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ORIGINAL ARTICLE

Cholesterol induces mitochondrial dysfunction and apoptosis in mouse pancreatic beta-cell line MIN6 cells

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Abstract Reduction of pancreatic β-cell mass is a key element leading to type 2 diabetes. Obesity and overweight with high levels of lipids including cholesterol are tightly linked to type 2 diabetes. The direct impact of cholesterol on pancreatic β-cells, however, has not been extensively studied. In this study, MIN6 mouse β-cell line was used to test the effect of cholesterol on pancreatic β-cell apoptosis over different doses and durations. It was found that cholesterol dose- and time-dependently induced cell death of MIN6 cells above 160 μM after 6 h treatment in vitro. Annexin-V staining revealed that cholesterol treatment significantly induced apoptosis in MIN6 cells. Cholesterol treatment resulted in the loss of the ability to retain Rhodamine 123, indicating mitochondrial damage in MIN6 cells. Cholesterol-induced cell apoptosis and mitochondrial damage were blocked by low-temperature condition. In addition, glutathione also protected MIN6 cells from cholesterol-induced cell death. It is concluded that high level of cholesterol induces cell apoptosis in MIN6 cells, which is in part due to mitochondrial dysfunction. We suggest that excessive uptake of cholesterol in β-cells may contribute to β-cell apoptosis and dysfunction and the deterioration of type 2 diabetes.

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Introduction

Hyperlipidemia plays an important role in pancreatic β-cell dysfunction, which is an important pathological component of type 2 diabetes and highly associated with obesity and overweight [1–3]. Hyperlipidemia in obesity and type 2 diabetes is characterized by high levels of free fatty acids, low-density lipoprotein (LDL), triglyceride, and cholesterol [4]. Prolonged elevation of fatty acids causes β-cell apoptosis, which is one of the main cause of the β-cell dysfunction under hyperlipidemia [5, 6]. Previous studies indicate that high levels of LDL may play a vital role in the induction of pancreatic β-cell dysfunction. Rat and human β-cells express high-affinity LDL receptors [7]. The internalization of LDL may lead to lipid accumulation in β-cells, which is particularly elevated in aging individuals [8]. LDL causes rat β-cell death and it was previously reported that low-temperature condition prevents LDL-induced β-cell death by reducing LDL uptake and metabolism [9]. However, the mechanism of toxic effect on β-cells by LDL is still not fully clarified.

Studies indicate that cholesterol is an important factor in mediating the toxic effect of LDL. LDL belongs to the lipoprotein family, which mainly transports cholesterol from the liver and small intestine to other peripheral tissues. Increased LDL levels are associated with atherosclerosis as LDL transports cholesterol to the arteries. Cholesterol induces cell death of vascular endothelial cells and macrophages that take part in the formation of atherosclerosis through apoptosis and/or necrosis [10–12]. Whether cholesterol induces β-cell death is, however,

unknown. In this study, we demonstrated that cholesterol induced apoptosis in MIN6 cells. Mitochondrial dysfunction may be involved in cholesterol-induced apoptosis of MIN6 cells.

Results

Effects of cholesterol on viability of MIN6 cells

Cholesterol significantly reduced cell viability in a dose- and time-dependent manner in MIN6 cells. In cultured MIN6 cells, it was observed under microscope that cholesterol at concentration above 160 μM caused decrease in cell viability and/or increase in cell apoptosis after 6 h incubation. MTT assay showed that MIN6 cell viability was significantly reduced by about 10 and 35% when treated with 160 and 320 μM cholesterol, respectively (Fig. 1a). Longer incubation to 12 h with 160 and 320 μM cholesterol decreased the viability of MIN6 cells to about 35 and 65% (Fig. 1b) and 24 h incubation with 160 and 320 μM cholesterol resulted in a further decrease to 40 and 90% when compared to control (Fig. 1c). Cholesterol at the concentration below or equal to 80 μM did not influence viability of MIN6 cells within 24 h treatment (Fig. 1).

Characterization of cholesterol-induced cell apoptosis in MIN6 cells

Annexin-V and propidium iodide (PI) stainings were used to identify apoptosis and necrosis in MIN6 cells. Neither annexin-V-positive cells nor PI-positive cells were observed in control group. In contrast, cholesterol treatment for 6 h (160 and 320 μM) induced a significant proportion of annexin-V-positive cells and more cells turned to annexin-V positive than that to PI-sensitive, suggesting increased number of cells undergoing apoptosis after cholesterol treatment (Fig. 2).

Effects of cholesterol on mitochondrial function

Retention of Rhodamine 123 was used to represent the mitochondrial function. The ability of mitochondria to retain Rhodamine 123 was significantly impaired in MIN6 cells after cholesterol treatment. Using confocal microscopy, we demonstrated that MIN6 cells of control group had retention of Rhodamine 123 within mitochondria, and the fluorescent signal was detected after 30 min incubation with Rhodamine 123 (Fig. 3a). The fluorescent signal was compartmental in cells, suggesting its retention in mitochondria. In contrast, MIN6 cells treated with 320 μM cholesterol for 6 h had a significantly reduced capacity to retain Rhodamine 123 (Fig. 3b), suggesting mitochondrial

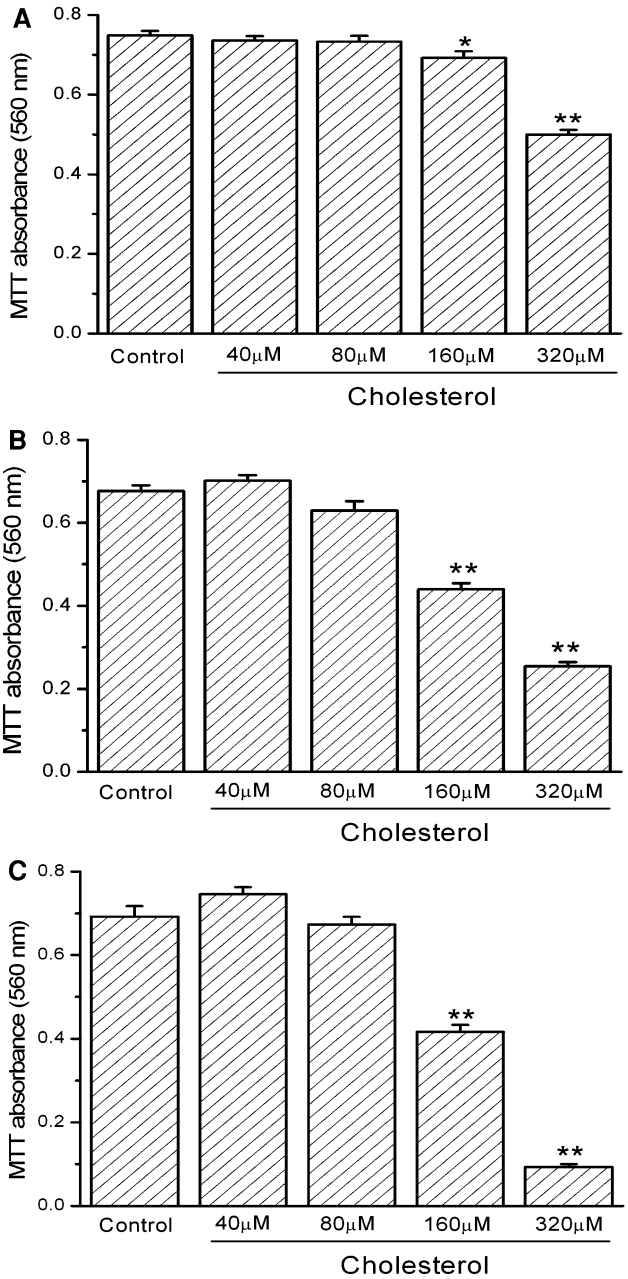

Fig. 1 Effects of cholesterol on viability of MIN6 cells. Cell viability was assessed by MTT assay. **a** 6 h incubation, **b** 12 h incubation, **c** 24 h incubation. * $P < 0.05$ and ** $P < 0.01$ vs. control ($n = 12$)

damage in cholesterol-treated group. This was confirmed by flow cytometry; cholesterol treatment led to ~40% reduction in mean fluorescent intensity (MFI) compared to control (Fig. 3c).

Effects of low temperature on cholesterol-induced cell death

To investigate whether the effects of cholesterol is a cellular membrane transport- and metabolism-related reaction,

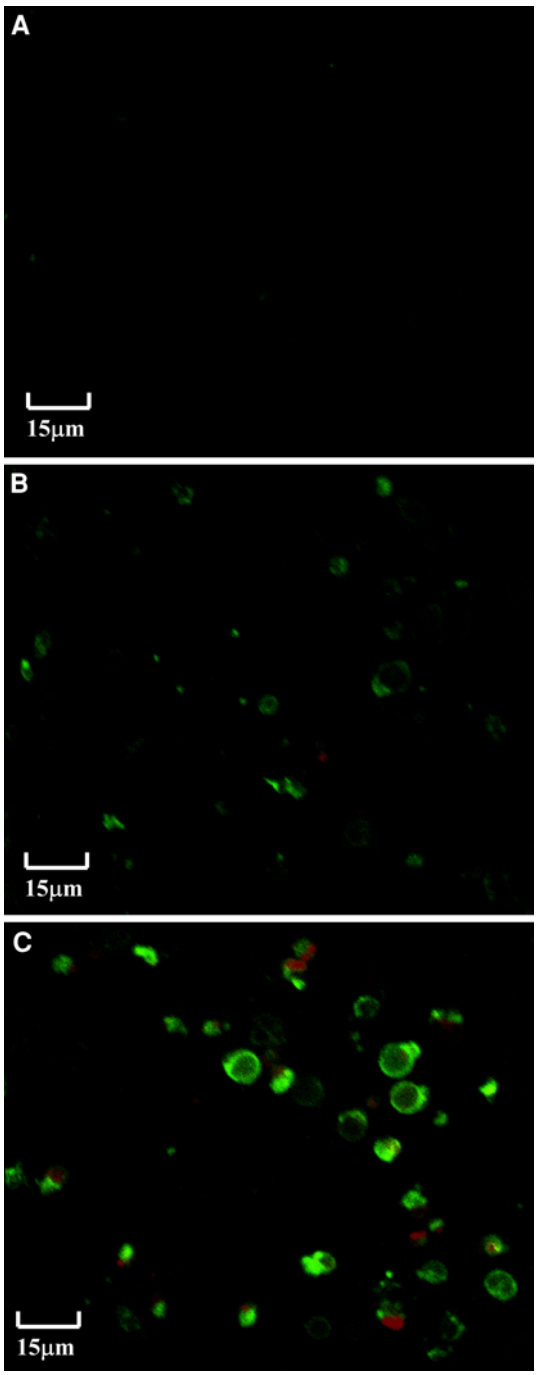

Fig. 2 Characterization of cholesterol-induced cell apoptosis in MIN6 cells. Apoptosis cells were stained by FITC-conjugated Annexin-V and detected as green fluorescence. **a** control, **b** 160 μM cholesterol treatment for 6 h, **c** 320 μM cholesterol treatment for 6 h

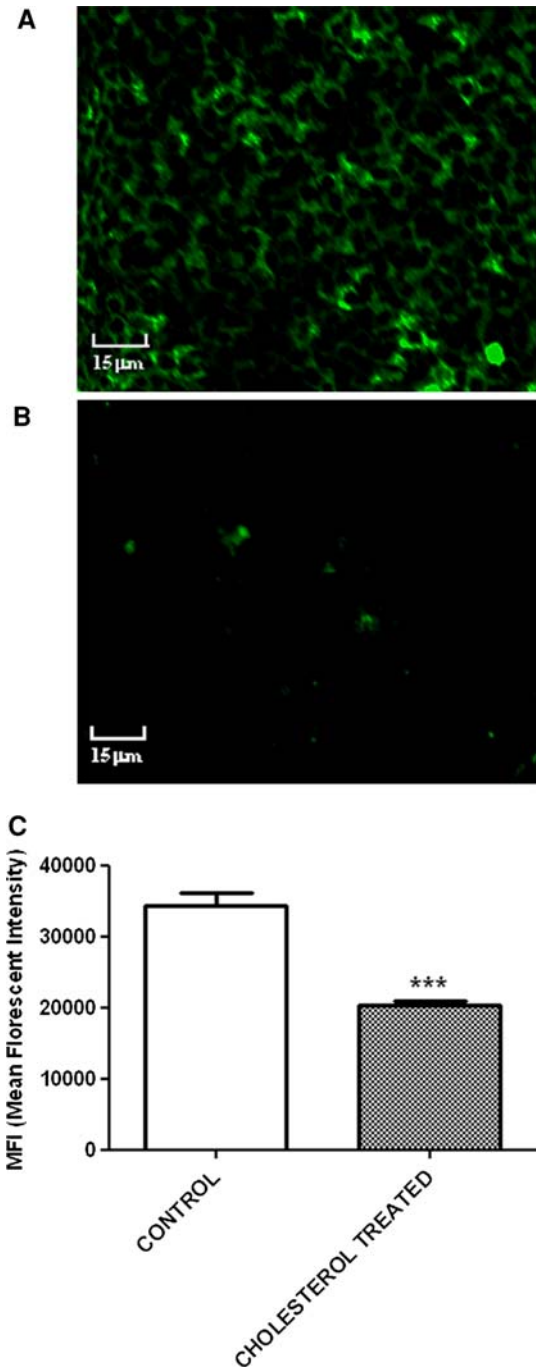

Fig. 3 Effects of cholesterol on mitochondrial function of MIN6 cells. Mitochondrial function was tested by Rhodamine 123, which is a green-fluorescent dye that is readily retained by active mitochondria. **a** Control, **b** 320 μM cholesterol treatment for 6 h, **c** the mean fluorescent intensity (MFI). *** $P < 0.001$ vs. control ($n = 4$)

MIN6 cells were incubated at 4°C during cholesterol treatment to decrease cholesterol transport and metabolism. Low-temperature treatment inhibited cholesterol-induced cell apoptosis in MIN6 cells. When MIN6 cells were cultured at 4°C, cholesterol up to 320 μM for 24 h treatment did not induce cell apoptosis (Fig. 4) whereas the same treatment at 37°C caused a significant increase in apoptosis (Fig. 2). In addition, MTT results showed that there were no changes in cell viability between control and cholesterol-treated groups ranging from 40 to 320 μM (Fig. 4a). Annexin-V staining showed that there was no significant

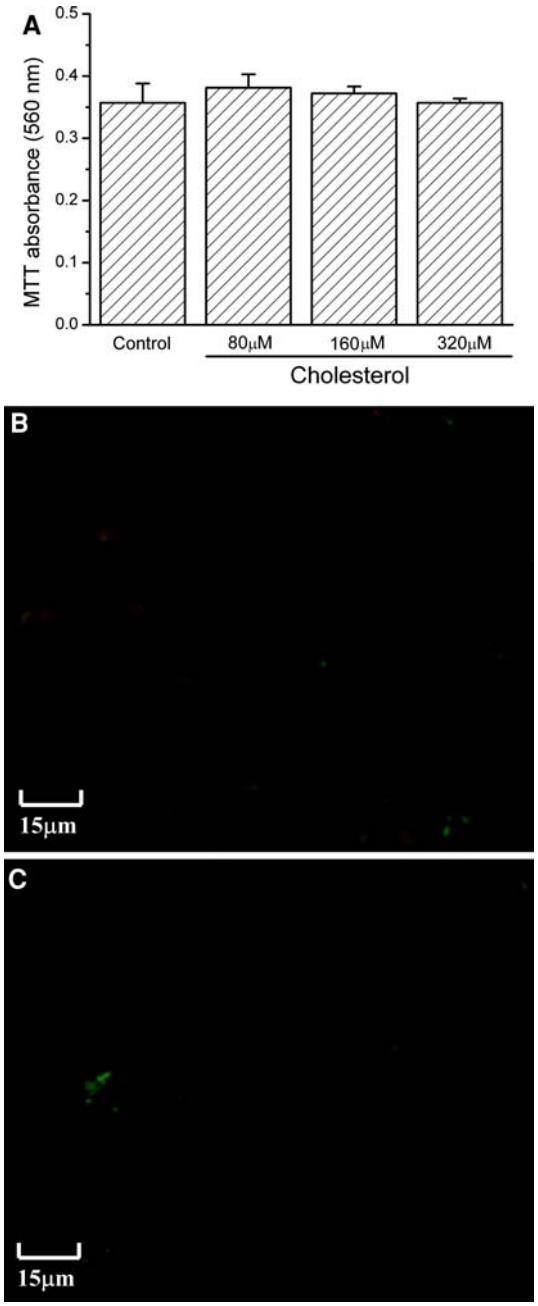

Fig. 4 Effects of low temperature on cholesterol-induced cell death of MIN6 cells. **a** MTT assay of cell viability ($n = 12$), **b** Annexin-V staining of control, **c** Annexin staining of cholesterol treatment

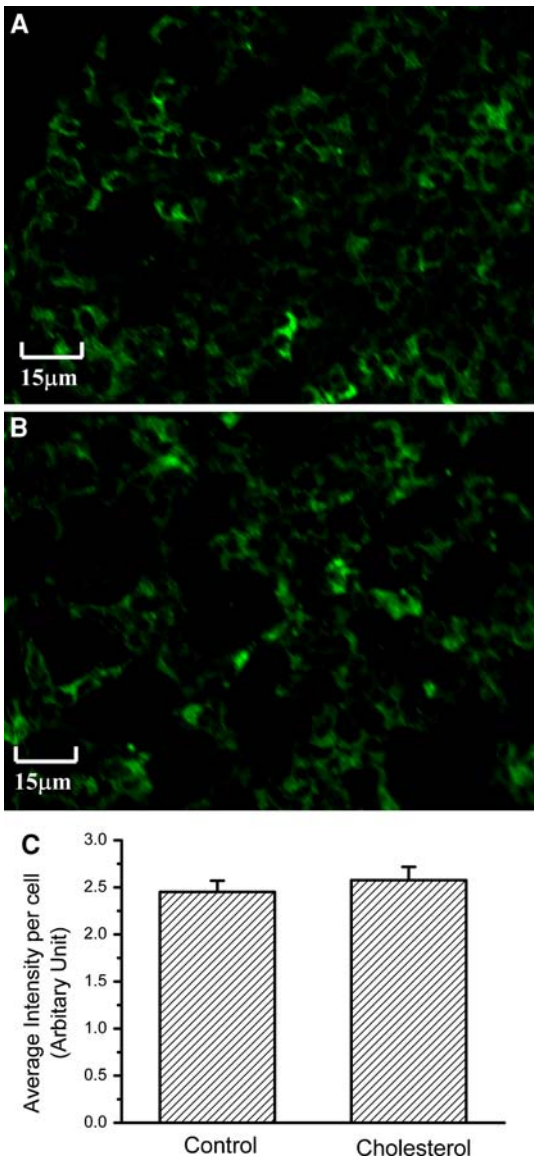

Fig. 5 Effects of low temperature on cholesterol-induced impairment of mitochondrial function. Mitochondrial function was tested by Rhodamine 123. **a** Control, **b** 320 μM cholesterol treatment for 12 h, **c** the average intensity of each cell. ** $P < 0.01$ vs. control ($n = 20$)

increase in annexin-V-positive cells and PI-positive cells compared to the controls (Fig. 4b, c).

Effects of low temperature on cholesterol-induced impairment of mitochondrial function

Low temperature also inhibited the impairment of cholesterol on mitochondrial function of MIN6 cells. After incubation for 12 h with 320 μM cholesterol, there were no changes in the capacity to sequester Rhodamine 123 compared to the controls (Fig. 5a, b). The fluorescence intensity of recorded cells was not significantly different between control and cholesterol treatment group (Fig. 5c).

Effects of antioxidant on cholesterol-induced cell death and impairment of mitochondrial function

We next observed whether antioxidant can protect MIN6 cells from cholesterol-induced cell death. MIN6 cells were treated with cholesterol either in the presence or absence of an antioxidant, glutathione (GSH). After 24 h incubation, GSH treatment (10 mmol/l) significantly increased cell

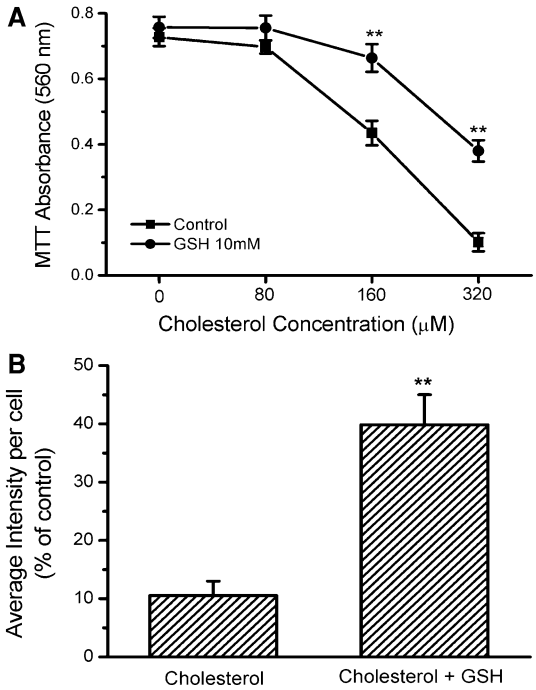

Fig. 6 Effects of anti-oxidative stress on cholesterol-induced cell death and impairment of mitochondrial function. **a** As shown by MTT assay, cholesterol-induced cell death was significantly inhibited by 10 mmol/l glutathione (GSH) after 24 h incubation (** $P < 0.01$ vs. cholesterol, $n = 12$). **b** The fluorescence intensity per cell after Rhodamine 123 loading increased from 10.54 ± 2.46% of control in cholesterol group to 39.86 ± 5.19% of control in GSH treatment group (** $P < 0.01$, $n = 30$)

viability of cholesterol-treated group as shown by MTT assay (Fig. 6a). In addition, Rhodamine 123 staining showed that the fluorescence intensity of recorded cells was significantly increased in cholesterol- and GSH-treated group when compared to cholesterol-only-treated group. The fluorescence intensity per cell increased from 10.54 ± 2.46% of control to 39.86 ± 5.19% of control (Fig. 6b).

Discussion

The present study demonstrates that cholesterol decreases cell viability and induces cell apoptosis of mouse pancreatic β-cell line, MIN6 in cultured condition. Cholesterol treatment significantly induced mitochondrial dysfunction and this may explain increased cellular apoptosis in cholesterol-treated groups.

Cholesterol has been related to apoptosis in different types of cells. It is well known that intracellular accumulation of free cholesterol to cause macrophage cell death, promotes advanced atherosclerotic lesions [13]. Cholesterol-induced macrophage apoptosis is mediated by mitochondria-dependent pathways, due to the loss of mitochondrial membrane potential [14–17]. Cholesterol also induces apoptosis of vascular smooth muscle cells and endothelial cells [12, 18–20], which also play an important role in the development of atherosclerosis. The present study demonstrates that cholesterol induces apoptosis in mouse pancreatic β-cell line, MIN6 cells and first places β-cell into the list of the cells that induced death by cholesterol. Cholesterol treatment above 160 μM decreased cell viability and increased cellular apoptosis in MIN6 cells in a time-dependent manner and our study suggests that mitochondria plays a vital role in cholesterol-induced cellular apoptosis [21]. The perturbation in mitochondrial membrane potential leads to the release of cytochrome C and activation of apoptosis [22, 23]. In this experiment, we demonstrated the loss of capacity to retain Rhodamine 123 after cholesterol treatment in MIN6 cells, confirming disturbed mitochondrial function, which in turn, led to apoptosis in MIN6 cells. This is similar to previous findings of cholesterol-induced macrophage apoptosis that was suggested to be due to mitochondrial dysfunction [21].

Blood cholesterol is mainly transported by LDL and is the toxic component of LDL to cell viability. LDL contains apolipoprotein B, apolipoprotein E, triglyceride, and importantly, cholesterol. Cellular LDL uptake is mediated by LDL receptors and cholesterol is rapidly released from LDL in the lysosome and utilized by cells [11, 24, 25]. It is demonstrated that pancreatic β-cells express LDL binding sites, and human and rat pancreatic β-cells can bind and uptake LDL [7]. LDL is degraded by β-cells in the lysosome. Another study showed that chronic exposure of β-cells to LDL causes cell death by increased LDL uptake and oxidation in rat β-cells [9]. Direct evidence for the role of cholesterol in LDL-induced β-cell death remains unknown and our present study provides such evidence. The present study indicates that cholesterol is an important factor leading to pancreatic β-cell death. A recent report shows that excessive cholesterol impairs insulin secretion from mouse islets [26], which provides a novel mechanism to the relationship between hyperlipidemia and pancreatic β-cell dysfunction. However, the influence of cholesterol on β-cell viability was not reported in the past study. Our study provides evidence to the induction of β-cell death. It is suggested that cholesterol may not only inhibit insulin secretion but also induce cell apoptosis of β-cells to deteriorate β-cell function, which opens another part of role of cholesterol in β-cell damage and the onset of type 2 diabetes.

Cholesterol-induced apoptosis of MIN6 cells is a cellular membrane transport- and metabolism-related reaction. Cholesterol at 37°C induced apparent cell death and apoptosis. However, there was no cell death or apoptosis at low temperature of 4°C, which may be due to reduced membrane transport and metabolism of cholesterol. It was reported that oxidized products of cholesterol may be responsible for cholesterol-induced cell apoptosis [16].

Low temperature may inhibit the transport and oxidation of cholesterol and block its toxic effects of cholesterol metabolites. It is suggested that cholesterol-mediated changes in membrane dynamics may have led to the depletion of mitochondrial GSH pool, a critical antioxidant that determines the susceptibility to stimuli that induce mitochondrial oxidative stress. Therefore, we examined whether increase in GSH pool can reduce or even prevent cholesterol-induced mitochondrial damage and apoptosis in MIN6. Our result demonstrated that GSH can protect MIN6 cells from cholesterol-induced cell apoptosis, which indicates oxidized products of cholesterol may have led to cholesterol-induced apoptosis in MIN6 cells.

The relationship of hyperlipidemia with the development of type 2 diabetes is complicated. Most studies on the link between hyperlipidemia and type 2 diabetes have focused on the effects of free fatty acids on β-cell function and insulin resistance of peripheral tissues. The present study demonstrates that cholesterol, besides free fatty acids, may also contribute to β-cell apoptosis occurring in hyperlipidemia. This study also applies to heart disease secondary to diabetes, which occur often in obese individuals with hyperlipidemia. The contribution of cholesterol to both atherosclerosis and β-cell dysfunction under hyperlipidemia suggest that two diseases may share similar cellular mechanism in the causes. It also justifies that control of cholesterol levels is an important way to prevent obesity-related diseases, including type 2 diabetes.

Materials and methods

Chemicals

Rhodamine 123, Annexin-V staining kit, MTT, isopropanol, and β-mercaptoethanol were purchased from Sigma (St. Louis, USA). Water soluble cholesterol (cholesterol: methyl-β-cyclodextrin) was purchased from Sigma (St. Louis, USA) and the concentration of cholesterol used in this study is based on previous studies [27–30]. Dulbecco's Modified Eagle's Medium (DMEM), fetal calf serum (FCS), penicillin, and streptomycin were from GIBCO BRL (Grand Island, NY, USA). Flask and plates for cell culture were purchased from NUNC (Denmark).

Cell culture

MIN6 cells were kindly provided by Dr. M Garry (Department of Biochemistry, Monash University, Clayton, Australia) with the approval of Dr. J. Miyazaki (Osaka University, Osaka, Japan). MIN6 cells were cultured in DMEM (25 mM glucose) supplemented with 10% heat-inactivated FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 50µM β-mercaptoethanol in a humidified atmosphere of 95% air and 5% CO_2. The culture medium was changed three times per week.

Cell viability assay

MIN6 cells were cultured in flat-bottomed 96-well plates. After the cells became 90% confluent, MTT was added into the medium at a final concentration of 0.5 mg/ml. Four hours later, the medium was discarded and 100 µl isopropanolol with 0.01 M HCl were added to each well. The plates were shaken at 300 rpm for 5 min to dissolve MTT crystals. Absorbance values were determined by ELISA plate reader (Perkin Elmer, USA) at wavelength of 560 nm and background wavelength of 690 nm and calculated by subtraction of absorbance values at 690 nm from that at 560 nm.

Apoptosis assay

Annexin-V assay was performed for determination of apoptosis. MIN6 cells were plated into 35 mm dishes. After 3 days growth, the cells were treated with cholesterol or control medium for 6 h after 70% confluence. Then the cells were washed with Hank's solution and stained with Annexin-V kit. Briefly, the cells were incubated for 10 min at room temperature with FITC-conjugated Annexin-V and propidium iodide (PI) under protection from light. After washing cells three times with Hank's solution, the FITC-positive cells and PI-positive cells were simultaneously observed by laser scanning confocal microscope. After excitation by laser at wavelength of 500 nm, Annexin-V-FITC was detected as a green fluorescence with an emission wavelength of 530 nm and PI was detected as a red fluorescence with an emission wavelength of 620 nm. Apoptosis cells were stained by Annexin-V and while necrosis cells were stained by PI.

Mitochondrial function assay

The positively charged Rhodamine analog Rhodamine 123 accumulates specifically in the mitochondria of living cells. The accumulation of Rhodamine 123 reflects the normal function of mitochondria, while mitochondria cannot retain Rhodamine 123 when its function is damaged with decline of membrane potential. (1) Confocal Microscopy: MIN6 cells were plated into 35 mm dishes to grow to 70% confluence. Six hours after the cholesterol treatment, Rhodamine 123 at concentration of 0.01 mg/ml was added to each dish for 20 min. After staining, the medium was discarded and the cells were rinsed with Hank's solution. Then the fluorescence was observed using confocal microscope. After activation at excitation wavelength of

500 nm, Rhodamine 123 was detected as a green fluorescence with emission wavelength of 530 nm. (2) Flow Cytometry: MIN6 cells were treated with cholesterol for 6 h and cells were collected by trypsinization. Cells were then centrifuged for 10 min at 1500 rpm, washed three times with PBS, and incubated with Rhodamine 123 (1 μg/ml) at 37°C for 15 min. After incubation, the cells were washed twice with PBS and fluorescent intensity was measured by BD LSR flow cytometer (Becton–Dickinson, NY, USA). Results are expressed as the mean fluorescent intensity (MFI), which represented the change in the mitochondrial potential.

Statistics methods

The data are represented as mean ± SEM for each group. The statistical significance between different groups was analyzed using unpaired Student's *t* test or ANOVA with a Bonferroni post test as appropriate. $P < 0.05$ was taken as the minimum level of significance.

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