Acetylcholinesterase is associated with apoptosis in β cells and contributes to insulin-dependent diabetes mellitus pathogenesis

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Acetylcholinesterase (AChE) expression is pivotal during apoptosis. Indeed, AChE inhibitors partially protect cells from apoptosis. Insulin-dependent diabetes mellitus (IDDM) is characterized in part by pancreatic β-cell apoptosis. Here, we investigated the role of AChE in the development of IDDM and analyzed protective effects of AChE inhibitors. Multiple low-dose streptozotocin (MLD-STZ) administration resulted in IDDM in a mouse model. Western blot analysis, cytochemical staining, and immunofluorescence staining were used to detect AChE expression in MIN6 cells, primary β cells, and apoptotic pancreatic β cells of MLD-STZ-treated mice. AChE inhibitors were administered intraperitoneally to the MLD-STZ mice for 30 days. Blood glucose, plasma insulin, and creatine levels were measured, and glucose tolerance tests were performed. The effects of AChE inhibitors on MIN6 cells were also evaluated. AChE expression was induced in the apoptotic MIN6 cells and primary β cells in vitro and pancreatic islets in vivo when treated with STZ. Induction and progressive accumulation of AChE in the pancreatic islets were associated with apoptotic β cells during IDDM development. The administration of AChE inhibitors effectively decreased hyperglycemia and incidence of diabetes, and restored plasma insulin levels and plasma creatine clearance in the MLD-STZ mice. AChE inhibitors partially protected MIN6 cells from the damage caused by STZ treatment. Induction and accumulation of AChE in pancreatic islets and the protective effects of AChE inhibitors on the onset and development of IDDM indicate a close relationship between AChE and IDDM.

Keywords: diabetes; acetylcholinesterase; apoptosis; β cell; streptozotocin

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Introduction

The classic function of acetylcholinesterase (AChE) is to hydrolyze acetylcholine and terminate impulse transmissions at cholinergic synapses [1]. AChE was also found in some non-cholinergic tissues and participates in other physiological processes, such as morphoregulation, adhesion, stress, and pathogenesis [2,3]. Previously, we reported that AChE was expressed during apoptosis induced by various stimuli in a number of cell lines [4]. AChE has been proposed to play a pivotal role in apoptosome formation [5,6], and synaptic AChE with an extended N-terminus induces apoptosis in neurons by interacting with other proteins [7,8]. Moreover, the appearance of this enzyme is considered as a marker for apoptosis under certain circumstances [9–11]. However, it is unknown whether AChE is induced in apoptotic cells in vivo.

Insulin-dependent diabetes mellitus (IDDM) is a complex disorder. The intricate etiology of IDDM and details of the pathogenic process are not well understood. Fortunately, an IDDM model of pathogenesis has been established by administration of multiple low-dose streptozotocin (MLD-STZ) in mice that results in a diabetic condition similar to that observed in human patients [12]. STZ is a diabetogenic chemical that can induce apoptosis in insulin-producing β cells by causing DNA and mitochondrial damage [13]. The apoptosis of these β cells is responsible for the development of pathology in the IDDM model [14]. Therefore, protecting the β cell from apoptosis would be a novel treatment strategy for diabetes.

AChE inhibitors (AChEIs) target AChE and protect cells from apoptosis through many mechanisms [15], but the contribution of AChE to IDDM pathology has not been reported. Here, our study explored the role of AChE in pancreatic β-cell apoptosis during the development of
IDDM because of the important role of AChE during apoptosis and the protective effects of AChEIs. Furthermore, we investigated whether AChEIs could alleviate IDDM symptoms in the mouse model.

**Materials and Methods**

**Materials**

STZ, BW284c51, iso-OMPA, and tacrine were obtained from Sigma (St Louis, USA). Huperzine A (HupA) was obtained from the Shanghai Institute of Materia Medica, Chinese Academy of Sciences (Shanghai, China). Bis-tacrine (BisT) was obtained from the Hong Kong University of Science and Technology (Hong Kong, China). The antibody for insulin was obtained from Dako (Carpinteria, USA). The antibody against actin was purchased from Sigma.

**Animal treatment**

Principles of Laboratory Animal Care (NIH publication No. 86-23, revised 1985) were followed. C57BL/6 J male mice between 6 and 8 weeks of age were used in all experiments. Animals were maintained in standard environmental conditions with free access to food and water. STZ was dissolved in sodium citrate buffer (pH 4.5) just before use and injected intraperitoneally (i.p.; 45 mg/kg body weight) for five daily doses. AChEIs were dissolved in 0.9% NaCl balanced buffer and injected intraperitoneally. HupA was administrated at the concentration of 0.25 mg/kg/day; tacrine of 0.5 mg/kg/day; and bis-tacrine of 0.25 mg/kg/day. The mice were observed for the onset of diabetes by measuring blood glucose concentration using Glucotrend Plus blood glucose test strips (Roche Diagnostics Science, Indianapolis, USA).

**Frozen tissue sections**

Mice were euthanized on the indicated day at approximately the same time. The mice pancreata were fixed overnight in 4% paraformaldehyde at 4°C, embedded in OCT (Richard-Allan Scientific, Kalamazoo, USA), and frozen in liquid nitrogen. Frozen tissues were sectioned to a thickness of 6 μm using a Microm HM520 Routine Cryostat (Richard-Allan Scientific).

**In situ AChE cytochemical staining**

AChE cytochemical staining was performed as previously described for detecting AChE cholinesterase activity [4]. The same sections were counter stained with hematoxylin, dehydrated with ethanol, and mounted in neutral balsam.

**Intraperitoneal glucose tolerance test**

Mice were fasted for 8 h by removal to a clean cage without food but with free access to water. After fasting, blood glucose levels were measured. Mice were weighed and injected i.p. with glucose (1 mg/g body weight). Blood glucose values were obtained at 10, 30, 60, 90, and 120 min.

**Determination of blood insulin and creatine concentrations**

Blood was collected when the mice were euthanized. The blood was allowed to clot and serum was separated by centrifugation. Serum insulin levels were detected using the Mercodia Mouse Insulin ELISA kit (Mercodia AB, Uppsala, Sweden). Serum creatine levels were analyzed by the routine biochemical analysis used in hospital.

**Isolation of pancreatic islets**

Pancreatic islets were isolated from C57BL/6 J male mice by digestion with collagenase P (Roche Applied Science, Indianapolis, USA) as previously described [17]. For detection of AChE expression during apoptosis of isolated islet cells, the islets were treated with 5 mM STZ for the indicated time, collected, and analyzed by western blot analysis. To clarify if AChE expression was linked to apoptosis of β cells, the islets were dispersed into single cells and cultured overnight to allow attachment to the plate. The cells were then treated with STZ and analyzed by immunofluorescent staining and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay.

**Cell culture and induction of apoptosis**

The MIN6 cell line was maintained in DMEM medium at 37°C in a 5% CO2 atmosphere. When ~70% confluent, the culture media was replaced with fresh media containing STZ at the indicated concentration and incubated for 24 h to induce apoptosis.

**Western blot analysis**

The pancreata from mice were homogenized in 100 mg/ml RIPA containing a cocktail of protease inhibitors (Roche Applied Science) on ice. The lysates were then centrifuged to collect the supernatant. MIN6 cells and isolated islets cells were resuspended in RIPA containing a cocktail of protease inhibitors. The cells were sonicated for 10 s and incubated on ice for 10 min. Equal amounts of the samples were resolved on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with Tris-buffered saline with Tween buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween-20) containing 5% fat-free dried milk for 1 h at 37°C and incubated with primary antibody overnight at 4°C. The membrane was then washed with TBST and
incubated with an HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, USA) for 2 h at room temperature. The membrane was washed again, and the immunoreactive protein was visualized using the chemiluminescent reagent ECL (Santa Cruz Biotechnology) according to the manufacturer’s protocol.

**MTT reduction cell viability assay**
MIN6 cells were seeded onto a 96-well plate and incubated for 24 h before the cells were treated with STZ to induce apoptosis. At the end of treatment, 20 μl MTT (5 mg/ml) was added to each well, and the plate was incubated for 4 h at 37°C; then 100 μl lysis buffer was added to each well. The color intensity was assessed with a Multiskan Mk3 microplate reader (Thermo Labsystems, Franklin, USA) at 590 nm.

**AChE immunofluorescence and TUNEL**
AChE immunofluorescence and fluorescein labeled TUNEL double staining protocols have been previously described [4,16,18]. TUNEL reaction mixture (Roche Diagnostics Corporation) was also used according to the manufacturer’s protocol. For each paraffin section, at least 6–10 fields were randomly selected and the frequency of TUNEL-positive cells was estimated at ×400 magnification.

**Results**

**Induction of pancreatic β-cell apoptosis during IDDM development**
Administration of MLD-STZ resulted in IDDM in C57BL/6 J mice. Hyperglycemia was detected in mice treated with MLD-STZ on day 8 post-treatment. Mice were diagnosed with diabetes when non-fasting blood glucose was >13.9 mM [Fig. 1(A)]. Most of the mice were considered diabetic by day 15 post-treatment. Insulin levels in the pancrea of the diabetic mice decreased dramatically compared with normal mice [Fig. 1(B), day 0 vs. day 15]. Apoptotic cells were detected in the pancreas by TUNEL staining [Fig. 1(B), lower panel]. TUNEL-positive cells were confirmed to be pancreatic β cells by a positive stain for insulin despite the decreased insulin content in the diabetic mice [Fig. 2(A), insulin], confirming that pancreatic β cells were eliminated by apoptosis during the development of IDDM.

**Induction and progressive accumulation of AChE in pancreatic islets during IDDM development**
AChE expression levels in the pancreata induced by MLD-STZ treatment were examined by western blot analysis. The amount of AChE protein increased concurrently with the degree of hyperglycemia [Fig. 1(C)]. A small amount of AChE was detected in mice sacrificed at day 0 due to AChE in the blood vessels and cholinergic innervation [Fig. 2(A), yellow and white arrows].

The pancreata from the mice killed at days 0, 8, 15, and 22 post-MLD-STZ treatment were stained for AChE enzymatic activity. No AChE positive staining was detected in the pancreatic islets from day 0 [Fig. 1(D), day 0], demonstrating that AChE is not expressed in the pancreatic islets of normal mice. Positive AChE activity emerged in the pancreatic islet area when the average blood glucose level reached slightly >13.9 mM [Fig. 1(D), day 8]. AChE activity in the islets of Langerhans intensified as the average blood glucose increased [Fig. 1(D), days 15 and 22]. These observations were consistent with the western blotting results showing that AChE expression was increased in the pancreas of the IDDM mice. Furthermore, AChE activity was confined within the area surrounding the islets of Langerhans and was not observed in other cell types, such as acinar cells [Fig. 1(D)]. These results show that AChE is induced and accumulates in the pancreatic islets during IDDM development.

**AChE expression was confined within the β cells during IDDM development**
We observed that β-cell apoptosis and AChE accumulation occurred in the pancreatic islets during IDDM development. More apoptotic cells were detected in the pancreata of diabetic mice than normal mice [Fig. 2(C), TUNEL]. The expression of AChE and insulin was detected simultaneously in the pancreatic insulin-producing cells [Fig. 2(A), lower panel/merge]. AChE activity was confined to the cells positive for insulin staining [Fig. 2(B)], with enzymatic activity mainly localized within the cell nuclei.

AChE expression was also detected in the blood vessels [Fig. 2(A), yellow arrows] and around the islets of Langerhans due to the cholinergic innervation [Fig. 2(A), white arrows]. To exclude interference of the AChE from cholinergic innervation and blood vessels, the islets of C57BL/6 J mice were isolated to detect AChE expression. The isolated islets were treated with 10 mM STZ for 24 h. Apoptosis was induced in the primary β cells as shown by TUNEL staining [Fig. 2(C), lower panel/TUNEL]. AChE expression was detected in the apoptotic primary β cells, but not in the non-apoptotic β cells [Fig. 2(C), insulin and merge], suggesting that the increase of AChE in the pancreas was due to the apoptosis of β cells during IDDM development.

**AChEIs prevented hyperglycemia and diabetes incidence caused by MLD-STZ**
To determine the effects of AChEIs on MLD-STZ diabetes progression, HupA, BisT, and tacrine were administered to the MLD-STZ mice i.p. from day 0 (1 day prior to
Figure 1 Induction and accumulation of AChE in pancreatic islets during IDDM development

(A) Multiple low-dose streptozotocin induced hyperglycemia in C57BL/6 J mice. The mice were induced with MLD-STZ from day 1 for 5 consecutive days and blood glucose levels were determined at the indicated time. Results were presented as the mean ± standard deviation for at least seven mice. (B) Pancreatic sections from mice sacrificed at day 0 (upper panel) or at day 15 (lower panel) were stained with immunofluorescence for insulin (red), and apoptotic cells were detected with TUNEL (green). The images were merged and parts of the merged image were magnified (original magnification ×100, magnified to ×400). (C) MLD-STZ mice were killed at the indicated time and the pancreata were homogenized. Equal amounts of protein were subjected to western blotting analysis using anti-AChE and anti-β-actin antibody. (D) Pancreatic sections from the mice sacrificed at the indicated times were first stained for AChE activity in the presence of 30 μM iso-OPMA, a BChE-specific inhibitor, and then the same sections were counterstained with hematoxylin. The brown precipitation represents AChE cholinesterase activity (original magnification ×200)
Progressive hyperglycemia was induced in the C57BL/6 J mice by MLD-STZ treatment [Fig. 3(A)], with a corresponding increase in diabetes incidence [Fig. 3(B)], blood glucose ≥ 13.9 mM]. Compared with the MLD-STZ group, the blood glucose levels of MLD-STZ mice treated with HupA and BisT were significantly reduced over a 30-day period (P < 0.05, STZ vs. STZ + HupA, STZ vs. STZ + BisT). Hyperglycemia in the tacrine group was also reduced, but the difference was not significant [Fig. 3(A)], P = 0.096, STZ vs. STZ + Tacrine]. Furthermore, the incidence of diabetes decreased significantly in response to treatment with AChEIs [Fig. 3(B)].

Figure 2 Expression of AChE in apoptotic β cells during IDDM development (A) Pancreatic sections from mice killed at day 0 (upper panel) and day 15 (lower panel) were stained for insulin (green) and AChE (red) by immunofluorescence staining. The red arrows represent induced AChE expressed in the insulin-producing cells (yellow arrows, blood vessels; white arrows, cholinergic innervation). (B) The presence of AChE was indicated by its cholinesterase activity (brown) and the β cells were identified by the presence of insulin (red). Red arrows showed that the activity of AChE is confined to the insulin-positive cells. (C) Primary cells from isolated islets were cultured for 24 h and 5 mM STZ was added to the culture media to induce the cell apoptosis. Normal cells (upper panel) or induced cells (lower panel) were subjected to immunofluorescence staining to detect expression of insulin (red) and AChE (blue), and apoptotic cells were evaluated by the TUNEL assay (green). White arrows indicate living β cells; red arrows, apoptotic β cells (original magnification ×400).
Intraperitoneal glucose tolerance test was performed at day 20 [Fig. 4(A)] and day 40 [Fig. 4(B)]. MLD-STZ treatment led to severely impaired glucose tolerance, but administration of HupA and BisT significantly restored glucose tolerance (*P*, 0.01, STZ vs. HupA, STZ vs. BisT).

AChEIs restored insulin levels and renal function in the MLD-STZ mice

Insulin is essential for responding to changes in blood glucose levels. The blood insulin levels in the normal mice were not affected by treatment with AChEIs (*P* > 0.05, saline vs. HupA, saline vs. BisT). Treatment with MLD-STZ severely reduced blood insulin values (*P* < 0.01, saline vs. STZ). Both HupA and BisT administration restored insulin content in the blood with significant differences compared with the MLD-STZ group, consistent with the intraperitoneal glucose tolerance test results [Fig. 5(A), *P* < 0.01, saline vs. HupA + STZ, saline vs. STZ + BisT]. These results showed that the secretion of insulin was diminished by MLD-STZ treatment but restored by treatment with AChEIs.

The ability to clear creatine is a measure of kidney function. Plasma creatine levels were determined to evaluate the toxicity of AChEIs. Blood creatine concentrations were not affected by injection of HupA and BisT, demonstrating that these chemicals were not biotoxic to the mice at this experimental dosage (*P* > 0.05, saline vs. HupA, saline vs. BisT). STZ injection strongly raised blood creatine levels, revealing that renal function was exceedingly impaired. HupA and BisT injection restored renal function as evidenced by the low blood creatine levels compared with the MLD-STZ group [Fig. 5(B), *P* < 0.01, saline vs. HupA + STZ, saline vs. STZ + BisT].

**HupA-protected MIN6-cell viability loss caused by STZ**

AChE is progressively expressed in pancreatic β cells during IDDM development, and AChEIs exert protective effects on IDDM. Therefore, we examined the effects of
AChEIs on STZ-induced apoptosis of MIN6 cells. AChE expression was induced in a dose-dependent manner in MIN6 cells when treated with STZ, as determined by western blot analysis [Fig. 6(A)]. After treatment with 5 mM STZ for 24 h, the MIN6 cells presented classical apoptotic morphology with chromatin condensed or segregated to form apoptotic bodies, as shown by Hoechst 33258 and TUNEL staining [Fig. 6(B), nuclei/TUNEL]. Most AChE-positive cells were also TUNEL positive [Fig. 6(B), merge].

To assess the effects of treatment with STZ and HupA on cell viability, MIN6 cells were pretreated with 1 mM HupA for 30 min before incubation with 5 mM STZ for 24 h. STZ decreased cell viability measured by MTT by 50% compared with control cells [Fig. 6(C)]. Pretreatment with HupA rescued cell viability by ~20% compared with the STZ group [Fig. 6(C), P < 0.01].

**Discussion**

In this study, we demonstrated that AChE expression was induced in apoptotic pancreatic β cells at the onset of IDDM. AChE expression accumulated in the islets of Langerhans during IDDM development, whereas AChEIs exhibited protective effects against IDDM in vivo and MIN6-cell apoptosis in vitro.

Much evidence has shown that AChE is induced during apoptosis in various cells [18], and that pancreatic β cells undergo apoptosis during IDDM development [14]. Here, we used the IDDM mouse model to demonstrate that AChE was expressed in both primary β cells and pancreatic β cells during IDDM development. These results showed that AChE expression during the cell apoptosis was a conserved phenomenon both in vitro and in vivo.

Increased AChE activity in pancreatic islets from diabetic rats was observed by Godfrey and Matschinsky [19]. They proposed that the relatively small islet volume in diabetic rats compared with control rats was responsible for these results. Consistent with their results, we found that AChE expression increased during IDDM development in mice. Furthermore, we found that AChE expression was associated with apoptotic β cells. Therefore, we suggest that the increase of AChE activity in rat pancreatic islets was relative with the β-cell apoptosis. However, in this study, AChE and insulin could be simultaneously detected only in a small number of cells. This may be due to the fact that AChE expression increases when apoptosis is induced in the cells, whereas insulin content decreases during the apoptosis process. Another possible explanation is that apoptosis could only be detected in a small number of cells at a specific time point due to the progressive process of pancreatic β cells elimination during IDDM development [14].

Along with the development of IDDM, AChE accumulated in pancreatic islets, implying that AChE was involved in IDDM pathogenesis. Cell apoptosis is a common and critical step in many diseases, such as Alzheimer’s disease (AD) [20] and diabetes [21]. The pathogenesis of AD is characteristic of a massive cholinergic neuron loss and the formation of senile plaques [22], and AChE has been proven to be a major component of senile plaques [23]. This process is very similar to what we observed during the development of IDDM. Furthermore, it has been reported that over-expression of AChE can lead to cell viability loss and even apoptosis [24], and that AChE expression exacerbated AD pathogenesis [23,25]. We proposed that AChE is induced in apoptotic cells and accelerated cell death due to toxicity. Thus, the increase and accumulation of AChE in the nidus facilitated disease development.

A previous report demonstrated that over-expression of an N-terminally extended ‘synaptic’ AChE variant led to neuronal death in AD [7]. In this study, we only detected expression of a 68-kDa protein, and the AChE variant necessary for the induction of β-cell apoptosis was unknown. Induced AChE was expressed mostly in the apoptotic nuclei [Fig. 2(A,B)], consistent with our previous report [4,26]. Whether the location of AChE in the nuclei is pivotal to cell apoptosis requires further investigation.

![Figure 5 AChE inhibitors restored plasma insulin levels and decreased creatine levels of the MLD-STZ mice](https://academic.oup.com/abbs/article-abstract/44/3/207/1045)
AChEIs have been intensively studied for the therapy of AD to maintain the AChE levels in the AD brain [27]. Accumulating evidence implies that the rationale for the demonstrable benefits of AChEIs in AD is likely to be more complex than simply replacement of lost AChE [15]. Zhang et al. [4] showed that the AChEI BW284c51 protected cells other than neurons from apoptosis. In this study, we also found that HupA prevented cell viability loss caused by STZ in MIN6 cells. Three AChEIs were administrated to MLD-STZ-treated mice to evaluate their effects on the onset and progression of IDDM. All three inhibitors decreased the hyperglycemia and incidence of IDDM induced by MLD-STZ, suggesting that AChEIs exerted a protective effect on the onset and development of IDDM.

AChEIs have been proposed to have anti-inflammatory properties [28–31], which could partially account for the protective effects exerted on IDDM progression. Moreover, pretreatment of MIN6 cells with HupA also prevented cell viability loss induced by STZ, indicating that AChEIs protected cells from apoptosis in vitro independent of in vivo immune responses. Thus, the protective effects of AChEIs on MLD-STZ-induced diabetes could be due to protection of β cells from apoptosis. Many mechanisms have been proposed to explain the effects of AChEIs on cellular apoptosis, including changing gene expression patterns, protecting cells against oxidative stress, or activation of nAChRs [15], but AChE is the potential target for the different inhibitors. The protective potency of the three inhibitors used in this study varied according to their specificity and capacity of AChE inhibition. This implies a close relationship between AChE and the protective efficacy of AChEIs.

We speculated that AChE was accumulated in pancreatic islets and contributed to IDDM pathogenesis by enhancing apoptosis. Administration of AChEIs may disrupt the activity of AChE during apoptosis and thus protect the cells and alleviate the symptoms of IDDM induced by MLD-STZ.

Figure 6 Huperzine A prevents cell viability loss caused by MLD-STZ treatment in MIN6 cells (A) MIN6 cells were treated with MLD-STZ at the indicated concentration for 24 h. The cells were collected for western blotting detection of AChE. Equal amounts of total protein for each sample were loaded. (B) Immunofluorescence staining was performed to detect the expression of AChE (red), apoptotic cells by TUNEL (green), and the nuclei by Hoechst33258 (blue). White arrows indicate living cells; red arrows, apoptotic cells (magnification ×400). (C) HupA partially prevents cell viability loss induced by STZ treatment. Cells were pretreated with 1 mM HupA for 30 min and then 5 mM STZ were added to the media for another 24 h. Cell viability was determined by MTT reduction assay. The results were presented as mean ± standard deviation of three independent experiments and the values of HupA group were normalized to 1. *P < 0.05 vs. STZ group by t-test.
Close relationship has been established between the AD and diabetes, and coincidence of AD and diabetes has long been noticed. AChEIs are the main drugs in combating AD [32,33]. Cholinergic connection between diabetes and AD has also been proposed [34,35]. This study showed that AChEIs have protective effect on the onset and development of IDDM. Thus, AChEIs could be potential candidates for dual AD/IDDM therapy.

Taken together, we observed that AChE expression increased with the development of IDDM. AChEIs exerted a protective effect on IDDM onset and development, demonstrating that AChE was closely associated with IDDM. These findings contribute to the understanding of the pathogenesis of IDDM and provide new pharmacological screening candidates for treatment.

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