Original Article

α1-Antitrypsin Protects β-Cells From Apoptosis

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β-Cell apoptosis appears to represent a key event in the pathogenesis of type 1 diabetes. Previous studies have demonstrated that administration of the serine proteinase inhibitor α1-antitrypsin (AAT) prevents type 1 diabetes development in NOD mice and prolongs islet allograft survival in rodents; yet the mechanisms underlying this therapeutic benefit remain largely unclear. Herein we describe novel findings indicating that AAT significantly reduces cytokine- and streptozotocin (STZ)-induced β-cell apoptosis. Specifically, strong antiapoptotic activities for AAT (Prolastin, human) were observed when murine insulinoma cells (MIN6) were exposed to tumor necrosis factor-α. In a second model system involving STZ-induced β-cell apoptosis, treatment of MIN6 cells with AAT similarly induced a significant increase in cellular viability and a reduction in apoptosis. Importantly, in both model systems, treatment with AAT completely abolished induced caspase-3 activity. In terms of its activities in vivo, treatment of C57BL/6 mice with AAT prevented STZ-induced diabetes and, in agreement with the in vitro analyses, supported the concept of a mechanism involving the disruption of β-cell apoptosis. These results propose a novel biological function for this molecule and suggest it may represent an effective candidate for attempts seeking to prevent or reverse type 1 diabetes. *Diabetes* 56:1316–1323, 2007

Type 1 diabetes is an autoimmune disease resulting from destruction of the insulin-producing islet β-cells (1). Multiple lines of evidence indicate that antigen-presenting cells (APCs), especially dendritic cells, are pathologically active in orchestrating the process of insulitis (2). APCs within islets likely respond to micro-environmental triggers, including β-cell death and apoptosis, and initiate the insulitis process by migrating out of the islet and into the peripheral pancreatic lymph nodes. These APCs trigger the activation and proliferation of β-cell–reactive T-cells, which destroy islet cells at a rate that eventually results in type 1 diabetes. It has been shown that both direct cytotoxic (T-cell mediated) and indirect cytokine-dependent (e.g., interleukin-1, tumor necrosis factor-α [TNF-α], and γ-interferon) mechanisms are responsible for β-cell apoptosis (3).

The direct cytotoxic mechanisms appear to involve the release of cytotoxic granule contents (e.g., perforin and granzymes) by cytotoxic T-cells (4). Granzymes play a critical role in triggering apoptotic cell death through mitochondrial pathways or by the activation of cellular caspases. Indeed, caspases (e.g., caspase-3) are key players in controlling the events leading to cellular apoptosis. Caspases are synthesized as inactive zymogens, which can be cleaved and activated by proteinases, including granzymes, cathepsins, and calpains. Granzymes and cathepsins are also members of a class of molecules known as serine proteinase inhibitor (serpin) proteinases. As for indirect mechanisms for β-cell apoptosis involving cytokines, signal transduction activities afforded by interactions between these molecules and specific receptors initiate activation of mitogen-activated protein kinase pathways, mobilization of transcription factors (e.g., nuclear factor-κB and signal transducer and activator of transcription-1), and upregulation or downregulation of downstream gene transcription (5). The mechanism(s) by which cytokines destroy β-cells are complex and under active investigation (3). However, it is possible that cytokine-induced cell death also involves caspase activation (6).

α1-Antitrypsin (AAT) is one of the major protective proteins in physiological circulation. As a member of the serpin family, AAT inhibits neutrophil elastase, proteinase-3, cathepsin G, thrombin, trypsin, and other proteinases. The protein also has anti-inflammatory properties, providing protection from tissue damage in the kidney (7), lung (8,9), and liver (10). AAT can suppress nuclear factor-κB translocation and increase inhibitor of κB levels in vivo (11). In terms of the actions of AAT for averting type 1 diabetes, we previously demonstrated that overexpression of AAT, afforded by gene delivery using recombinant adeno-associated virus, significantly reduced insulitis and prevented the development of overt hyperglycemia in NOD mice (12,13). Studies by Lewis and coworkers (14,15) have shown that administration of clinical-grade human AAT prolongs islet allograft survival and exhibits islet-related cytoprotective effects. Such findings have led to the proposal that AAT may represent a novel form of therapy for disorders (i.e., autoimmunity and transplantation) involving adverse immune responses (15). However, the mechanism by which AAT administration provides these beneficial therapeutic outcomes remains largely unclear. Given its designation as a serpin, we

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AAT, α1-antitrypsin; APC, antigen-presenting cell; MTT, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide; STZ, streptozotocin; TNF-α, tumor necrosis factor-α; TUNEL, terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling.

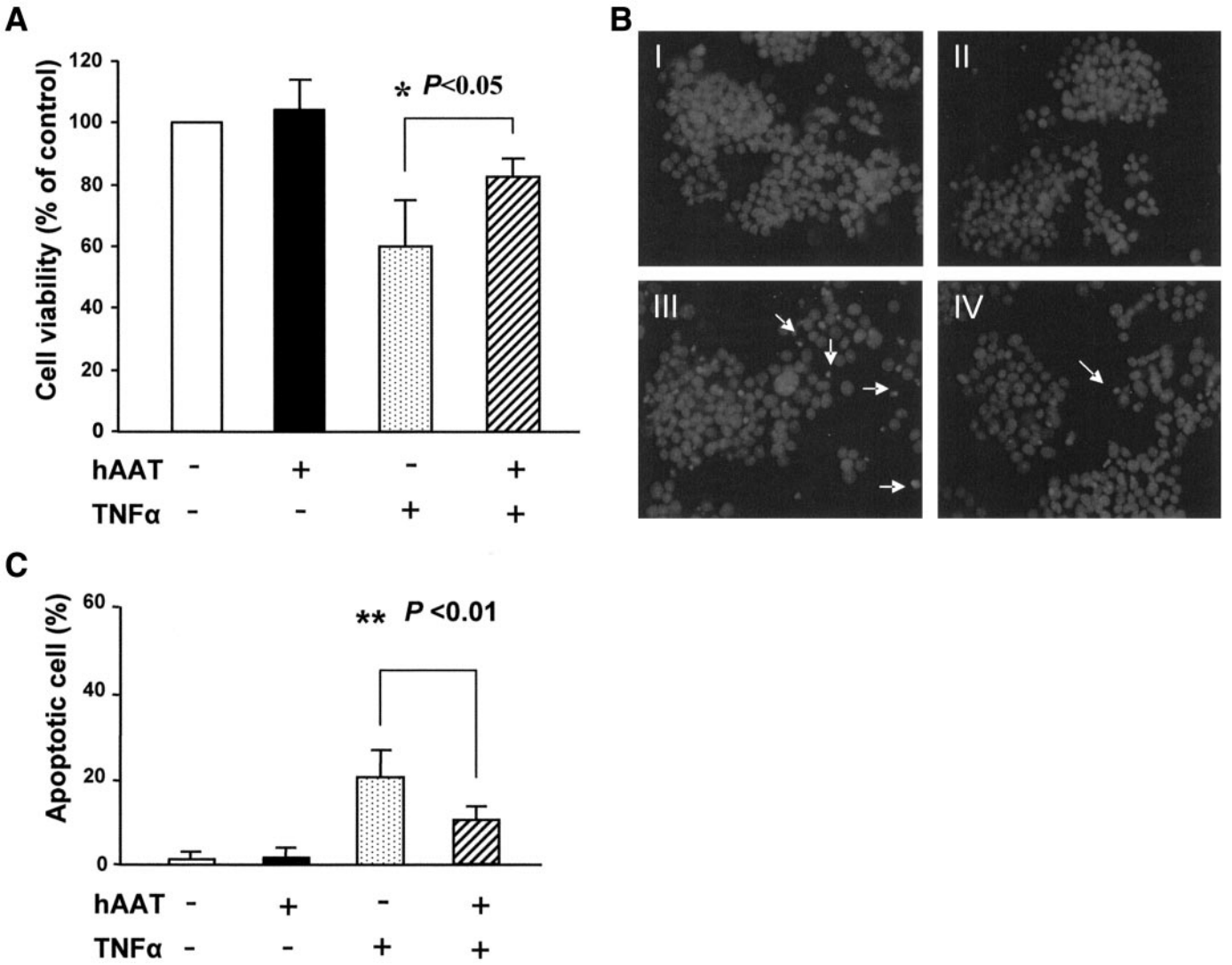

FIG. 1. AAT protects β-cells against TNF-α–induced apoptosis. MIN6 cells were pretreated with or without AAT for 4 h and treated with or without TNF-α for 48 h. *A*: Cell viability was estimated by MTT assay and expressed as a percentage of the values of control. Each bar represents the mean ± SE of three independent experiments. *B*: Apoptotic nuclear changes (arrows) detected by Hochest staining. Representative photomicrographs of untreated (I), AAT-treated (II), TNF-α–treated (III), and AAT- and TNF-α–treated (IV) cells are shown. *C*: The percentage of apoptotic cells was estimated by counting at least 1,200 cell nuclei in each of three separate experiments. hAAT, human AAT.

thought it beneficial to address the hypothesis that in a model setting (in vitro and in vivo) for type 1 diabetes, AAT, through pathways related to caspase activity, protects against islet β-cell apoptosis. These studies not only support that hypothesis but, in addition, suggest that strategies seeking to protect β-cells from apoptosis (i.e., attempts to prevent or reverse type 1 diabetes) might benefit by the administration of AAT.

RESEARCH DESIGN AND METHODS

Cell culture. The mouse insulinoma cell line MIN6 was maintained in Dulbecco's modified Eagle medium (Cellgro) with 15% FBS (Cellgro) at 37°C in 5% CO_2. To eliminate effect of bovine AAT in the serum, MIN6 cells were cultured in serum-free medium for 12 h before AAT treatment. Cells were treated with clinical-grade human AAT (Prolastin; Bayer) at a final concentration of 0.5 mg/ml. At 4 h after addition of AAT, TNF-α at a final concentration of 40 ng/ml or streptozotocin (STZ) at a final concentration of 1 or 5 mmol/l was added into the culture medium. Controls included cells with no AAT pretreatment along with TNF-α addition or AAT treatment with no TNF-α supplementation. For cell viability or apoptosis assay, cells were harvested 48 h after TNF-α treatment or 24 h after STZ treatment. For caspase-3 activity assays, cells were harvested 12 h after TNF-α or STZ treatment.

MTT assay. Cell viability was estimated by the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (16). Cells (50,000 cells/well) were seeded in 96-well flat-bottom plates with serum-free Dulbecco's modified Eagle medium. After culture with testing agents (AAT, TNF-α, or STZ as described above), the medium was removed. MTT (200 μl, 0.5 mg/ml) was added into the wells, and the plates were incubated for 3 h at 37°C. After removal of the MTT solution, cells were treated with 100 μl isopropyl alcohol containing 0.04 mol/l HCl for 1 h. The plates were then read at 550 nm.

Apoptosis assay. Apoptotic nuclear changes were assessed by Hoechst staining followed by fluorescent microscopic examination (17). MIN6 cells (10,000 cells/well) were seeded in 8-well chamber slides. After incubation with testing agents (AAT, TNF-α, or STZ as described above), cells were stained with 20 mg/ml Hoechst 33342 (Sigma-Aldrich) at 37°C for 20 min, washed three times with PBS, and fixed with 4% formaldehyde for 15 min at room temperature. Morphological changes of nuclei were observed under a fluorescent microscope. Apoptotic cells were assessed by nuclear shrinkage, fragmentation, and chromatic condensation. At least 1,200 cells were assessed in 30 contiguous fields. The percentage of apoptotic cells was calculated for each field and averaged for the treatment group.

Detection of AAT cellular entry by fluorescent labeling and immunostaining. MIN6 cells were cultured on chamber slides with serum-free medium before incubation with AAT. In fluorescent-labeling studies, AAT was labeled with DyLight547 using a Protein Labeling Kit (Pierce). After 6 h of incubation with labeled AAT (0.5 mg/ml), cells were washed with PBS, fixed with 4% formaldehyde, and mounted on slides without permeabilization.

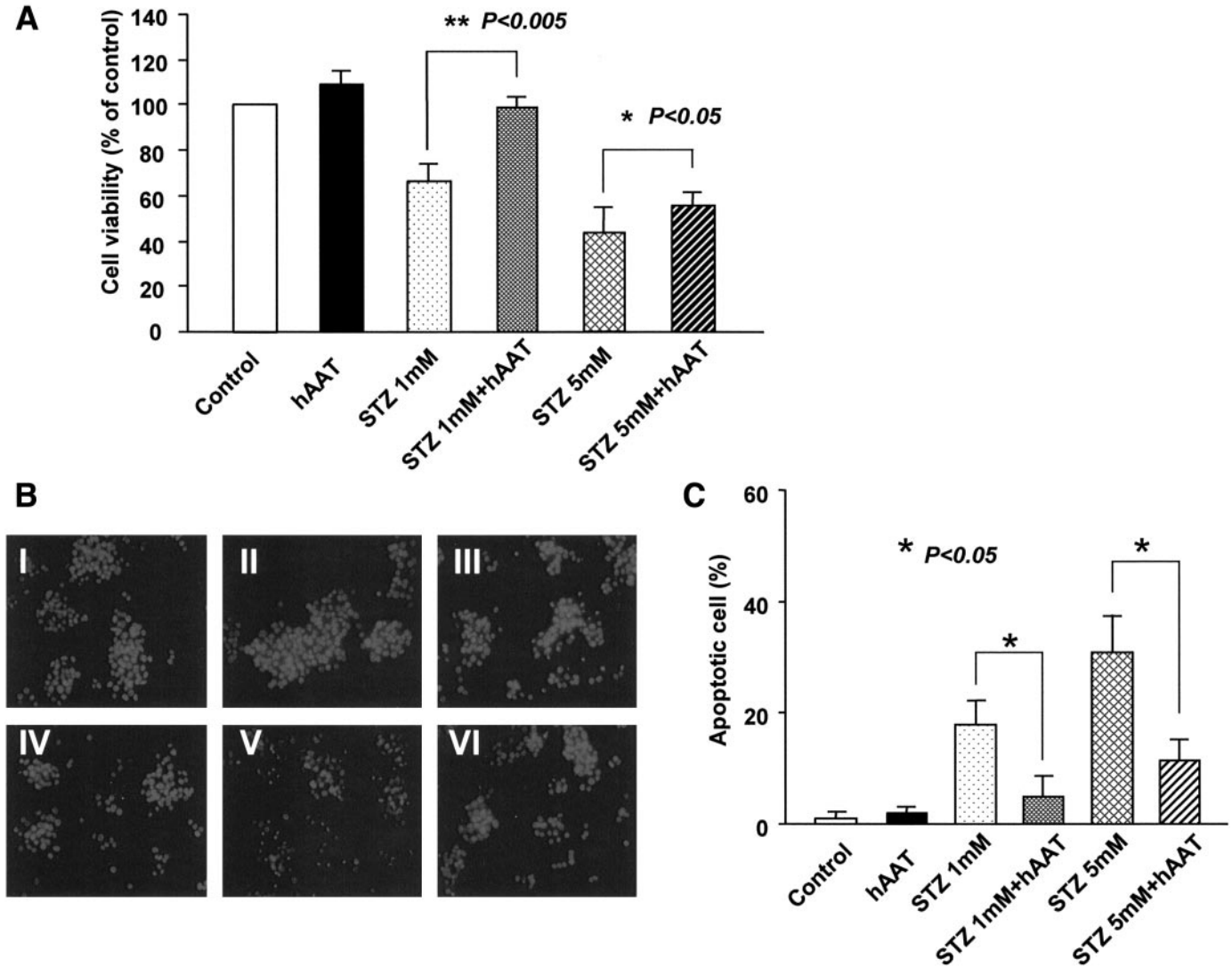

FIG. 2. AAT protects β-cells against STZ-induced apoptosis. MIN6 cells were pretreated with or without AAT for 4 h and treated with or without STZ for 24 h. *A*: Cell viability was estimated by MTT assay and presented as a percentage of the control values. Each bar represents the mean ± SE of three independent experiments. *B*: Apoptotic nuclear changes detected by Hochest staining. Representative photomicrographs of MIN6 cells untreated (I) or treated with AAT (II), 1 mmol/l STZ (III), AAT and 1 mmol/l STZ (IV), 5 mmol/l STZ (V), or AAT and 5 mmol/l STZ (VI) are shown. *C*: The percentage of apoptotic cells was estimated by counting at least 1,200 cell nuclei in each of three separate experiments (each bar represents the mean ± SE). hAAT, human AAT.

Images were captured using a Bio-Rad confocal microscope. In immunostaining studies, cells were fixed after incubating with/without AAT for 6 h. AAT was detected by a rabbit anti-AAT antibody (1:100; Research Diagnostics), followed by incubation with fluroescein isothiocyanate–conjugated goat anti-rabbit IgG (1:200; Sigma-Aldrich) and mounting with fluorescent mounting media with DAPI (Vector). Images were taken using a Zeiss fluorescent microsope.

Caspase-3 activity assay. Caspase-3 activity was determined by using the EnzChek Caspase-3 Assay Kit #2 (Molecular Probes) according to the manufacturer's instructions. Briefly, cells were washed with PBS and lysed and caspase-3 activity in the extracts measured by fluorometric assay. Fluorescent product of the substrate Z-DEVD-rhodamine 110 generated by caspase-3 in the cell extract was detected by a SPECTRA max fluorometer with excitation of 496 nm and emission of 520 nm. Background fluorescence was determined by including a specific caspase-3 inhibitor (Ac-DEVD-CHO) in the reaction mixtures (100 μl). To test the direct inhibitory effect of AAT on caspase-3 in cell-free conditions, AAT was diluted into various concentrations and incubated with 0.5 units of recombinant active caspase-3 (Biovision) at room temperature for 1 h before the addition of caspase-3 substrate.

Animals. Eight-week-old male C57BL/6 mice were purchased from The Jackson Laboratory. All animals were housed in a specific pathogen-free room and handled as approved by the University of Florida Institutional Animal Care and Use Committee. STZ (Sigma-Aldrich) was intraperitoneally injected (50 mg · kg^{-1} · day^{-1} for 5 days) as a freshly prepared solution in 0.1 mmol/l sodium citrate, pH 4.5. Clinical-grade human AAT (Prolastin) was intraperitoneally injected every 3 days continuously from 6 days before STZ injection until the end of the experiment.

Intraperitoneal glucose tolerance test. After overnight fasting, animals received an intraperitoneal injection of a 50% glucose solution (1 mg/kg). Blood glucose was measured at baseline as well as at 30, 60, 90, 120, and 180 min after glucose challenge.

Histology and immunohistochemistry. Pancreata from all mice were fixed in Fekete's solution, embedded in paraffin, and sectioned at 4 μ. Sections were stained with hematoxylin and eosin for routine morphology. Apoptotic cells were detected using a terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) assay according to the manufacturer's recommendations (Apotag Kit; Chemicon). Briefly, sections were deparaffinized, rehydrated, and incubated with 20 μg/ml proteinase K at room temperature for 15 min. Sections were incubated in labeling solutions containing terminal deoxynucleotidyl transferase and dUTP. Labeled nuclei were detected using an anti-digoxigenin antibody conjugated with peroxidase and the peroxidase reaction visualized with 3,3′-diaminobenzidine tetrahydrochloride and hydrogen peroxide. To determine insulin expression in β-cells, sections were immunostained with an anti-insulin antibody (1:200; DAKO) as previously described (12).

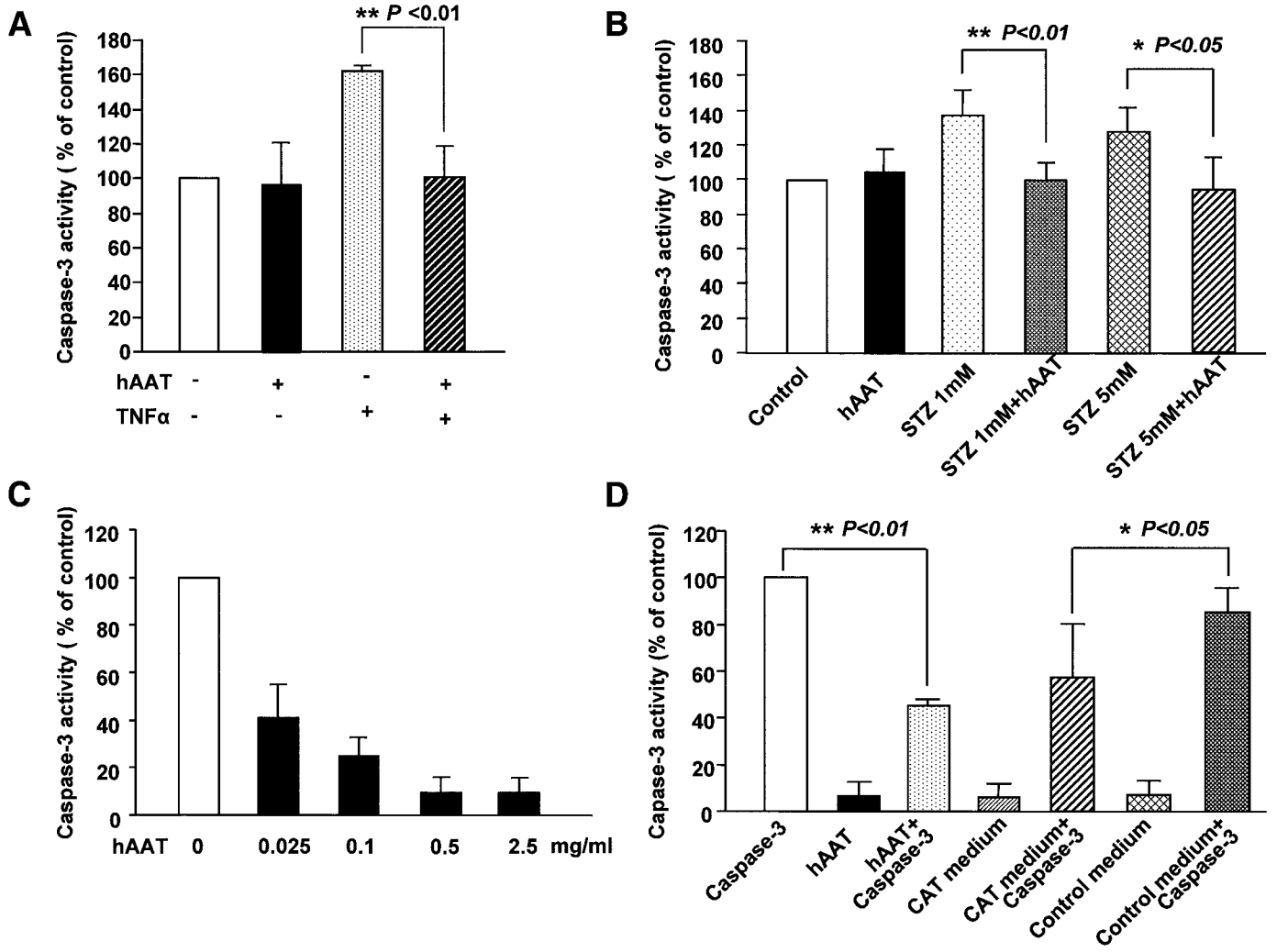

FIG. 3. AAT directly inhibited caspase-3 activity. *A*: AAT inhibits TNF-α–induced caspase-3 activity. Caspase-3 activity was measured in cell lysates following treatment with and without AAT and TNF-α, as indicated. *B*: AAT inhibits STZ-induced caspase-3 activity. Cellular caspase-3 activity was measured following treatment with and without AAT treatment and STZ injection. *C*: AAT directly inhibited caspase-3 activity in vitro in a dose-dependent manner. Recombinant active caspase-3 (0.5 units in 100 μl reaction volume) was incubated with AAT for 1 h before the detection of its activity. Each bar represents the means ± SE of three experiments. *D*: A total of 293 cells expressing AAT inhibited caspase-3 activity similarly to purified AAT (Prolastin); 293 cells were transfected with or without AAT-expressing plasmid. Choline acetyltransferase (CAT) plasmid containing hAAT cDNA driven by cytomegalovirus promoter has been previously tested for AAT expression (20). AAT concentration in the culture medium was determined by enzyme-linked immunosorbent assay. Final AAT concentrations in the reactions labeled as hAAT (human ATT) or CAT (choline acetyltransferase) medium were 0.025 mg/ml. Data are means ± SE of experiments (three to five repeats in each experiment).

RESULTS

AAT inhibits cytokine-induced β-cell apoptosis. Previous studies have demonstrated that TNF-α induces apoptosis in the MIN6 mouse insulinoma cell line, especially at concentrations >34 ng/ml (18). To identify the mechanisms underlying the beneficial effects of AAT, we evaluated its influence on TNF-α–induced β-cell death using the MIN6 cell line. Following treatment of MIN6 cells with TNF-α for 48 h, the addition of AAT (4 h) significantly increased cell viability as determined by MTT assay (Fig. 1*A*). Similar studies using Hochest staining demonstrated that AAT treatment significantly reduced MIN6 cell apoptosis (Fig. 1*B* and *C*).

STZ-induced β-cell apoptosis is inhibited by AAT. STZ is a commonly used agent for induction of β-cell death and experimental diabetes in rodent models. In terms of its properties, previous studies have demonstrated that STZ induces caspase-3 activity in β-cells (19). To test for potential effects of AAT in terms of modifying the degree of STZ-induced β-cell apoptosis, we performed a series of in vitro experiments. These studies suggested that treatment of MIN6 cells with AAT significantly increased both cellular viability (Fig. 2*A*) and reduction in STZ-induced apoptosis (Fig. 2*B* and *C*).

The antiapoptotic effects of AAT involve an inhibition of caspase-3 activity. To identify the means underlying the antiapoptotic effects of AAT, cellular caspase-3 activity was determined in both of the aforementioned cytokine- and STZ-induced apoptosis models. Interestingly, AAT completely abolished TNF-α– and STZ-induced caspase-3 activity in MIN6 cells (Fig. 3*A* and *B*). In addition, in vitro assays involving cell-free conditions demonstrated that AAT directly inhibited caspase-3 activity in a dose-dependent manner (Fig. 3*C*). To confirm that this inhibition did not result from contaminants in the purified AAT preparation (Prolastin), we transfected 293 cells with or without AAT-expressing plasmid (20). Results from these experiments demonstrated that the inhibitory effects of 293 cells

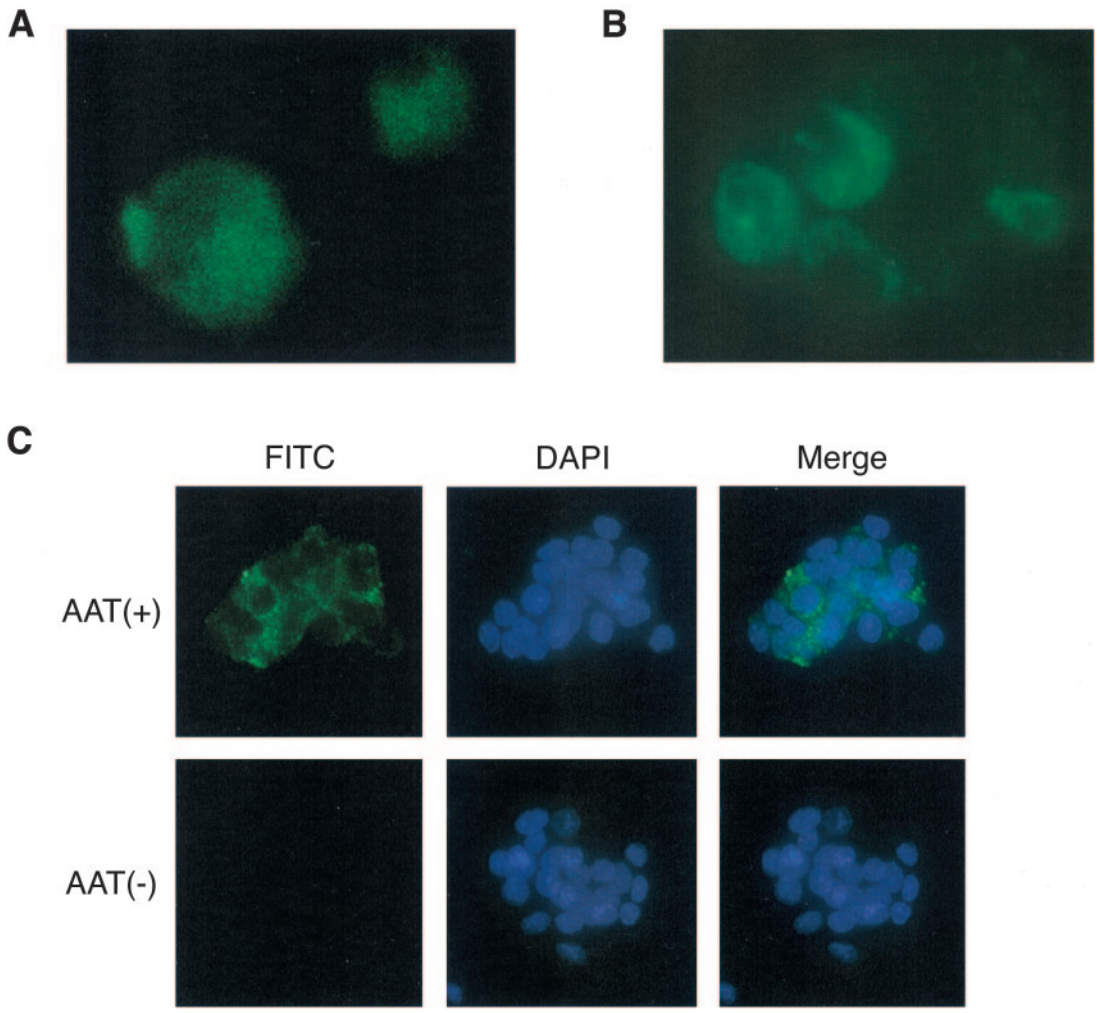

FIG. 4. Intracellular localization of AAT in MIN6 cells. *A*: Fluorescent-labeled AAT entered MIN6 cells and showed a punctuated pattern in the cytoplasm. To observe this, cells were incubated with labeled AAT for 6 h and washed three times with saline. *B*: Immunostaining with rabbit anti-hAAT antibody. For this procedure, cells were incubated with AAT for 6 h. *C*: Immunostaining for AATα and nuclear staining showed that the majority of hAAT was in cytoplasm. FITC, fluorescein isothiocyanate.

expressing AAT were comparable with those of Prolastin, while the control medium did not provide inhibitory activity (Fig. 3*D*). These data indicate that the actions of AAT involve blockage of a general apoptotic pathway that functions through inhibition of caspase-3.

AAT localizes to intracellular locations in MIN6 cells. While the above data strongly suggest that AAT inhibited β-cell apoptosis by direct inhibition of caspase-3 activity, a demonstration of the ability of AAT to physically enter MIN6 cells was not shown. To test the possibility that AAT acts in an intracellular fashion following direct entry, leading to caspase-3 inhibition, we performed a series of studies involving incubation of MIN6 cells with fluorescently labeled AAT. These experiments demonstrated that labeled AAT enters cells and resides in cytoplasm, providing a punctuated pattern of visualization (Fig. 4*A*). Additional studies using AAT-specific immunostaining were consistent with these results (Fig. 4*B* and *C*). These data support our hypothesis that AAT enters β-cells and inhibits caspase-3 activity directly, leading to protection against apoptosis in models involving induction of this pathway.

The therapeutic administration of AAT prevents STZ-induced diabetes in C57BL/6 mice. With in vitro studies indicating protective effects against β-cell apopotosis, at least through representation provided with studies of MIN6 cells, we thought it vital to test for potential protective effects of AAT in vivo. To test this, C57BL/6 mice were treated with this serpin (1 mg · mouse^{-1} · 3 days^{-1} throughout the experiment) while control animals received saline under similar injection schedules. Following a second AAT or saline injection, all mice were treated with low-dose STZ (50 mg · kg^{-1} · day^{-1} for 5 days). AAT-treated mice showed significantly lower blood glucose levels (Fig. 5*A*) and a reduced rate of diabetes (Fig. 5*B*) than saline-injected animals. In addition, glucose tolerance testing of mice 30 days following STZ injection demonstrated that AAT-treated mice demonstrated improved 2-h metabolic response profiles in comparison with control animals not treated with AAT (Fig. 5*C*).

TUNEL assays showed that the number of apoptotic β-cells in AAT-treated mice was significantly lower than that in saline-injected animals (Fig. 6*A*). Furthermore, insulin immunostaining study demonstrated that AAT-treated mice had more β-cells than saline-injected mice (Fig. 6*B*).

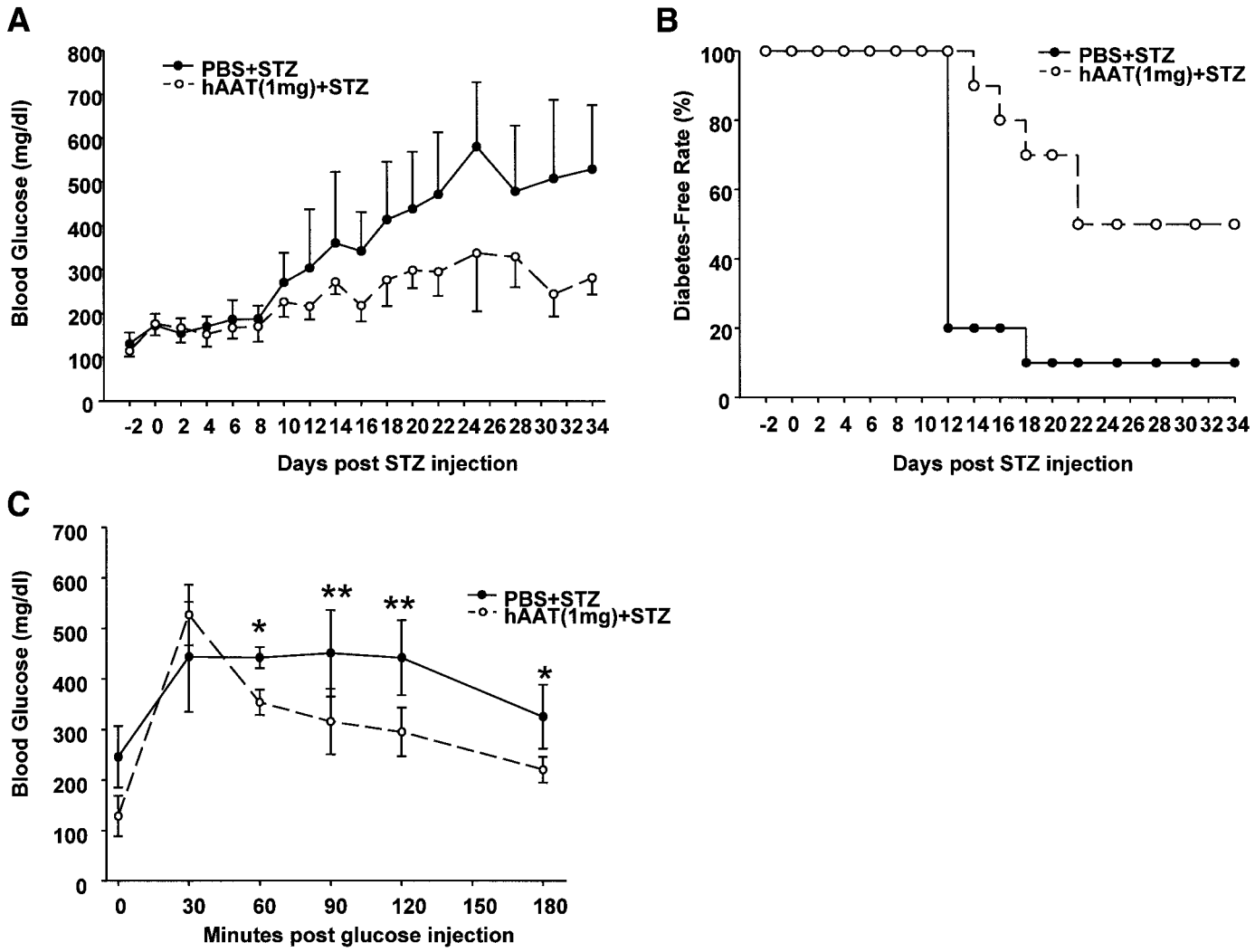

FIG. 5. AAT prevents STZ-induced type 1 diabetes and β-cell death in vivo. Adult C57BL/6 mice were treated with or without AAT (0.5 mg · kg^{-1} · 3 days^{-1}) and subjected to low-dose STZ injection (50 mg · kg^{-1} · day^{-1} for 5 days). *A*: Blood glucose levels in AAT-treated group were significantly lower than those in the control group 12 days after STZ injection ($P < 0.05$). *B*: Life-table analysis showed that AAT treatment significantly reduced diabetes development ($P < 0.05$). *C*: Blood glucose levels during intraperitoneal glucose tolerance testing. At 30 days after STZ injection, mice were fasted overnight and injected intraperitoneally with glucose (1 mg/kg). Blood glucose levels were measured at the time indicated. $n = 5$, *$P < 0.05$, ** $P < 0.01$. hAAT, human AAT.

DISCUSSION

AAT is the most abundant proteinase inhibitor in the circulation. It is well accepted that AAT is functional in extracellular fluids by inhibition of proteinases. Recent studies have indicated that AAT can directly interact with various cells (9,13,21). In the present study, we have now shown that AAT can also enter cells and function intracellularly. The results provided in this study have important implications and identify an additional mechanism for understanding observations regarding the multiple immunomodulatory functions of AAT, such as our previous studies of type 1 diabetes in NOD mice indicating that AAT administration in vivo attenuated cell-mediated immunity and altered T-cell repertoire and autoantibody formation. Indeed, a further understanding of the mechanism of AAT cellular entry (e.g., pinocytosis and receptor-mediated endocytosis) would improve and widen the use of AAT.

As a member of the serpin family, AAT inhibits not only serin proteinases but also cysteine proteinases including caspases (22). It has been reported that the viral serpin CrmA inhibits caspase-1, -3, and -8 (23,24). Studies have shown that AAT can be modified by nitric oxide and gain cysteine proteinase inhibitor activity (25,26). In the present study, we have shown that AAT inhibits caspase-3 activity in vitro and in vivo. Consistent with the observations that AAT protects lung endothelial cells, our finding extends the knowledge of AAT functions and uncovers a novel protective mechanism for the molecule (9). Further studies on the molecular interactions between AAT and caspase-3 may provide an improved understanding of the mechanisms underlying this inhibition.

As AAT demonstrates a marked ability to prevent both diabetes formation (in vivo) and β-cell apoptosis, one could speculate as to whether, in a setting of human type 1 diabetes, deficiencies in the quantity of AAT would be noted with the disease. Regarding this notion, it has also been reported that type 1 diabetic patients have significantly lower levels of AAT (27,28). We have recently observed that serum AAT levels in NOD mice are twofold lower than in C57BL/6 mice, but our preliminary studies of human type 1 diabetic patients have not revealed such an association (data not shown). We have also shown that

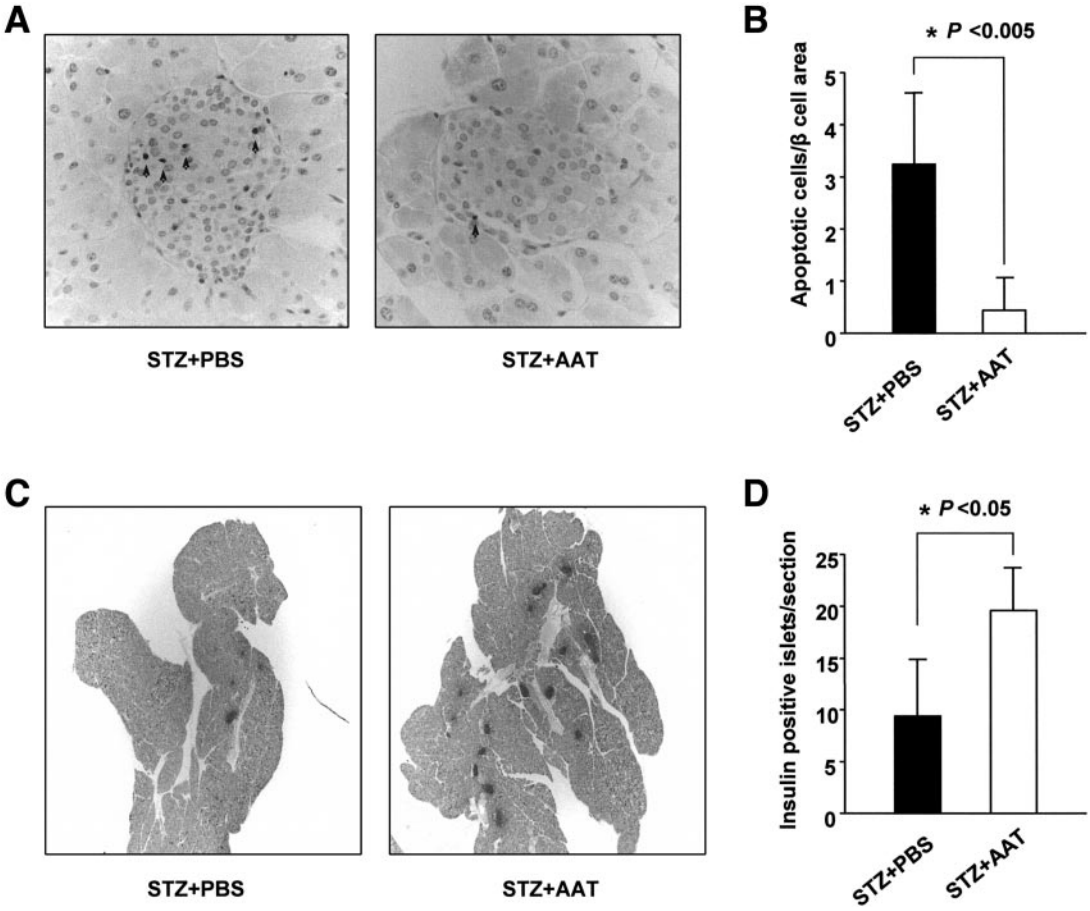

FIG. 6. ***A*****: Apoptotic cells in the islet were detected by TUNEL assay. Representative images from each group are shown.** ***B*****: Average numbers of apoptotic cells in each islet area were plotted. Insulitis in each group was scored (described in RESEARCH DESIGN AND METHODS section) and presented as representative images (*** ***C*****) and percentage of total islets in each animal (*** ***D*****).**

AAT gene expression in pancreatic islet cells is driven by multiple promoters and regulated differently from liver (29). With this, we would posit that additional studies linking the expression of AAT and its function in type 1 diabetes are warranted—especially those that could associate AAT levels with the natural history of progression to the disease. These results also support the potential that AAT could represent a novel form of therapy for the prevention and reversal of type 1 diabetes. This notion not only exists for settings of direct administration, but also as supported under indications involving islet transplantation, as suggested in the aforementioned studies of Lewis and coworkers (14,15). Indeed, those investigators showed that in the presence of AAT, islet cells respond with a variety of beneficial characteristics including but not limited to enhanced viability, inducible insulin secretion, and reduced TNF-α release (under interleukin-1β/γ-interferon stimulation).

Progressive β-cell failure and apoptosis is not only thought to represent a key event in the pathogenesis of type 1 diabetes but also of type 2 diabetes. Although the signals and their pathways for β-cell apoptosis in these two disorders are different, caspase-3 is one of the common enzymes for many pathways and may play an important role in both diseases (30). Given that in the present study we demonstrated that treatment with AAT protects β-cells against apoptosis through inhibition of caspase-3 activity, these results may imply a potential of AAT for the treatment of type 2 diabetes as well. Indeed, taken collectively, we believe these studies provide strong rationale for additional studies seeking to identify the potential role of AAT in the pathogenesis of type 1 (and possibly type 2) diabetes and support efforts with the goal of examining the potential therapeutic benefits of AAT administration for reversing and/or preventing these disorders.

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