

**Alpha 1 Antitrypsin Protects  $\beta$ -Cells from Apoptosis**

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Abbreviations: AAT, alpha 1 antitrypsin; MIN, murine insulinoma cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; rAAV, recombinant adeno-associated virus; STZ, streptozotocin.

$\beta$ -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 alpha 1 antitrypsin, 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 alpha 1 antitrypsin significantly reduces cytokine- and streptozotocin-induced  $\beta$ -cell apoptosis. Specifically, strong anti-apoptotic activities for alpha 1 antitrypsin (Prolastin®, human) were observed when murine insulinoma cells (Min6) were exposed to TNF- $\alpha$ . In a second model system involving streptozotocin induced  $\beta$ -cell apoptosis, treatment of Min6 cells with alpha 1 antitrypsin similarly induced a significant increase in cellular viability and a reduction in apoptosis. Importantly, in both model systems, treatment of alpha 1 antitrypsin completely abolished induced caspase-3 activity. In terms of its activities in vivo, treatment of C57BL/6 mice with alpha 1 antitrypsin prevented streptozotocin-induced diabetes and in agreement with the in vitro analyses, supported the concept of a mechanism involving the disruption of  $\beta$ -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.

Type 1 diabetes is an autoimmune disease resulting from destruction of the insulin producing islet  $\beta$  cells (1). Multiple lines of evidence indicate that antigen-presenting cells (APC), especially dendritic cells, are pathologically active in orchestrating the process of insulinitis (2). Antigen presenting cells within islets likely respond to micro-environmental triggers including  $\beta$ -cell death & apoptosis, and initiate the insulinitis process by migrating out of the islet and into the peripheral pancreatic lymph nodes. These APC trigger the activation and proliferation of  $\beta$ -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., IL-1, TNF- $\alpha$ , IFN- $\gamma$ ) mechanisms are responsible for  $\beta$ -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 serpin proteinases. As far as indirect mechanisms for  $\beta$ -cell apoptosis involving cytokines, signal transduction activities afforded by interactions between these molecules and specific receptors initiate activation of MAPK pathways, mobilization of transcription factors (e.g., NF- $\kappa$ B, and STAT-1) and upregulation or downregulation of downstream gene transcription (5). The

mechanism(s) by which cytokines destroy  $\beta$ -cells are complex and under active investigation (3). However, it is possible that cytokine induced cell death also involves caspase activation (6).

Alpha 1 antitrypsin (AAT) is one of the major protective proteins in physiological circulation. As a member of serine proteinase inhibitor (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 NF- $\kappa$ B translocation and increase I- $\kappa$ B levels in vivo (11). In terms of the actions of AAT for averting type 1 diabetes, we previously demonstrated that over expression of AAT, afforded by gene delivery using recombinant adeno-associated virus, significantly reduced insulinitis and prevented the development of overt hyperglycemia in NOD mice (12; 13). Studies by Lewis et al have shown that administration of clinical grade human AAT prolongs islet allograft survival and exhibits islet related cytoprotective effects (14; 15). Such findings have led to the proposal that AAT may represent a novel form of therapy for disorders (i.e., autoimmunity, 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 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  $\beta$ -cell apoptosis. These studies not only support that hypothesis but in addition, suggest strategies seeking to protect  $\beta$ -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 (DMEM; Cellgro) with 15% fetal bovine serum (Cellgro) at 37 °C in 5% CO<sub>2</sub>. To eliminate effect of bovine AAT in the serum, Min6 cells were cultured in serum free medium for 12 hr prior to AAT treatment. Cells were treated with clinical grade human AAT (Prolastin®, Bayer) at the final concentration of 0.5 mg/ml. Four hr after addition of AAT, TNF- $\alpha$  at final concentration of 40 ng/ml or STZ at the final concentration of 1 or 5 mM was added into the culture medium. Controls included cells with no AAT pre-treatment along with TNF- $\alpha$  addition, or AAT treatment with no TNF- $\alpha$  supplementation. For cell viability or apoptosis assay, cells were harvested 48 hr after TNF- $\alpha$  treatment or 24 hr after STZ treatment. For caspase-3 activity assays, cells were harvested 12 hr after TNF- $\alpha$  or STZ treatment.

**MTT assay.** Cell viability was estimated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (16). Cells (50,000 cells/well) were seeded in 96-well-flat-bottom plates with serum free DMEM medium. After culture with testing agents (AAT, TNF- $\alpha$  or STZ as described above), the medium was removed. MTT (200  $\mu$ l, 0.5 mg/ml) was added into the wells and the plates were incubated for 3 hr at 37 °C. After removal of the MTT solution, cells were treated with 100  $\mu$ l of isopropyl alcohol containing 0.04 M HCl for 1 hr. 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- $\alpha$  or STZ as described above), cells were stained with

Hoechst 33342 (20 mg/ml, Sigma) at 37 °C for 20 min, washed 3 times with PBS, and fixed with 4% formaldehyde for 15 min at room temperature. Morphological changes of nuclei were observed under a fluorescence 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 prior to incubation with AAT. In fluorescent labeling studies, AAT was labeled with DyLight<sup>TM</sup>547 using a Protein Labeling Kit (Pierce). After 6 hr of incubation with labeled AAT (0.5mg/ml), cells were washed with PBS, fixed with 4% formaldehyde, and mounted on slides without permeabilization. Images were captured using Bio-Rad confocal microscope. In immunostaining studies, cells were fixed after incubating with/without AAT for 6 hr. AAT was detected by a rabbit anti-AAT antibody (1:100, RDI), followed by incubation with FITC-conjugated goat anti-rabbit IgG (1:200, Sigma) and mounting with fluorescent mounting media with DAPI (Vector). Images were taken using a Zeiss fluorescent microscope.

**Caspase-3 activity assay.** Caspase-3 activity was

determined using the EnzChek Caspase-3 Assay Kit #2 (Molecular Probes) according to the manufacturer's instructions. Briefly, cells were washed with PBS, 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 Perkin-Elmer LS50B 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  $\mu$ l). 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 hr prior to 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. Streptozotocin (Sigma) was intraperitoneally (IP) injected (50 mg/kg/day, for 5 days) as a freshly prepared solution in 0.1 mM sodium citrate, pH 4.5. Clinical grade human AAT (Prolastin®, Bayer) was IP injected every 3 days continuously from 6 days before STZ injection until the end of the experiment.

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

**Histology and immunohistochemistry.** Pancreas from all mice were fixed in Fekete's solution, embedded in paraffin, and sectioned at 4 microns. Sections were stained with hematoxylin and eosin for routine morphology. Apoptotic cells were detected using the TUNEL (Tdt-mediated dUTP nick-end labeling) assay using according to manufacturer's recommendations (Apotag kit, Chemicon). Briefly, sections were deparaffinized, rehydrated, and incubated with 20  $\mu$ g/ml proteinase K at room temperature for 15 min. Sections were incubated in labeling solutions containing TdT and dUTP. Labeled nuclei were detected using an anti-digoxigenin antibody conjugated with peroxidase, and the peroxidase reaction visualized with 3,3'-diaminobenzidine

tetrahydrochloride & hydrogen peroxide. To determine insulin expression in  $\beta$ -cells, sections were immunostained with an anti-insulin antibody (1:200, DAKO) as previously described (12).

## RESULTS

Alpha 1 antitrypsin inhibits cytokine-induced  $\beta$ -cell apoptosis. Previous studies have demonstrated that TNF- $\alpha$  induces apoptosis in the Min6 mouse insulinoma cell line, especially at concentrations above 34 ng/ml (18). In order to identify the mechanisms underlying the beneficial effects of AAT, we evaluated its influence on TNF- $\alpha$  induced  $\beta$ -cell death using the Min6 cell line. Following treatment of Min6 cells with TNF- $\alpha$  for 48 hr, the addition of AAT (4 hr) significantly increased cell viability as determined by MTT assay (Fig. 1A). Similar studies using Hoechst staining demonstrated that AAT treatment significantly reduced Min6 cell apoptosis (Fig. 1B, C).

STZ-induced  $\beta$ -cell apoptosis is inhibited by AAT. STZ is a commonly used agent for induction of  $\beta$ -cell death and induction of experimental diabetes in rodent models. In terms of its properties, previous studies have demonstrated that STZ induces caspase-3 activity in  $\beta$ -cells (19). To test for potential effects of AAT in terms of modifying the degree of STZ-induced  $\beta$ -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. 2A) as well as a reduction in STZ induced apoptosis (Fig. 2B,C).

The anti-apoptotic effects of AAT involve an inhibition of caspase-3 activity. To identify the means underlying the anti-apoptotic 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- $\alpha$  and STZ induced caspase-3 activity in Min6 cells (Fig. 3 A, B). In addition, *in vitro* assays involving cell-free conditions demonstrated that AAT directly inhibited caspase-3 activity in a dose dependent manner (Fig. 3C). To confirm this inhibition did not result from contaminants in the purified AAT preparation (i.e., Prolastin®), we transfected 293 cells with or without AAT expressing plasmid (20). Results from these experiments demonstrated that the inhibitory effects of 293 cell expressed AAT were comparable to that of Prolastin®, while the control medium did not provide inhibitory activity (Fig. 3D). These data indicate that the actions of AAT involve blockage of a general apoptotic pathway that functions through inhibition of caspase-3.

Alpha 1 antitrypsin localizes to intracellular locations in Min6 cells. While above data strongly suggest that AAT inhibited  $\beta$ -cell apoptosis by direct inhibition of caspase-3 activity, a demonstration of the ability for 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. 4A). Additional studies utilizing AAT specific immunostaining were consistent with these results (Fig. 4B, C). These data support our hypothesis that AAT enters  $\beta$ -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  $\beta$ -cell apoptosis, at least through representation provided with studies of Min6 cells, we thought it vital to test for potential the protective effects of AAT *in vivo*. To test this, C57BL/6 mice were treated with this serpin (1mg/mouse/3 days 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 (50mg/kg/day, 5 days). AAT treated mice showed significantly lower blood glucose levels (Fig. 5A) and a reduced rate of diabetes (Fig. 5B) than saline treated animals. In addition, glucose tolerance testing of mice 30 days following STZ induction demonstrated that AAT treated mice demonstrated improved 2 hour metabolic response profiles in comparison to non-AAT treated control animals (Fig. 5C).

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

## 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 studies, 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, altered T cell repertoire, and autoantibody formation. Indeed, a further understanding the mechanism of AAT cellular entry (e.g., pinocytosis, receptor mediated endocytosis) would improve and widen the utilization of AAT.

As a member of 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 (NO) and gain cysteine proteinase inhibitor activity (25; 26). In the present studies, 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  $\beta$ -cell apoptosis, one could speculate as to whether in a setting of human type 1 diabetes, deficiencies in the quantity of AAT were noted with the disease. To this notion, it has also been reported that type 1 diabetes patients have significantly lower levels of AAT (27; 28). We have recently observed that serum AAT levels in nonobese diabetic (NOD) mice are two-fold lower than C57BL/6 mice, but our preliminary studies of human type 1 diabetes patients have not revealed such an association (data not shown). We have also shown that 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, suggested in the aforementioned studies of Lewis et al. (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 and inducible insulin secretion, and reduced TNF- $\alpha$  release (under IL-1 $\beta$ /IFN $\gamma$  stimulation).

Progressive  $\beta$ -cell failure and apoptosis is not only thought to represent a key event in the pathogenesis of type 1 diabetes, but in addition, with type 2 diabetes. Although the signals and their pathways for  $\beta$ -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 studies, we demonstrated treatment of AAT protect  $\beta$ -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 AAT may play in the pathogenesis of type 1 (and possibly, type 2) diabetes and support efforts whose goals involve examination of the potential therapeutic benefits of AAT administration for reversing and/or preventing these disorders.

## ACKNOWLEDGEMENTS

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Figure Legends

FIG. 1. AAT protects  $\beta$ -cells against TNF- $\alpha$  induced apoptosis. Min6 cells were pretreated with or without AAT for 4 hr and treated with or without TNF- $\alpha$  for 48 hr. *A*: Cell viability was estimated by MTT assay and expressed as a percentage of the values of control. Each bar represents the mean ( $\pm$  S.E.) of 3 independent experiments. *B*: Apoptotic nuclear changes (arrows) detected by Hoechst staining. Representative photomicrographs of untreated (I), AAT (II), TNF- $\alpha$  (III), and AAT & TNF- $\alpha$  treated (IV) are shown. *C*: The percentage of apoptotic cells was estimated by counting at least 1200 cell nuclei in each of 3 separate experiments.

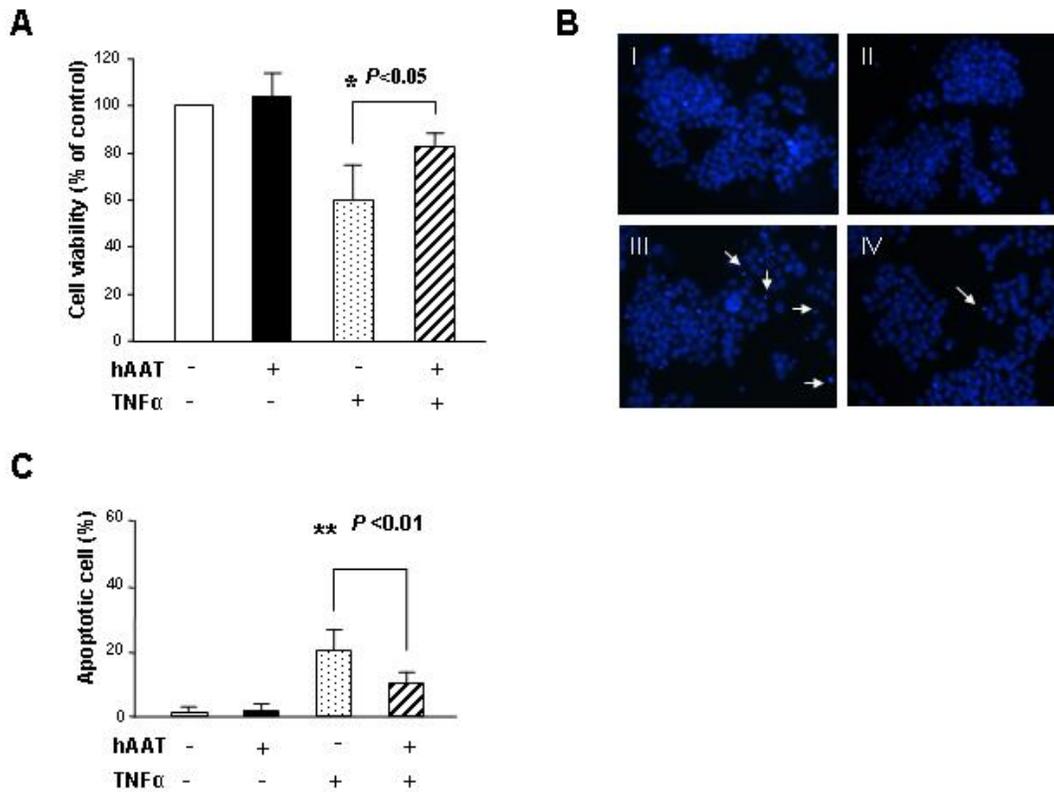


Figure 1

FIG. 2. AAT protects  $\beta$ -cells against STZ induced apoptosis. Min6 cells were pretreated with or without AAT for 4 hr and treated with or without STZ for 24 hr. **A**: Cell viability was estimated by MTT assay and presented as a percentage of the values control. Each bar represents the mean ( $\pm$  S.E.) of 3 independent experiments. **B**: Apoptotic nuclear changes detected by Hoechst staining. Representative photomicrographs of untreated (I), AAT (II), STZ (1 mM) (III), AAT & STZ (1 mM) (IV), STZ (5mM) (V), and AAT & STZ (5 mM) treated (VI) are shown. **C**: The percentage of apoptotic cells was estimated by counting least 1200 cell nuclei in each of 3 separate experiments (each bar represents the mean ( $\pm$  S.E.)).

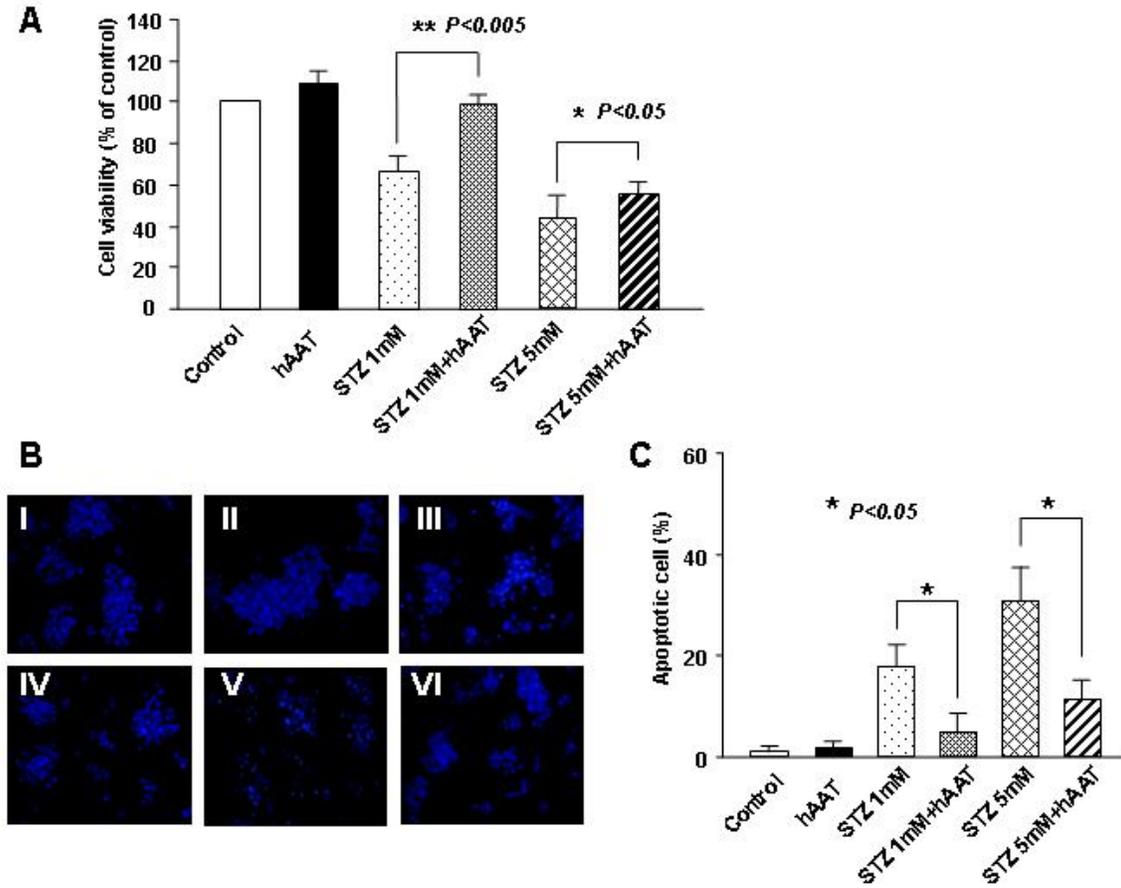


Figure 2

FIG 3. AAT directly inhibited caspase-3 activity. *A*: AAT inhibits TNF- $\alpha$ -induced caspase-3 activity. Caspase-3 activity was measured in cell lysates following treatment with and without AAT & TNF- $\alpha$  treatment, as indicated. *B*: AAT inhibits STZ-induced caspase-3 activity. Cellular caspase-3 activity was measured following treatment with and without AAT & STZ treatment. *C*: AAT directly inhibited caspase-3 activity *in vitro* in dose-dependent manner. Recombinant active caspase-3 (0.5 unit in 100  $\mu$ l reaction volume) was incubated with AAT for 1 hr before the detection of its activity. Each bar represents the means ( $\pm$  S.E) of 3 experiments. *D*: 293 cells expressed AAT inhibited caspase 3 activity similarly as purified AAT (Prolastin). 293 cells were transfected with or without AAT expressing plasmid. CAT plasmid containing hAAT cDNA driven by CMV promoter has been previously tested for AAT expression (20). AAT concentration in the culture medium was determined by ELISA. Final AAT concentrations in the reactions labeled as hAAT or CAT medium were 0.025 mg/ml. Data represents the mean ( $\pm$  S.E) of experiments (3 to 5 repeats in each experiment).

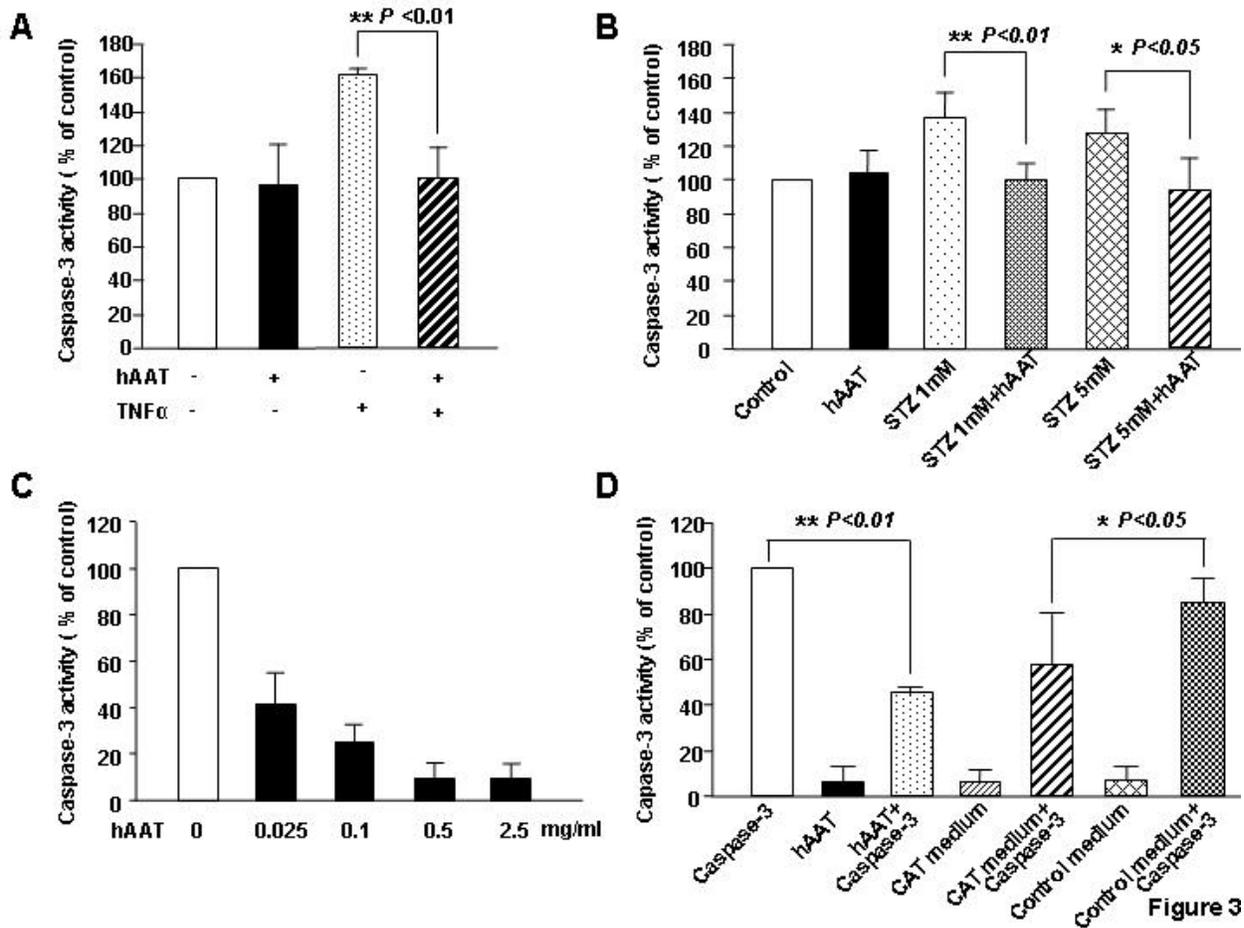


Figure 3

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 hr and washed for 3 times with saline. *B*: Immunostaining with rabbit anti-hAAT antibody. For this procedure, cells were incubated with AAT for 6 hr. *C*: Immunostaining for hAAT and nuclear staining showed that majority of hAAT was in cytoplasm.

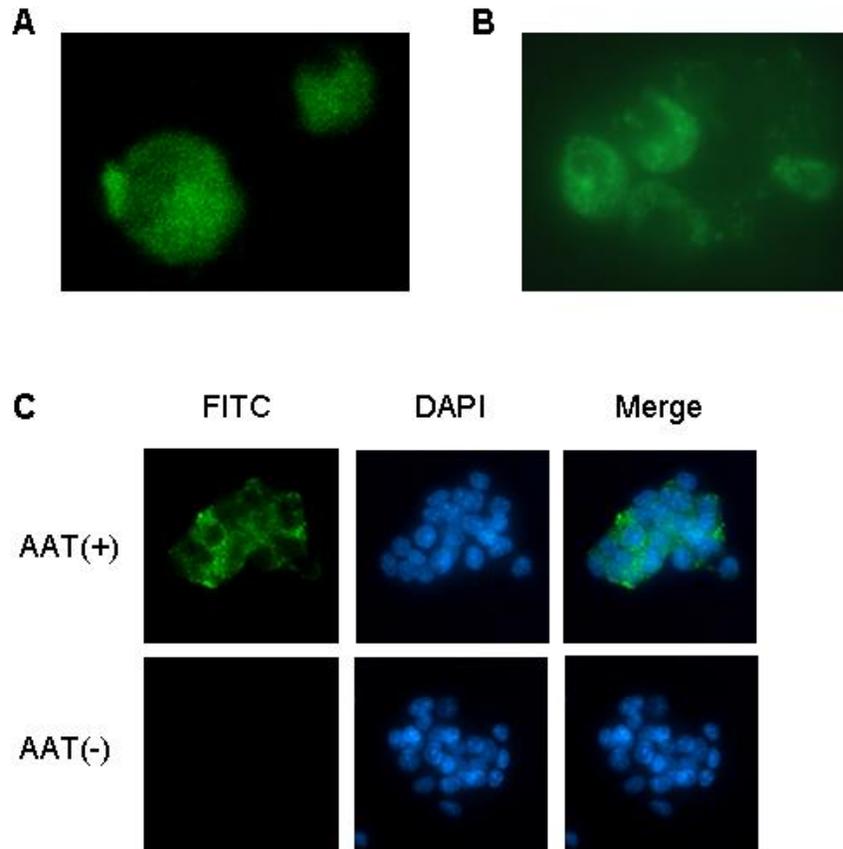


Figure 4

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

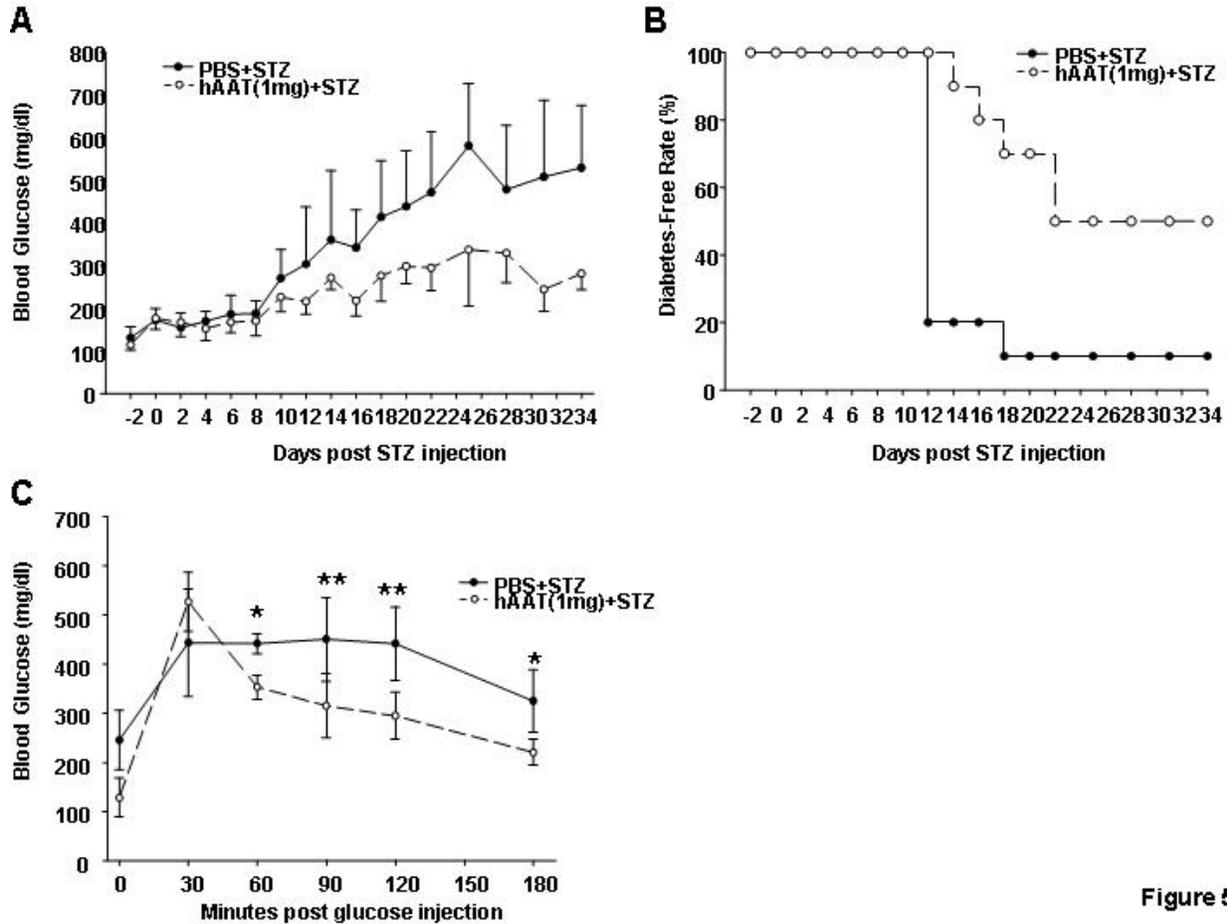


Figure 5

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. Insulinitis in each group was scored (described in methods section) and presented as *C*: representative images and *D*: percentage of total islets in each animal.

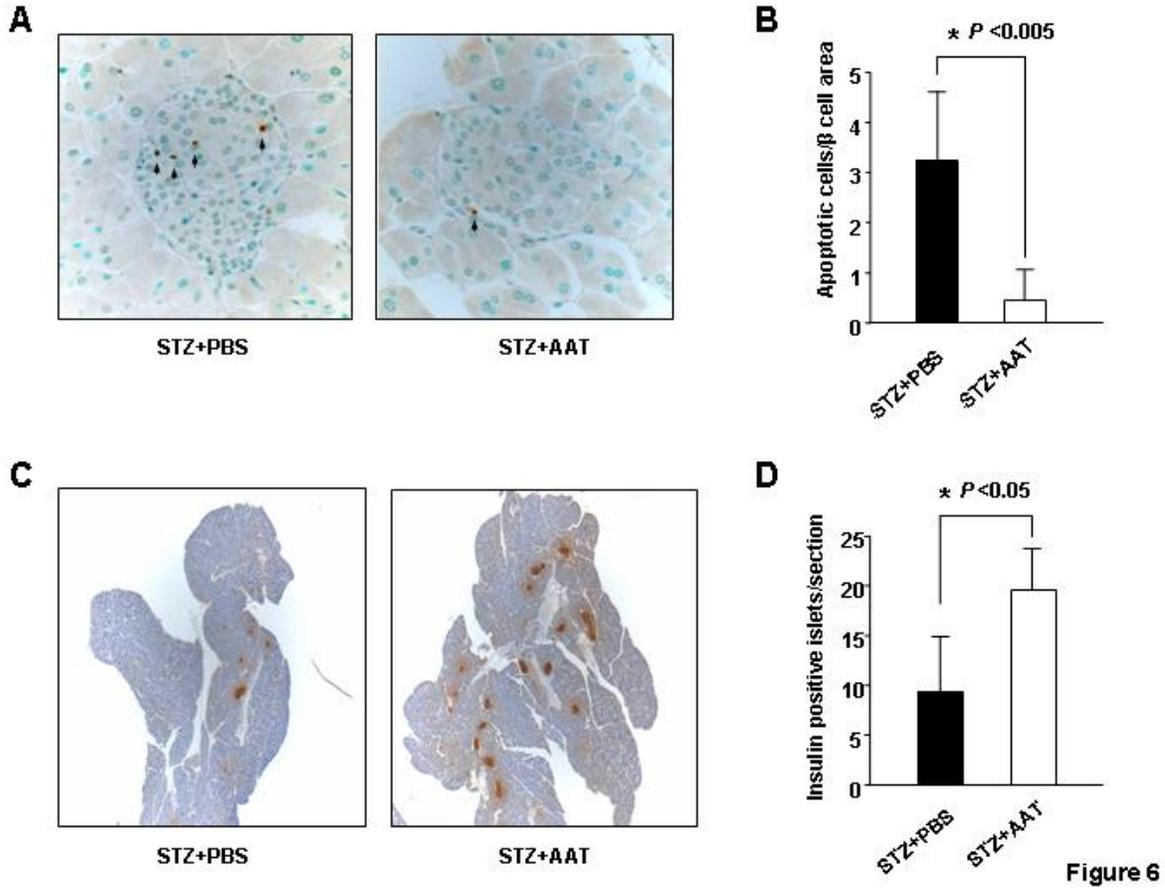


Figure 6