0.32 mg/ml type XI collagenase was injected into the common bile duct. The intrathoracic aorta was transected immediately before HBSS injection to minimize interstitial haemorrhage in the pancreas. The distended pancreas was removed and incubated for 20 min at 37 °C. Cold HBSS was added to stop the digestion reaction, and the digested pancreas was dispersed into small fragments by pipetting. The tube was then centrifuged at 60 ***g*** for 1 min at 4 °C. The supernatant was discarded and the pellet was washed twice with cold HBSS. The tissue suspension was passed through a mesh filter to remove the large undigested tissues, which usually consisted of mesenteric lymph nodes and large vessel fragments. The filtered tissue was washed again and the pellet was resuspended in 25 % Ficoll solution. The tissue suspension was layered under a discontinuous Ficoll gradient (23, 20.5 and 11 %) followed by centrifugation at 30 ***g*** for 3 min. The tissue at the 20.5 %/11 % Ficoll interface was collected and washed twice with cold HBSS. These islets were precultured overnight in RPMI medium supplemented with 10 % fetal calf serum, 100 units/ml penicillin and 0.1 mg/ml streptomycin sulphate in a humidified atmosphere of 5 % CO_2 at 37 °C before exposure to the reagents.

Determination of internucleosomal DNA cleavage

After incubation with the reagents described, internucleosomal DNA cleavage was detected as reported previously [19]. HIT cells were washed twice with PBS, scraped off the plates and precipitated by centrifugation at 120 ***g*** for 10 min. Lysis buffer (0.2 M Tris/HCl, pH 8.0, 0.1 M Na_2EDTA, 1 % SDS and 100 μg/ml proteinase K) was added and the cells were incubated for 4 h at 55 °C. The nuclear lysates were extracted twice with phenol and then with an equal volume of phenol/chloroform/3-methylbutan-1-ol (25:24:1, by vol.). DNA was precipitated with 0.05 vol. of 5 M NaCl and 2.5 vol. of ethanol overnight at −35 °C, and sedimented at 5000 ***g*** for 10 min at 4 °C. The DNA pellet was dried and dissolved in 10 mM Tris/HCl, pH 7.5, containing 1 mM Na_2EDTA and 20 μg/ml RNase A and incubated for 1 h at 37 °C. The DNA was finally extracted with an equal volume of phenol/chloroform/3-methylbutan-1-ol (25:24:1, by vol.). DNA samples were analysed by electrophoresis on 1.5 % agarose gels and visualized by staining with 1 μg/ml ethidium bromide. *Pst*I-digested λDNA was used as a molecular size marker.

Fluorescence microscopy

After incubation with the reagents, fluorescence microscopy was carried out as reported previously [20,21]. HIT cells were washed twice with PBS, scraped off the plates and precipitated by centrifugation at 120 ***g*** for 10 min. Cells were fixed for 5 min with 3 % paraformaldehyde buffered with PBS, washed with PBS and stained with H33258 (16 μg/ml) for 15 min at room temperature. Cells were also stained with 0.5 μg/ml 4′,6-diamidino-2-phenylindole hydrochloride (DAPI) in PBS for 60 min at room temperature for quantitative analysis of apoptosis. Cells containing highly condensed chromatin and irregular DNA inclusions were defined as apoptotic; non-apoptotic cells showed a more homogeneous and moderate DNA staining throughout the entire nucleus. After each treatment, 400–500 cell nuclei were observed, and apoptotic and total nuclei were counted in fluorescent mode. The percentage of apoptotic nuclei was calculated after each treatment.

Scanning electron microscopy

After incubation with the reagents, scanning electron microscopy was carried out as follows. HIT cells on a cover glass attached to culture dishes were fixed with 2.5 % glutaraldehyde buffered with 0.1 M sodium phosphate buffer (pH 7.4). The cover glass was removed from the dishes, and the cells on the cover glass were post-fixed with 1 % OsO_4 in the same buffer and dehydrated with a graded ethanol series. The specimen was further dried in a freeze dryer using t-butyl alcohol, and then coated with platinum and carbon.

Transmission electron microscopy

After incubation with the reagents, transmission electron microscopy was carried out as follows. HIT cells on culture dishes were fixed with 2.5 % glutaraldehyde buffered with 0.1 M sodium phosphate buffer (pH 7.4), post-fixed with 1 % OsO_4 in the same buffer for 1 h and stained with 3 % aqueous uranyl acetate for 1 h. After dehydration with increasing concentrations of ethanol, the cells were embedded in Epon 812 resin. Ultra-thin sections mounted on copper grids were stained with 3 % aqueous uranyl acetate and Reynolds' lead citrate for 5 min and 3 min respectively.

Immunological determination of proteins glycated by fructose in HIT cells

After incubation of HIT cells with several reagents, cells were washed twice with PBS and homogenized in PBS containing 1 mM *p*-amidinophenylmethanesulphonyl fluoride hydrochloride, 10 mM benzamidine, 10 μg of antipain and 10 μg/ml aprotinin. The homogenates were centrifuged at 10000 ***g*** at 4 °C for 20 min. Supernatant fractions were obtained and the protein concentration was measured with a BSA protein assay reagent kit (Pierce). Aliquots of 50 μl of glycated samples, diluted to 50 μg/ml with PBS, were added to the wells of an immunoplate, incubated for 2 h at room temperature and washed four times with 0.05 % Tween-20 in PBS. A 250 μl portion of 1 % BSA in PBS was added to block non-specific binding, with incubation at room temperature for 2 h followed by washing four times with PBS.

We have recently established a polyclonal antibody that can recognize proteins glycated by fructose, but not by glucose or ribose (N. Miyazawa, J. Fujii, T. Myint, A. Hoshi, T. Teshima and N. Taniguchi, unpublished work), by a procedure similar to that used for purification of a hexitol-lysine antibody which recognizes proteins glycated by glucose, but not by fructose or ribose [22]. In brief, the anti-Amadori product antibody recognizing fructosylated lysine used in the present study was raised in rabbits by immunization with fructosylated lysine conjugated to keyhole-limpet haemocyanin in complete adjuvant, and was purified by ammonium sulphate fractionation and affinity chromatography on fructosylated lysine coupled to activated CH-Sepharose-4B. The affinity-purified polyclonal antibody was diluted 1:10000 with PBS. Aliquots (50 μl/well) were added to the immunoplate, followed by incubation at room temperature for 2 h. Unbound antibody was removed by washing four times with washing buffer, and the plate was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2000 dilution in PBS) for 2 h at room temperature. The unbound second antibody was removed and peroxidase activity was

detected by adding 50 μl of an *o*-phenylenediamine dihydrochloride solution containing H_2O_2, prepared according to manufacturer's instructions (Sigma Chemicals Co.). The reaction was stopped by the addition of 50 μl of 1 M H_2SO_4 to each well. The absorbance of each well was determined at 490 nm with an Immunoreader NJ 200 (Intermed). The results are expressed in relative units, with the aborbance of untreated cells arbitrarily set at 1.0.

Measurement of intracellular peroxide levels by flow cytometry analysis

Intracellular peroxide levels were assessed using the oxidant-sensitive fluorescent probe H_2DCF-DA [23]. In the presence of intracellar peroxides, H_2DCF is oxidized to a highly fluorescent compound, 2′,7′-dichlorofluorescein. Cells, treated with reagents, were incubated with 5 μM H_2DCF-DA. The cellular fluorescence intensity, which was directly proportional to the intracellular peroxide level, was measured after 30 min of H_2DCF-DA oxidation using a FACScan instrument (Becton Dickinson). For each analysis, 10000 events were recorded. For image analysis, cells were analysed for fluorescence intensity using a lysis cell analysis system.

Nitrite determination

NO formation was measured spectrophotometrically as nitrite formed during exposure, using the Griess reaction [24]. Briefly, 100 μl aliquots were removed from the culture medium and incubated with 100 μl of Griess reagent [1 % sulphanilamide in 0.1 mol/l HCl and 0.1 % *N*-(1-naphthyl)ethylenediamine dihydrochloride] at room temperature for 10 min. The absorbance was measured at 550 nm using an Immunoreader NJ 2000 (Intermed).

Gel-mobility shift assay

After incubation of HIT cells with reagents, nuclear extracts were prepared using the procedure reported previously [25]. Samples of 6 μg of nuclear extract were incubated with 2 μg of poly(dI-dC) at 4 °C in a 20 μl of reaction buffer (25 mM Hepes, pH 7.9, 5 mM EDTA, 50 mM KCl, 10 % glycerol, 1 mM dithiothreitol and 0.5 mM *p*-amidinophenylmethanesulphonyl fluoride hydrochloride). The binding reaction was initiated by addition of 5′-end-^{32}P-labelled oligonucleotide of wild-type or mutated nuclear factor κB (NF-κB) probe and, when required, non-radioactive competitor double-stranded oligonucleotide, followed by incubation at room temperature for 30 min. The wild-type NF-κB probe was 5′-GGCCCAACTGGGGACTCTCCC TTTG-3′ (the NF-κB binding site is underlined), and the mutated probe was 5′-GGCCCAACTGCTCACTCTCC CTTTG-3′ (mutated nucleotides are underlined). Products were electrophoresed at 150 V for 1 h on 5 % polyacrylamide gels in 1 × TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA), and dried gels were analysed by autoradiography.

Measurement of carbonyl levels

After incubation of HIT cells with fructose or ribose, the carbonyl level was measured as reported previously [26]. Cells were washed twice with PBS, homogenized in 0.5 ml of lysis buffer (10 % Triton X-100 in PBS) and centrifuged to remove debris. Then 1 vol. of 10 % (w/v) streptomycin solution was added to 9 vol. of supernatant and allowed to stand for 15 min. Centrifugation was carried out at 5000 ***g*** for 10 min, and the supernatant was taken for assay of protein-bound carbonyl groups. Proteins were pelleted by precipitation with 10 % trichloroacetic acid and centrifugation at 5000 ***g*** for 5 min. The precipitate was resuspended in 0.5 ml of 2 M HCl, with or without 10 mM 2,4-dinitrophenylhydrazine, and stirred at 5 min intervals for 1 h at room temperature. Proteins were precipitated again with 10 % trichloroacetic acid and pelleted at 5000 ***g*** for 5 min. Pellets were washed three times with 1 ml of ethanol/ethyl acetate (1:1, v/v) to remove free reagent, allowing the sample to stand for 10 min before centrifugation and discarding the supernatant each time. Pellets were resuspended in 0.6 ml of 6 M guanidine/10 mM phosphate buffer/trifluoroacetic acid (pH 2.3), incubated at 37 °C overnight and centrifuged at 5000 ***g*** for 3 min. The absorbance of supernatants was measured in a spectrophotometer at 280 nm to assess protein concentration and at 366 nm to assess carbonyl content. Carbonyl content was caluculated using a molar absorption coefficient of 22.0×10^3 $M^{-1} \cdot cm^{-1}$ for aliphatic hydrazones.

Measurement of malondialdehyde levels

After incubation with the reagents, malondialdehyde levels were measured as reported previously [27]. Cells were homogenized in 0.5 ml of PBS, and then 1 ml of TBA reagent (0.375 % thiobarbituric acid, 0.25 M HCl, 0.06 % 2-6-di-t-butyl-4-hydroxytoluene and 22 % ethanol) was added. The solution was incubated at 100 °C for 15 min and centrifuged at 500 ***g*** for 10 min. The absorbance of supernatants was measured in a spectrophotometer at 535 nm to assess the malondialdehyde level, which was calculated using a molar absorption coefficient of 1.56×10^5 $M^{-1} \cdot cm^{-1}$ and expressed as nmol/mg of protein.

RESULTS

Internucleosomal DNA cleavage and morphological changes induced by reducing sugars

After incubation of HIT cells with 50 mM fructose for 3 days, cleavage of DNA into nucleosomal fragments was observed (Figure 1A), suggesting apoptosis, although this was not clearly visible after 2 days of incubation. DNA cleavage was greater after incubation with 100 mM fructose and after incubation with $>$ 25 mM ribose for 3 days, DNA cleavage was also observed (Figure 1A). These results demonstrate that reducing sugars induce HIT cells to undergo internucleosomal DNA cleavage in a dose- and time-dependent manner. To investigate the mechanism by which DNA is damaged in β-cell nuclei, several reducing sugars were employed. Glucose is also a reducing sugar, but its reducing capacity is not as high as that of fructose or ribose. DNA cleavage was not clearly detected after 3 days of incubation with 100 mM glucose. After incubation with sorbitol, a non-reducing sugar, cleavage was not observed at all. These results indicate that DNA cleavage was dependent on the the reducing capacity of the sugar.

Internucleosomal DNA cleavage is often accompanied by morphological changes in the cells. Thus morphological changes were also examined by fluorescence microscopy. Nuclear fragmentation, a change characteristic of apoptosis, was observed after 3 days of treatment with 100 mM fructose (Figure 1B, parts a and c) or 25 mM ribose (Figure 1B, parts b and d). The percentage of cells undergoing apoptosis was also measured after 1–7 days of treatment with 100 mM fructose or 25 mM ribose (Figure 1C). After treatment with fructose or ribose, some cells were lysed. Thus the cell number was also counted after 1–7 days of treatment with 100 mM fructose or 25 mM ribose (Figure 1D).

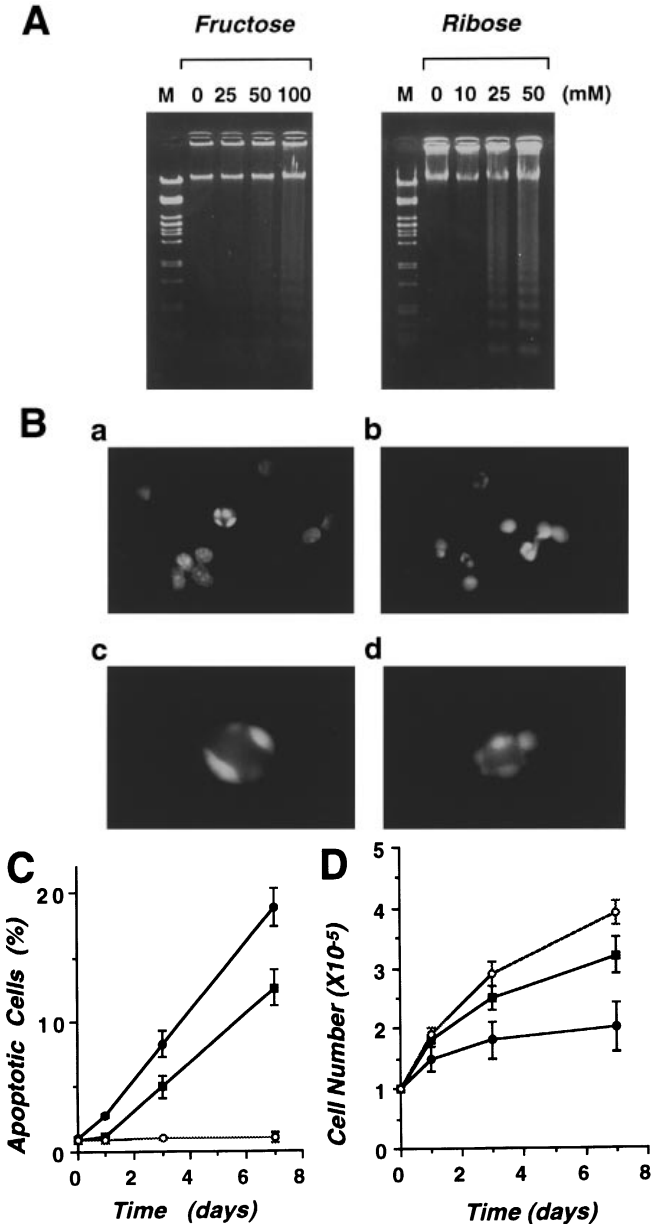

Figure 1 Internucleosomal DNA cleavage and morphological changes in HIT cells treated with reducing sugars

(**A**) After incubation of HIT cells with several concentrations of fructose (0–100 mM) or ribose (0–50 mM) for 3 days, DNA from the cells was extracted and subjected to electrophoresis on a 1.5% agarose gel. Lane M contains molecular mass markers. (**B**) After incubation of HIT cells with the reagents described below for 3 days, morphological changes were examined by fluorescence microscopy. Parts (**a**) and (**c**), 100 mM fructose; (**b**) and (**d**), 25 mM ribose. Magnification: (**a**) and (**b**), ×128; (**c**) and (**d**), ×640. (**C**) After incubation of HIT cells with 100 mM fructose (■) or 25 mM ribose (●) for 1–7 days, the cells were stained with DAPI and the percentage of cells undergoing apoptosis was measured. ○, Control. Results are means ± S. D. of three experiments. (**D**) After incubation of HIT cells with 100 mM fructose (■) or 25 mM ribose (●) for 1–7 days, the number of cells per 6-well plate was counted. ○, Control. Results are means ± S.D. of three experiments.

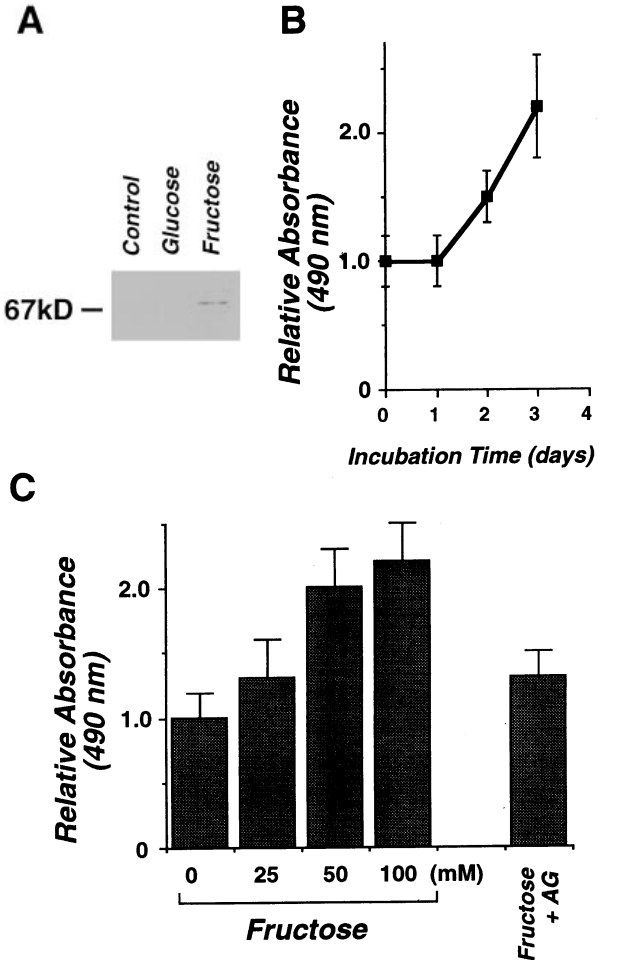

Figure 2 Determination by ELISA of glycated proteins in HIT cells treated with fructose

(**A**) After incubation of BSA with fructose or glucose, Western blot analysis was carried out with an antibody specific for proteins glycated by fructose. A single band indicates glycated BSA (67 kDa). (**B**) After incubation of HIT cells with 100 mM fructose for 1–3 days, glycated proteins were detected by ELISA with the antibody specific for proteins glycated by fructose. Results are means ± S.D. of three experiments. (**C**) After incubation with several concentrations of fructose (0–100 mM) or 100 mM fructose + 1 mM aminoguanidine (AG) for 3 days, glycated proteins were detected by ELISA. Results are means ± S.D. of three experiments.

Immunological determination of glycated proteins in HIT cells treated with fructose

We examined whether cytosolic proteins were actually glycated in HIT cells after incubation with fructose (Figures 2B and 2C) by using an antibody specific for proteins glycated by fructose (confirmed by Western blot analysis; Figure 2A). After incubation with 100 mM fructose for 2 days, glycated proteins were detected in HIT cells by ELISA with the antibody (Figure 2B). The extent of glycation was increased after 3 days of incubation (Figure 2B). Glycated proteins were detected after incubation with 25 mM fructose, but the degree of glycation was greater after incubation with 50 mM or 100 mM fructose (Figure 2C). These results show that the proteins of HIT cells were glycated by fructose in a time- and dose-dependent manner. These changes were suppressed by the addition of 1 mM aminoguanidine, an inhibitor of the glycation reaction (Figure 2C).

Reducing sugars increase intracellular peroxide levels in HIT cells

We assessed the intracellular peroxide level by flow cytometric analysis using the peroxide-sensitive fluorescent probe H_2DCF-DA. Considerable production of intracellular peroxide in HIT cells was observed after 3 days of incubation with 100 mM

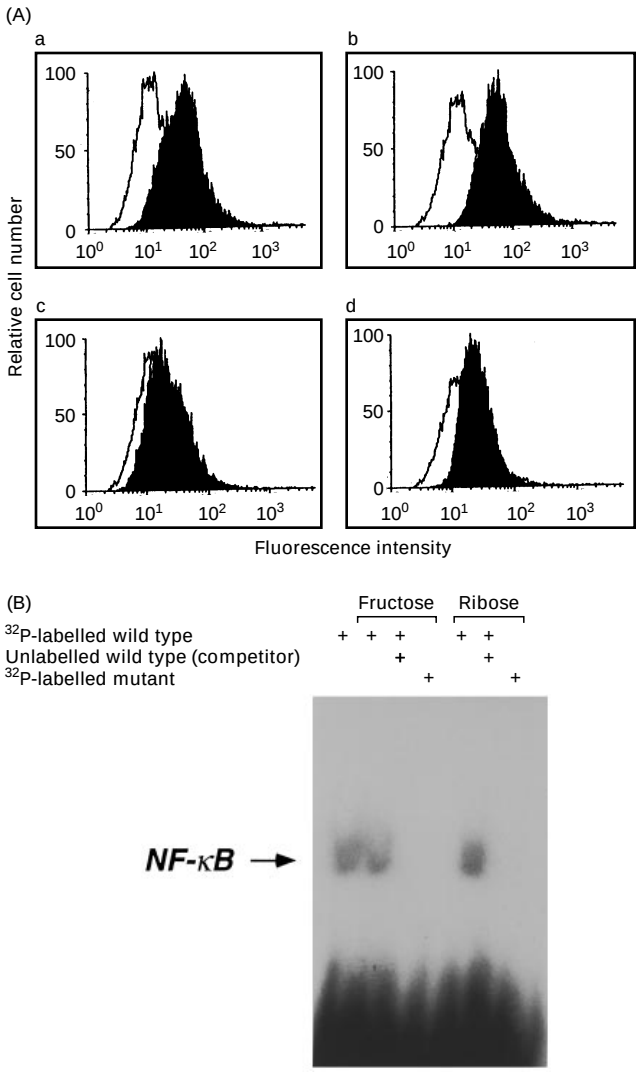

Figure 3 Flow cytometric analysis of intracellular peroxide levels, and analysis of protein binding to NF-κB

(**A**) Relative peroxide concentrations in the cells were quantified by flow cytometry using the peroxide-sensitive dye H_2DCFH-DA. Cells were incubated with H_2DCF-DA (5 μM) during the final 30 min of each treatment. Cells were treated for 3 days with (black area) or without (white area) 100 mM fructose (**a**), 100 mM fructose + 10 mM NAC (**b**), 25 mM ribose (**c**) or 25 mM ribose + 10 mM NAC (**d**). (**B**) After treatment of HIT cells for 3 days without (lane 1) or with 100 mM fructose (lanes 2–4) or 25 mM ribose (lanes 5–7), a gel-mobility shift assay was performed using the NF-κB consensus binding site or a mutated oligonucleotide as a probe. Probes were used as shown.

fructose (Figure 3A, part a) or 25 mM ribose (Figure 3A, part b); peroxide formation was also detected, to a lesser extent, after 2 days of incubation. The increase in intracellular peroxide was suppressed by the addition of 10 mM NAC (Figure 3A, parts c and d) or 500 units/ml catalase (results not shown). Thus intracellular peroxide is a possible mediator of DNA cleavage induced by fructose or ribose.

NO is a free radical and could induce apoptosis in β-cells, as we and others have reported previously [19,28]. Therefore we measured NO production spectrophotometrically as nitrite formed during exposure to fructose or ribose using the Griess reaction. However, there was no increase in nitrite in the medium after incubation with ribose or fructose (results not shown). We also examined whether NF-κB is activated by reducing sugars, since this factor is known to be regulated by oxidative stress. NF-κB was activated to some extent in untreated HIT cells; after incubation with fructose or ribose, however, the extent of activation was not changed significantly, contrary to our expectations (Figure 3B). Thus NF-κB does not seem to be involved in reducing-sugar-induced apoptosis. Furthermore, apoptosis was not blocked by $ZnCl_2$, an endonuclease inhibitor (results not shown), which indicates that the sugar-induced apoptosis is not triggered by activation of an endonuclease.

Table 1 Increases in carbonyl and malondialdehyde levels in HIT cells treated with fructose

After incubation of cells with reagents for 3 days, carbonyl and malondialdehyde levels were measured. Results are means ± S.E.M. of three experiments.

	Content (nmol/mg of protein)	
Treatment	Carbonyl	Malondialdehyde
Control	8.6 ± 0.8	0.3 ± 0.1
Fructose	15.7 ± 1.0	2.6 ± 0.3
Fructose + NAC	9.8 ± 0.6	0.8 ± 0.2
Fructose + aminoguanidine	10.2 ± 0.8	1.4 ± 0.2

Increases in carbonyl and malondialdehyde levels induced by reducing sugars

An increased content of carbonyl groups is an index of the oxidative modification of amino acids, including the glycation reaction. Amadori rearrangement products have carbonyls in their structures. We measured the carbonyl levels in HIT cells after incubation with reducing sugars. After 3 days of incubation with 100 mM fructose, carbonyl formation was increased; this increase was suppressed by the addition of 1 mM aminoguanidine or 10 mM NAC (Table 1). Furthermore, in order to confirm that β-cells are modified by oxidative stress after treatment with reducing sugars, we measured malondialdehyde levels. Malondialdehyde is an abundant aldehyde resulting from lipid peroxidation and has been used as an index of the extent of lipid peroxidation. After 3 days of incubation with 100 mM fructose, the malondialdehyde level was greatly increased; again, this increase was suppressed by the addition of 1 mM aminoguanidine or 10 mM NAC (Table 1). These results indicate that reducing sugar induces oxidative modification by provoking oxidative stress through the glycation reaction. Furthermore, in order to rule out the possibility that NAC directly reduces malondialdehyde without affecting its generation, NAC was added at the end of the incubation period immediately before assay. The increase in malondialdehyde content was not suppressed in this case (results not shown).

Involvement of oxidative stress in induction of apoptosis by reducing sugars

In order to understand the mechanism by which reducing sugars induce apoptosis, experiments were performed with several possible inhibitors. Fructose and ribose may interfere with the redox status of the cell. Because the increase in oxidative stress, including the production of hydrogen peroxide, was implicated in the induction of internucleosomal DNA cleavage by reducing sugars, we tested whether the induction of apoptosis was suppressed by NAC, which can raise intracellular GSH levels and thereby protect cells from the effects of ROS [29,30]. Addition

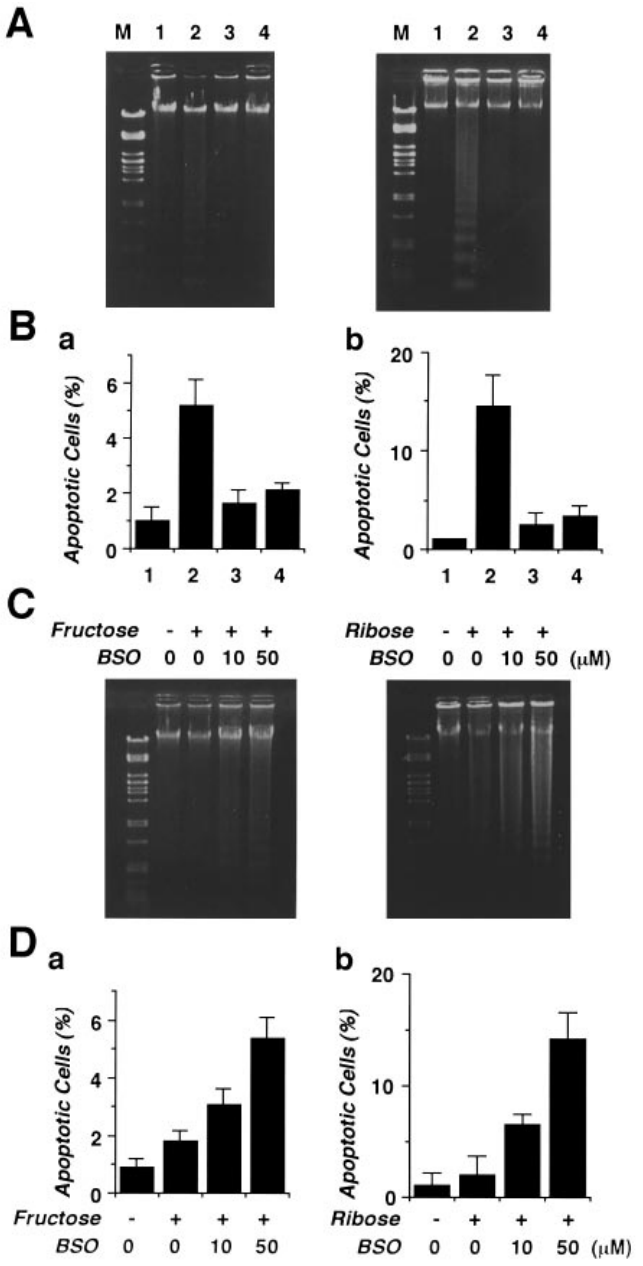

Figure 4 Effects of NAC, aminoguanidine and BSO on internucleosomal DNA cleavage

(**A**) After incubation of HIT cells with reagents for 3 days, DNA from the cells was extracted and subjected to electrophoresis on a 1.5% agarose gel. Lane M contains molecular mass markers. Left panel: lane 1, control; lane 2, 100 mM fructose; lane 3, 100 mM fructose + 10 mM NAC; lane 4, 100 mM fructose + 1 mM aminoguanidine. Right panel: lane 1, control; lane 2, 25 mM ribose; lane 3, 25 mM ribose + 10 mM NAC; lane 4, 25 mM ribose + 1 mM aminoguanidine. (**B**) After incubation of HIT cells with reagents for 3 days, the cells were stained with DAPI and the percentage of cells undergoing apoptosis was measured. Left panel: 1, control; 2, 100 mM fructose; 3, 100 mM fructose + 10 mM NAC; 4, 100 mM fructose + 1 mM aminoguanidine. Right panel: 1, control; 2, 25 mM ribose; 3, 25 mM ribose + 10 mM NAC; 4, 25 mM ribose + 1 mM aminoguanidine. Results are means ± S.D. of three experiments. (**C**) Effect of BSO on internucleosomal DNA cleavage induced by reducing sugars. After incubation of HIT cells with different concentrations (0–50 μM) of BSO for 1 day, the cells were incubated with 100 mM fructose or 25 mM ribose for 2 days and the DNA from the cells was extracted and subjected to electrophoresis on a 1.5% agarose gel. Lane M contains molecular mass markers. (**D**) After incubation of HIT cells with different concentrations (0–50 μM) of BSO for 1 day, the cells were incubated with 100 mM fructose or 25 mM ribose for 2 days, the cells were stained with DAPI and the percentage of cells undergoing apoptosis was measured. Results are means ± S.D. of three experiments.

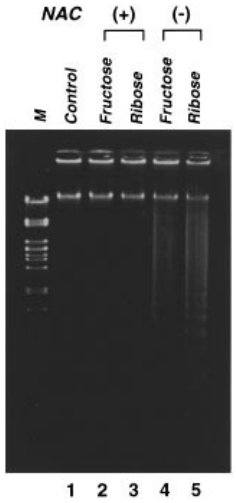

Figure 5 Internucleosomal DNA cleavage induced by reducing sugars in isolated rat pancreatic islets

After incubation of isolated pancreatic islets with reagents for 3 days, DNA from the cells was extracted and subjected to electrophoresis on a 1.5% agarose gel. Lane M contains molecular mass markers. Lane 1, control; lane 2, 100 mM fructose + 10 mM NAC; lane 3, 25 mM ribose + 10 mM NAC; lane 4, 100 mM fructose; lane 5, 25 mM ribose.

of 10 mM NAC inhibited internucleosomal DNA cleavage induced by fructose or ribose (Figure 4A). DNA cleavage was also suppressed by addition of 1 mM glutathione ethyl ester (results not shown). Furthermore, experiments were performed with DL-buthionine sulphoximine (BSO), a reagent which depletes intracellular GSH levels. After preincubation with BSO, the extent of DNA cleavage induced by fructose or ribose was increased (Figure 4C), although cleavage was not detected after incubation with BSO alone (0–50 μM). DNA damage in BSO-treated cells was also prevented by NAC (results not shown). These results indicate that oxidative stress is involved in DNA cleavage.

In order to confirm the participation of the glycation reaction in DNA cleavage, aminoguanidine, an inhibitor of the glycation reaction, was employed [31–33]. Addition of 1 mM aminoguanidine to the incubation medium suppressed internucleosomal DNA cleavage (Figure 4A). The percentage of cells undergoing apoptosis was also measured after addition of 10 mM NAC or 1 mM aminoguanidine (Figure 4B), and after preincubation with BSO (Figure 4D). All of these results were consistent with the conclusion that oxidative stress provoked through the glycation reaction by reducing sugars was responsible for internucleosomal DNA cleavage.

To examine the possible involvement of a protein kinase in apoptosis [21,35], we examined the effects of several protein kinase inhibitors on internucleosomal DNA cleavage induced by reducing sugars. The protein kinase C inhibitor H7 and the protein kinase A inhibitor H8, however, had no inhibitory effect on DNA cleavage (results not shown). Thus protein kinases do not seem to have a role in reducing-sugar-induced apoptosis.

Internucleosomal DNA cleavage triggered by reducing sugars in isolated rat pancreatic islets

The pancreatic β-cell-derived cell line HIT is known to possess some β-cell functions such as insulin secretion, but may differ from normal β-cells in other aspects. Therefore we performed the experiments with freshly isolated rat pancreatic islets in order to

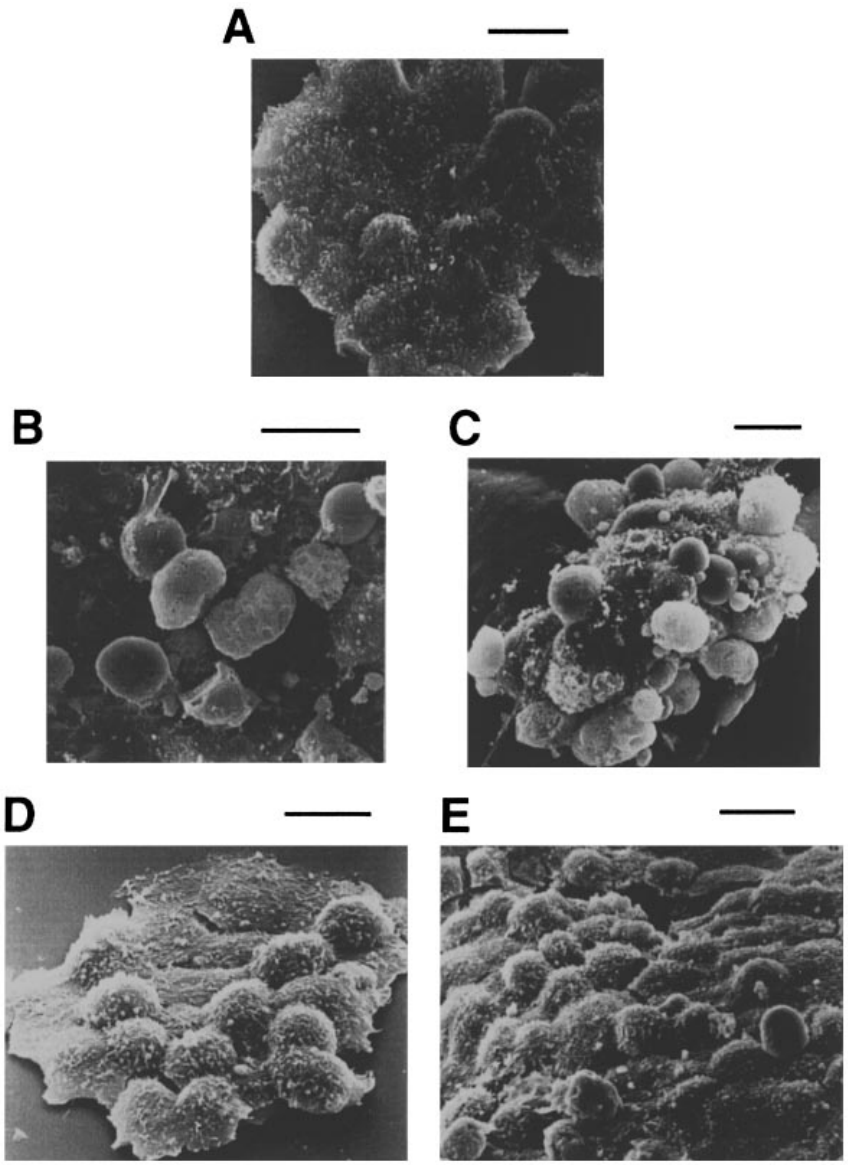

Figure 6 Morphological evidence for apoptosis by scanning electron microscopy

After incubation of HIT cells with reagents for 3 days, morphological changes were examined by scanning electron microscopy. (**A**) Control; (**B**) 100 mM fructose; (**C**) 25 mM ribose; (**D**) 100 mM fructose + 10 mM NAC; (**E**) 25 mM ribose + 10 mM NAC. Bars = 2 μm.

validate the significance of sugar-induced apoptosis in the deterioration of β-cells. After incubation of isolated pancreatic islets with 100 mM fructose or 25 mM ribose for 3 days, cleavage of DNA into nucleosomal fragments was observed, as seen in pancreatic β-cell line HIT (Figure 5). Furthermore, addition of 10 mM NAC to the incubation medium suppressed DNA cleavage (Figure 5), as was observed in HIT cells (Figure 4A). Thus the results obtained using HIT cells seem to be applicable to freshly isolated pancreatic islets.

Morphological evidence for apoptosis by electron microscopy

We examined morphological changes by electron microscopy. Scanning electron microscopic examination showed that untreated HIT cells were covered with microvilli and ruffles (Figure 6A). After 3 days of treatment with 100 mM fructose or 25 mM ribose, the numbers of microvilli and ruffles were decreased. Instead, extensive surface blebbing and granular protrusions were often observed. Spherical bodies with a smooth surface were sometimes found to be attached to the cells. These are thought to correspond to apoptotic bodies (Figures 6B and 6C). These morphological changes were suppressed by the addition of 10 mM NAC (Figures 6D and 6E). Furthermore, after incubation with fructose or ribose, some vacuoles, many small vesicles and nuclear fragmentation with condensed nuclear chromatin were observed by transmission electron microscopy (Figures 7B and 7C), although these morphological features were not observed in untreated cells (Figure 7A). These morphological changes were also suppressed by the addition of 10 mM NAC (Figures 7D and 7E). These results indicate that oxidative stress

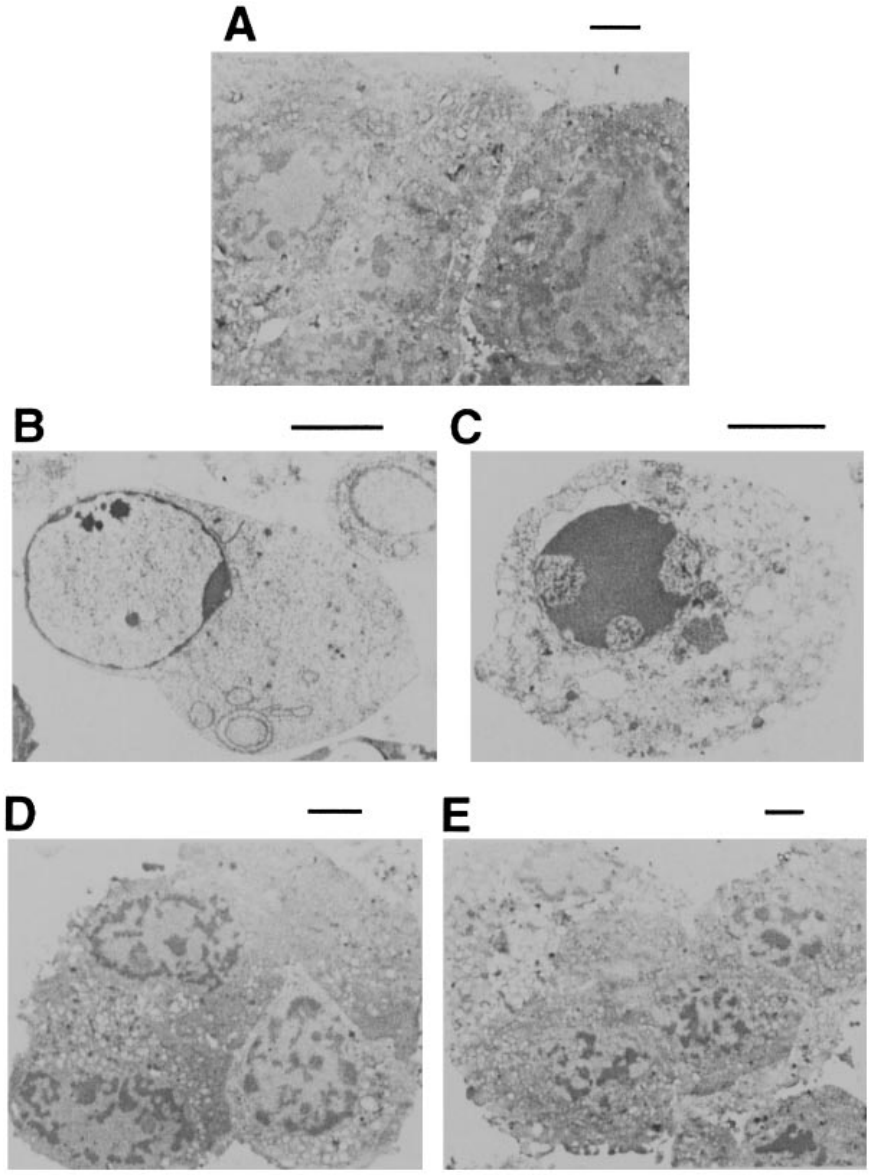

Figure 7 Morphological evidence for apoptosis by electron transmission microscopy

After incubation of HIT cells with reagents for 3 days, morphological changes were examined by transmission electron microscopy. (**A**) Control; (**B**) 100 mM fructose; (**C**) 25 mM ribose; (**D**) 100 mM fructose + 10 mM NAC; (**E**) 25 mM ribose + 10 mM NAC. Bars = 2 μm.

is involved in the morphological changes as well as in DNA damage.

DISCUSSION

ROS have been demonstrated to cause apoptotic cell death, which can be blocked by antioxidants [2–5]. In the present study we have shown that several reducing sugars caused internucleosomal DNA cleavage leading to apoptosis in pancreatic β-cells; this effect depended on the reducing capacity of the sugars. Under diabetic conditions, the production of several reducing sugars is increased through glycolysis or the polyol pathway, and oxidative stress is provoked. There are several ways in which intracellular levels of ROS could be increased under diabetic conditions. One is an increase in the production of ROS through the glycation reaction. Another would be the inhibition of mechanisms for ROS elimination. The activities of superoxide dismutase and GSH peroxidase are reported to be decreased under diabetic conditions [36,37]. Consequently, several organs are exposed to a large amount of ROS. We have reported that DNA cleavage and protein fragmentation are induced by ROS produced through the glycation reaction [36,38].

An involvement of oxidative stress in sugar-induced apoptosis is supported by the inhibitory effect of NAC. NAC raises intracellular GSH levels and thereby provides GSH peroxidase with the co-substrate required to eliminate hydroperoxide [29]. Sugar-induced apoptosis was also suppressed by aminoguanidine, an inhibitor of the glycation reaction [31–33]. All of these data indicate that oxidative stress provoked through the glycation reaction triggers the apoptosis of β-cells, leading to

deterioration of their function. It has been reported, however, that aminoguanidine has antioxidant properties in addition to its effects on glycation, and may interfere with the measurement of lipid peroxidation [38a]. Thus we cannot eliminate the possibility that aminoguanidine had effects other than as an inhibitor of glycation in our experiments. After treatment of β-cells with reducing sugars an increase in intracellular peroxide levels was observed, and the levels of carbonyl and malondialdehyde in β-cells were also increased. Carbonyl levels are increased as a result of the glycation reaction [39]. Amadori rearrangement products, but not Schiff bases, have carbonyls in their structures. Taken together, these observations indicate that reducing sugars induce lipid peroxidation in membranes as well as DNA cleavage in nuclei by producing ROS.

We could not clearly detect internucleosomal DNA cleavage after treatment of β-cells with glucose. Glucose is, however, also a reducing sugar (although its reducing capacity is not as great as that of fructose or ribose) and thus the function of β-cells is thought to deteriorate after exposure to glucose itself for a long duration, as seen under diabetic conditions. Glucose is converted into fructose by the polyol pathway, and the fructose level is increased in several organs under diabetic conditions [11–13]. The levels of two major enzymes in the polyol pathway (aldose reductase and sorbitol dehydrogenase) are very low in pancreatic β-cells [40]. Thus we believe that glucose is not easily converted into fructose in β-cells themselves. It seems that fructose produced in other tissues is transported into β-cells. It is reported that fructose is transported into β-cells via the glucose transporter GLUT2 which is present in β-cells [41], and β-cells are exposed to a greater amount of fructose under diabetic conditions. Finally, β-cells are thought to undergo oxidative modification (e.g. lipid peroxidation) and apoptosis. The actual concentration of fructose *in vivo* (normal range 0.05–0.3 mM) is not as high as those used in our experiments, but the function of β-cells is thought to deteriorate after exposure to a low concentration of fructose for a long duration. After treatment with fructose, some cells were lysed and the increase in cell number was suppressed (Figure 1D). Thus the actual extent of cell damage seems to be greater than the percentage of apoptotic cells. Furthermore, the percentage of apoptotic cells increased gradually in a time-dependent manner (Figure 1D). Therefore, since under diabetic conditions β-cells are exposed to fructose for a long period and their function gradually deteriorates, the present study is useful for understanding the mechanisms of deterioration of β-cells under diabetic conditions.

There are several possible mechanisms by which apoptosis can be triggered. Some proteases or endonucleases may be activated and facilitate apoptosis. The activation of NF-κB is known to be regulated by ROS [42], but NF-κB activation was not clearly detected after treatment of β-cells with reducing sugars. NF-κB was activated to some extent even in untreated HIT cells, and moreover ROS are thought to accumulate gradually, mainly through the glycation reaction. This may be why we could not clearly detect the activation of NF-κB. There may be another mechanism by which apoptosis is triggered by reducing sugars.

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