Intraperitoneal co-administration of thymosin α-1 ameliorates streptozotocin-induced pancreatic lesions and diabetes in C57BL/6 mice

LONGXIN QIU1,2, CUILIN ZHANG1, JUN ZHANG1, JIAXIN LIANG1, JUN LIU3, CISHU JI3 and JAMES Y. YANG1,3

1Ministry of Education Key Laboratory for Cell Biology and Tumor Cell Engineering and Department of Biomedical Sciences, School of Life Sciences, Xiamen University, Xiamen 361005; 2School of Life Sciences, Longyan University, Longyan 364000; 3Xiamen University Laboratory Animal Center, Xiamen University, Xiamen 361005, P.R. China

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Abstract. We investigated the effects of the in vivo administration of thymosin α-1 (Tα-1) on streptozotocin (STZ)-induced pancreatic lesions and diabetes. Mice were randomly divided into four experimental groups: normoglycemic control, STZ-treated, STZ plus 0.1 μg/kg body weight/day Tα-1-treated, and STZ plus 1 μg/kg/day Tα-1-treated. Blood glucose was assayed periodically, and serum insulin was determined at the end of the experiment using the ELISA Kit. Aldehyde fuchsin staining was used for histopathological examination of the pancreas. Parameters for oxidative stress were measured with pancreatic malondialdehyde (MDA) level, glutathione (GSH) content and enzymatic activities of superoxide dismutase and catalase. Fourteen days after the initiation of Tα-1 treatment and up to day 35 when the treatment was stopped, both of the two STZ and Tα-1-co-treated mouse groups had significant lower levels of blood glucose than the STZ-treated but Tα-1-untreated mice, although both remained higher than that of the normoglycemic controls. At the end of the Tα-1 treatment, the serum insulin level for STZ-treated mice receiving 1 μg/kg/day Tα-1 for 35 days was 2-fold (P<0.001) as much as that of the Tα-1-untreated STZ-diabetic mice, although not completely restored to the normal level. Pancreatic aldehyde fuchsin staining showed that STZ treatment caused significant pancreatitis, islet atrophy, and a significant reduction in the number of pancreatic β cells. These histological lesions, however, were significantly alleviated by 1 μg/kg/day Tα-1 treatment for 35 days. Furthermore, compared with the Tα-1-untreated STZ-diabetic mice, the pancreatic GSH level of the 1 μg/kg/day Tα-1-treated STZ-induced mice was 1.92-fold that of the untreated STZ-induced mice (P<0.01), whereas the pancreatic MDA level was only 81.9% that of the untreated STZ-diabetic mice (P<0.05). Together these results demonstrate that co-administration of Tα-1 leads to significant protection against STZ-induced pancreatic damage and diabetes, and part of the protection might be achieved through enhancing pancreatic antioxidative capability.

Introduction

Thymosins are a group of polypeptides that are primarily produced in the thymus gland, the site where T lymphocytes mature. Initially, these polypeptides were extracted from the thymus of bovine or cow. One of the early crude preparations, thymosin fraction 5 (TF5), contains at least 40 types of small acidic polypeptides, with most having molecular weights ranging from 1-15 kilodaltons. Based on isoelectric focusing, these small polypeptides were classified as α, β and γ thymosins, respectively. Since their discovery, a great number of studies have been carried out to investigate the distribution, expression regulation, biological function and physiological and pathophysiological roles as well as the clinical usage of these small proteins (1).

Three of the most well-studied thymosins/prothymosins include prothymosin-α (ProTα), thymosin β4 and thymosin α-1 (Tα-1). Both ProTα and thymosin β4 were initially considered to be thymic hormones but were later found to be widely distributed in many types of mammalian cells. Studies have shown that ProTα is a nuclear protein that plays various important roles such as nucleosome assembly/disassembly, regulation of the cell cycle and apoptosis, nucleocytoplasmic
transport and the production, processing or export of RNA transport and the production, processing or export of RNA (2,3). Thymosin B4, on the other hand, plays important roles in wound healing and tissue remodeling and has potential use in the treatment of myocardial infarction, chronic pressure ulcers and venostasis ulcer (4).

Tα-1 is one of the components of TFS with diverse potent biological activities (5). The known molecular and biological functions of Tα-1 include activation of the p38 MAPK and NFκB pathways, inhibition of steroid-induced thymocyte apoptosis, stimulation of various interleukins and interferons and relevant receptors, regulation of inflammation and immunity, antifungal, bacterial and viral infections. Tα-1 is a short peptide containing only 28 amino acids, and it can be easily synthesized chemically. Tα-1 is now approved worldwide in many countries for the treatment of hepatitis B and C and as immune adjuvant in the treatment of a number of other diseases such as non-small cell lung cancer, melanoma and HIV/AIDS (6,7).

Type I diabetes (T1DM) is largely considered to be a chronic disorder that results from autoimmune destruction of the insulin-producing pancreatic β cells. Experimentally, pancreatitis or T1DM models can be easily created by treating mice or rats with pancreatic toxins such as alloxan, STZ or taurocholate. Since thymosins have potent activities against a number of diseases, it is of interest to find out whether they can be beneficial for the protection against the development of T1DM. In 1983, in an effort to investigate whether thymosin administration can boost the immune response to enhance resistance to fungal infections in mice, Salvin and Tanner found that TFS administration to alloxan-induced diabetic mice significantly improved parameters for resistance to fungal infections (8). In another study, Okimoto et al observed that TFS treatment in vitro improved the impaired maturation of T lymphocytes from STZ-diabetic mice (9). This TFS-mediated T lymphocyte protection was also further confirmed by Winter et al (10). Of particular interest, however, was that when thymus gland extract TFS was administered subcutaneously into spontaneous hypertensive rats, blood pressure in the animals was significantly decreased in comparison with the controls (11). Based on changes in mitogen reactivity, rosette formation, prostaglandin E levels, immune complex deposition and blood pressure following TFS treatment, Strausser (11) suggested that TFS might be used as an anti-autoimmune disease agent, useful in treating autoimmune diseases such as hypertension, lupus and diabetes. Indeed, when TFS was used to treat STZ-treated CD-1 mice, the induction of insulinitis and hyperglycemia was significantly prevented (12). Mizina also reported that thymosin (TFS) treatment restored blood insulin levels to improve blood glucose in alloxan-induced diabetic mice (13). While the above two studies established that TFS is beneficial for the protection of pancreatic tissues, it is not clear which thymosin plays the major roles since TFS is a mixture of more than 40 thymic peptides. In a recent study, Yao et al demonstrated that co-administration of Tα-1, the first small peptide component isolated from TFS, appeared to improve sodium taurocholate-induced severe acute pancreatitis in rats, based on a few parameters such as changes in peripheral T cell number and CD3+/CD4+/CD8- subtypes as well as serum levels of cytokines including interleukin (IL)-1β and tumor necrosis factor-α (TNF α) (14). This study suggests that Tα-1 is beneficial for the protection against chemical toxin-induced pancreatic damage in part through Tα-1-mediated immunomodulation. In our current study, we aimed to determine whether Tα-1 is the major molecule responsible for the TFS-mediated protection against the development of STZ-induced diabetes in C57BL/6 mice. We also wanted to ascertain whether Tα-1-mediated antioxidative mechanisms are involved in pancreatic protection.

Materials and methods

Animal experiments. Male C57BL/6 mice, 6-8 weeks of age, were used for this study. All animals were maintained on standard laboratory chow under a 12 h:12 h light-dark schedule, and experiments were conducted according to protocols and guidelines approved by the Xiamen University Institutional Animal Care and Use Committee. The mice were randomly divided into four experimental groups (each containing 6 animals): normoglycemic control (A), STZ-treated (B), STZ + 0.1 μg/kg/day Tα-1-co-treated (C), STZ + 1 μg/kg/day Tα-1-co-treated (D). Diabetes was induced for B, C and D groups of mice by multiple low doses of STZ (Sigma, St. Louis, MO, catalog no. S0130) (40 μg/kg body weight, intraperitoneal (i.p.) injection once daily for 5 days) administered as a freshly prepared solution in 0.1 mM sodium citrate (pH 4.5). For C and D groups of mice, 0.1 μg/kg body weight or 1 μg/kg body weight of Tα-1 (Sigma, catalog no. T3410) (dissolved in saline) was co-administered, respectively, as a single daily i.p. injection co-initiated with STZ injection and continued for 5 weeks, after which (day 35) the experiment was terminated. The same volumes of saline were also co-administered to the A and B groups of mice.

Determination of blood glucose and serum insulin. The blood glucose level was measured periodically throughout the experimental period using a glucometer (OneTouch Ultra, LifeScan). At the end of the Tα-1 treatment, mice were sacrificed, and blood was collected from the sinus orbit. Serum insulin was measured using a mouse insulin enzyme-linked immunosassay kit (ADL, USA).

Determination of superoxide dismutase (SOD) and catalase (CAT) activity. At the end of the experiment, mice were sacrificed. Pancreases were removed quickly, perfused with chilled phosphate buffer (0.1 M, pH 7.4) and stored in liquid nitrogen until use. Tissues were homogenized in cold phosphate buffer (0.1 M, pH 7.4) containing 1.17% KCl using a Polytron homogenizer. The homogenate was centrifuged at 10,500 x g for 15 min at 4°C. The supernatant was used to assay for enzyme activities.

SOD activity was assayed using a commercial kit (Jianchen, Nanjing, P.R. China). Enzyme activity was expressed as units/mg protein, and 1 unit of enzyme was defined as the enzyme activity required for suppressing the production of nitrite by 50%. CAT activity was assayed by following the method of Sinha (15). The reaction mixture (3 ml) contained 1.95 ml of phosphate buffer (0.1 M, pH 7.0), 1.0 ml of 0.019 M H₂O₂, and 0.05 ml of tissue homogenate (supernatant). Changes in absorbance were recorded at 240 nm...
and calculated as μmoles of H$_2$O$_2$ consumed/min/mg of protein.

**Determination of malondialdehyde (MDA) and glutathione (GSH).** An aliquot of 1 ml of the suspension medium was taken from the supernatant obtained after the centrifugation of tissue homogenate (10% w/v) at 10,500 × g for 15 min. Approximately 0.5 ml of 30% trichloroacetic acid (TCA) followed by 0.5 ml of 0.8% thiobarbituric acid (TBA) was then added. The tubes were kept in a shaking water bath for 30 min at 80˚C. After 30 min of incubation, the tubes were taken out and kept in ice-cold water for 10 min. These were then centrifuged at 800 × g for 15 min. The absorbance of the supernatant was read at 540 nm at room temperature against an appropriate blank. The concentration of MDA was measured from the standard calibration curve prepared by using tetraethoxypropane. Lipid peroxidation was expressed as nmoles of MDA/mg of protein. The GSH level in pancreatic tissue was assayed using a commercial kit (Jianchen) and was expressed as nmoles of GSH/mg of protein.

**Aldehyde fuchsin staining.** Immediately following dissection, pancreatic tissues were washed with ice-cold saline. They were then fixed with 4% buffered paraformaldehyde and embedded in paraffin. Sections (5-μm) were stained with Gomori’s aldehyde fuchsin solution [pararosaniline (0.5 g), 70% ethanol (100 ml), paraldehyde (1 ml) and hydrochloric acid (1 ml)] as described previously (16). Briefly, sections were dewaxed and rehydrated, and then oxidized with Lugol’s iodine, decolourised with sodium thiosulphate. Processed sections were stained with aldehyde fuchsin solution and counterstained with haematoxylin and light green/orange G.

**Statistical analysis.** Quantitative data were expressed as the mean ± SEM. The Student’s t-test was used for pairwise comparisons and one-way ANOVA with Newman-Keuls multiple comparison test for multigroup analyses. Differences were considered significant when probability values were <0.05.

**Results**

*Tα*-1 co-administration led to reduced blood glucose levels, partially restored plasma insulin levels and significant improvement in pancreatic morphology in STZ-induced diabetic mice. To investigate the effect of *Tα*-1 on the development of diabetes, we co-treated two groups of mice with *Tα*-1 at the doses of 0.1 or 1 μg/kg/day, respectively, for 5 weeks. Fig. 1a shows the effect of *Tα*-1 co-treatment on blood glucose levels. Following 5 days of STZ injections, hyperglycemia developed quickly in the STZ-treated but *Tα*-1-untreated mice (B); blood glucose rose to ~16.4 mM by day 14, reached 24.3 mM at day 21 and sustained a relatively stable level up to day 35, while that of the normoglycemic control (A) remained constant at <8 mM. In contrast, in two groups that were STZ-treated but received *Tα*-1 co-treatment at the doses of 0.1 and 1 μg/kg, respectively (C and D), blood glucose levels were significantly lower than that of the B group of mice for all the time points assayed (starting from day 14 to day 35). The blood glucose levels for both the C and D groups of mice, however, remained significantly higher than that of the normal mice (A). Despite this, *Tα*-1 co-treatment was demonstrated to significantly correct STZ-induced hyperglycemia. Furthermore, these changes in blood glucose were accompanied by corresponding changes in body weight among the mouse groups. At the end of the *Tα*-1 treatment, significant weight loss was observed for all STZ-treated mouse groups (B, C and D) (Fig. 1b). However, for the two mouse groups that were STZ-treated but received *Tα*-1 co-treatment at the doses of 0.1 and 1 μg/kg, respectively (C and D), the body weights were significantly higher than that of the B group mice, suggesting overall improvement in metabolism following *Tα*-1 administration.
To assess the impact of Tα-1 treatment on insulin secretion, serum insulin levels were assayed in normal, diabetic and Tα-1-treated diabetic mice. As shown in Fig. 2, STZ treatment resulted in a significant decrease in serum insulin levels by 66.1% in STZ-diabetic mice (B) when compared with normoglycemic mice (0.93±0.19 ng/ml for B vs 2.74±0.13 ng/ml for A, \(P<0.001\)). Following co-administration of 1 μg/kg/day Tα-1 for 35 days, however, serum insulin levels were partially restored and were nearly 2-fold higher than that of diabetic mice (1.86±0.11 ng/ml for D vs 0.93±0.19 ng/ml for B, \(P<0.001\)).

To evaluate the effects of Tα-1 on STZ-induced pancreatic histological damage, we performed aldehyde fuchsin staining on three treatment groups of mice. As shown in Fig. 3, in normal mice, aldehyde fuchsin staining showed typical normal morphology and pancreatic islet shape and size with substantial large number of β cells (purple-stained). In contrast, significant pancreatitis, islet atrophy, and almost complete loss of β cells were found for mice that were STZ-treated but not Tα-1 co-treatment. Notably, in STZ-treated mice receiving 1 μg/kg/day Tα-1 for 35 days, all STZ-induced lesions appeared to be significantly alleviated, which included restoration of pancreatic shapes and sizes and the number of β cells and the disappearance of characteristic histological lesions in diabetic mice.

The significant improvement in blood glucose levels, insulin secretion and pancreatic damage conferred by Tα-1 co-administration strongly suggest that Tα-1 is one of the major components present in the TF5 preparation that mediates the protection against STZ-induced pancreatic lesions and development of T1DM.

**Tα-1 co-administration improved pancreatic antioxidative defense in STZ-treated mice.** MDA, GSH, SOD and CAT levels for pancreatic tissues following STZ diabetic induction and Tα-1 treatment are summarized in Table I. At day 35, pancreatic MDA for mice that were STZ-treated but not Tα-1 was 1.68-fold that of the normal controls (0.39±0.03 nmol/mg for B vs 0.23±0.01 nmol/mg for A; \(P<0.001\)), indicating greatly increased lipid peroxidation. Tα-1 treatment at 1 μg/kg/day for 5 weeks, however, resulted in a significantly lower level of MDA (0.31±0.02 nmol/mg for D vs 0.39±0.03 nmol/mg for B; \(P<0.05\)). In accordance with the changes in MDA levels, the pancreatic tissue GSH level was dramatically decreased following STZ treatment (17.19±2.11 nmol/mg for B vs 52.93±3.25 nmol/mg for A; \(P<0.001\)) but was greatly alleviated as a consequence of Tα-1 treatment (32.92±3.57 nmol/mg for D vs 17.19±2.11 nmol/mg for B; \(P<0.01\)). Furthermore, although not statistically significant, Tα-1 treatment to a certain degree restored the activities of both SOD (17.13±0.8 U/mg protein for D vs 14.32±0.85 U/mg protein for B; \(P<0.05\) with the Student’s t-test but \(P>0.05\) with one-way ANOVA) and CAT (0.7±0.06 U/mg protein for D vs 0.42±0.06 U/mg protein for B; \(P<0.05\) with the Student’s t-test but \(P>0.05\) with one-way ANOVA). While Tα-1 co-treatment to a certain degree improved the above-mentioned antioxidative parameters, the treatment did not completely correct the STZ-induced deterioration in antioxidative capabilities of the pancreatic tissues. Together, these data strongly indicate that Tα-1 treatment at 1 μg/kg/day for 5 weeks leads to reduced MDA, increased GSH level and increased activities of both SOD and CAT, suggesting that Tα-1 treatment greatly enhances the overall antioxidative capability of pancreatic tissues which might contribute, in part, to its protection against STZ-induced pancreatic lesions and diabetes.

![Figure 2](image1.png)  **Figure 2.** Effect of Tα-1 treatment on serum insulin levels in STZ-induced diabetic mice. Mouse groups used were normoglycemic control, STZ-treated and STZ + 1 μg/kg Tα-1-treated, respectively (n=6/group). Data are expressed as the mean ± SEM. ***\(P<0.001\).

![Figure 3](image2.png)  **Figure 3.** Aldehyde fuchsin staining of pancreatic tissues from three mouse groups. Pancreatic tissues from three mouse groups, i.e., normoglycemic control (a), STZ-treated (b) and STZ + 1 μg/kg Tα-1-treated (c), were collected and stained as described. Results are typical for 3 mice/group. Insulin-secreting β cells (arrow) were stained purple. Pancreatic α cells and other peripheral cells appear brown-yellow. Original magnification, ×400.
In spite of this, the activation and subtype switch of T lymphocytes alone might not be sufficient to completely explain the overall protective effects of TF5 or Tα-1. Apparently, the actual mechanisms involved might be much more complicated and might include mechanisms involved in angiogenesis (21,22), wound healing (21) and oxidative defense.

**Oxidative stress has been implicated in the pathophysiology of various diseases including diabetes mellitus (23-25).** Immune cells, particularly macrophages and T cells, are cytotoxic to islet β cells, in part, by generating cytokines and free radicals. Pancreatic β cells are very susceptible to oxidative damages because of their low antioxidative capacity (26). β cell injury in animals has been found to be provoked by chemicals that increase cellular oxidative stress, which include STZ and alloxan. During STZ metabolism, various toxic intermediates are produced, including methyl radicals, reactive oxygen species (ROS) and nitric oxide (NO) (27-29).

A number of studies have shown that thymosins might be involved in antioxidative defense. For instance, Li et al (30) reported that in the liver of young adult thymectomized rats, MDA, a metabolite of lipid peroxidation, was significantly increased. In these thymectomized rats, both the activities of MDA and the hepatic content of GSH were also significantly decreased. Administration of thymosins to these animals, on the other hand, led to decreased MDA levels and increased GSH content in the liver. Moreover, Lau et al also found that thymosin treatment protected vascular endothelial cells from oxidative injury (31). Together, these results suggest that, in addition to their well-established immunomodulatory roles, thymosins might also play important roles in antioxidative defense. For Tα-1, Ademoglu et al (32) reported that plasma and erythrocyte MDA levels were significantly decreased in Tα-1-injected rats. Armcut et al (33) reported that liver MDA levels were significantly decreased after Tα-1 treatment in high fructose-induced steatohepatitis rats. In addition, Tα-1 was also found to be able to protect liver and aorta from oxidative damage in atherosclerotic rabbits through increasing liver and aorta GSH levels and decreasing liver and aorta MDA levels (34). Tα-1, therefore, might confer pancreatic protection against toxin-induced β cell damage and the development of hyperglycemia, in part, by improving cellular defense against oxidative stress.

In our study, pancreatic MDA levels were greatly increased while GSH levels were greatly decreased in STZ-induced diabetic mice. Additionally, the activities of two major
enzymes for antioxidative defense, SOD and CAT, were greatly reduced. STZ treatment, therefore, caused severe loss of antioxidative capabilities and increased lipid peroxidation. These lesions, however, were significantly alleviated in mice which received both STZ and Tα1. As a consequence of co-administration of 1 μg/kg/day Tα1 for 5 weeks, pancreatic MDA levels were significantly reduced and GSH levels were significantly increased. Furthermore, although not statistically significant, improvement in enzyme activities were also observed for SOD and CAT. While these data are consistent with previous studies showing the antioxidative roles of Tα1 in a number of other tissues (32-34), our results indicate that in vivo administration of Tα1 greatly enhances the antioxidative capability of β cells and pancreatic tissues in STZ-induced diabetic mice, suggesting that indeed the pancreatic protection provided by Tα1 is, in part, mediated via enhanced pancreatic antioxidative defense.

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References


