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Thymulin and peroxiredoxin 6 have protective effects against streptozotocin-induced type 1 diabetes in mice

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Abstract

Protective effects of peroxiredoxin 6 (PRDX6) in RIN-m5F β-cells and of thymulin in mice with alloxan-induced diabetes were recently reported. The present work was aimed at studying the efficiency of thymulin and PRDX6 in a type 1 diabetes mellitus model induced by streptozotocin in mice. Effects of prolonged treatment with PRDX6 or thymic peptide thymulin on diabetes development were evaluated. We assessed the effects of the drugs on the physiological status of diabetic mice by measuring blood glucose, body weight, and cell counts in several organs, as well as effects of thymulin and PRDX6 on the immune status of diabetic mice measuring concentrations of pro-inflammatory cytokines in blood plasma (TNF-α, interleukin-5 and 17, and interferon-γ), activity of NF-κB and JNK pathways, and Hsp90α expression in immune cells. Both thymulin and PRDX6 reduced the physiological impairments in diabetic mice at various levels. Thymulin and PRDX6 provide beneficial effects in the model of diabetes via very different mechanisms. Taken together, the results of our study indicated that the thymic peptide and the antioxidant enzyme have anti-inflammatory functions. As increasing evidences show diabetes mellitus as a distinct comorbidity leading to acute respiratory distress syndrome and increased mortality in patients with COVID-19 having cytokine storm, thymulin, and PRDX6 might serve as a supporting anti-inflammatory treatment in the therapy of COVID 19 in diabetic patients.

Keywords

cytokines, peroxiredoxin 6, signaling, streptozotocin-induced diabetes, thymulin

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Introduction

The prevalence of diabetes in the world has increased dramatically in recent years.[1] This is a serious social problem because diabetes is a complex disease, involving many organs and systems, and the risk of death for people with diabetes is at least double compared to their age-matched healthy peers.[2] Besides, patients with diabetes mellitus seem to be prone to develop more severe symptoms of COVID-19 and appear to have an increased mortality rate.[3,4]

In animal models, diabetes is most commonly chemically induced following alloxan or streptozotocin (STZ) administration. These substances can be used to induce both type 1 and type 2 diabetes mellitus; however, they are generally used to induce type 1 diabetes because they do not directly lead to insulin resistance. Both alloxan and STZ are toxic glucose analogues that are transferred

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into pancreatic β-cells by a GLUT2 glucose transporter.[5] Although alloxan and STZ induce diabetes through different mechanisms, they both lead to the destruction of β-cells. Alloxan induces production of reactive oxygen species (ROS) and, through glutathione reduction, of hydroxyl radicals. The radicals are responsible for the death of β-cells, whose antioxidant defense is less effective compared to that of other tissues.[6] Although alloxan destroys β-cells, alloxan-induced hyperglycemia is a non-persistent and reversible condition, wherein the blood glucose levels normalize over time.[1]

Instead, STZ-induced diabetes is a more persistent model that can be used for both short-term and prolonged experimental studies. After its absorption by β-cells, STZ is cleaved into glucose and methyl nitrosourea components.[3] Due to its alkylating properties, the latter component leads to DNA fragmentation and death of the β-cells, and, ultimately, to an insulin-dependent diabetes-like disease. STZ is a glucosamine-nitrosourea drug that was isolated from *Streptomycetes achromogenes*[7,8] and shows similar properties to alloxan. Both STZ and alloxan are hydrophilic substances and can be considered as toxic beta-glucose analogues.[7] Interestingly, STZ has antibiotic activity and is also used as an alkylating agent in a cancer chemotherapy.[9] Like alloxan, STZ is transferred through the cell membrane by GLUT2 transporters.[9] Of note, GLUT2 are also expressed in the kidneys and liver; therefore, STZ is also harmful for these organs. The pivotal mechanism underlying STZ-induced β-cell death is DNA alkylation.[10] Furthermore, STZ may produce low amounts of ROS, most notably superoxide and hydroxyl radicals. Although STZ-associated formation of ROS is usually considered insignificant, these oxygen radicals may contribute to the death of β-cells and the development of diabetes mellitus.

Considering that STZ-induced diabetes is mediated by DNA degradation in combination with relatively low oxidative stress, the potential of the thymic peptide thymulin and the antioxidant enzyme peroxiredoxin 6 (PRDX6) as anti-diabetic drugs was assessed in the present study. The immunomodulatory and anti-inflammatory effect of thymulin was previously shown in mice with septic-type inflammation[11,12] and in mice with experimental autoimmune encephalomyelitis.[13,14] Furthermore, our previous study showed beneficial effects of PRDX6 in RIN-m5F β-cells in vitro[15] and of thymulin in mice with diabetes induced by alloxan administration in vivo.[16,17] In addition, a significant stimulatory effect of PRDX6 on the insulin-producing activity of pancreatic β-cells was demonstrated, and, most importantly, the effect of PRDX6 was detected during culturing the cells under both normal and diabetes-modeling conditions.[15] This phenomenon highlights the important role of the antioxidant protein for the mammalian insulin status. The present work aimed at evaluating the efficiency of thymulin and PRDX6 in mice with STZ-induced type 1 diabetes. The effects of thymulin and PRDX6 on the development of immune imbalance in diabetic mice were analyzed by measuring the blood cytokine profile, the activity of the signaling cascades: nuclear factor-κB (NF-κB) and c-Jun N-terminal kinase (JNK), as well as the expression of heat shock protein (Hsp)90α. In addition, the effect of thymulin and PRDX6 on the physiological status of diabetic mice was evaluated by measuring the blood glucose concentration, cell counts in thymus, spleen and pancreas, and body weight.

Materials and methods

Animal diabetes model, and PRDX6 or thymulin treatments

Six- to 8-week-old male Balb/c mice (22–25 g) were maintained under standard laboratory conditions (temperature in the range of 20°C–21°C, 10–14-h light/dark cycles, and 65% humidity), with food and water provided ad lib. Food pellets, containing a balanced composition of proteins, vitamins, and minerals were used. The mice were divided into four groups of seven mice in each: streptozotocin-treated mice (STZ), STZ mice treated with PRDX6, STZ mice treated with thymulin, and controls (intact mice). All measurements were carried out individually for each mouse, with six-nine replicates; the value showed is an average mean ± std. error.

Experimental protocols were approved by the Ethical Committee of the Institute of Cell Biophysics (approval #57, 30/12/2011). The experiments with animals were performed in accordance with the Guidelines for Ethical Conduct in the Care and Use of Animals.

BALB/c mice were injected intraperitoneally with freshly made STZ (45 mg/kg body weight) in

0.01 M citrate buffer, pH 4.3-4.6, for five consecutive days. Meanwhile, mice in the control group were given an equal amount of citrate buffer. Fasting blood glucose in mice was measured for 20 days starting on day 8 after the first STZ injection. Experiments were started 20 days after the STZ injection, when the blood glucose level was consistently greater than 12 mM, indicating the successful establishment of diabetes model. PRDX6 (20 mg/kg body weight) was administered intravenously on the first and eighth days of diabetes development. To prepare the thymulin solution, Serum Thymic Factor peptide (Abcam, Cambridge, MA, USA) was used, to which an equimolar concentration of $ZnCl_2$ was added. Thymulin was applied intraperitoneally at a dosage of 5 μg/mouse every other day in a volume of 100 μl/mouse. In total, animals of this group received eight injections of the thymulin solution. The dosages of PRDX6 and thymulin were adjusted in our early studies.[13,18]

Procedures of PRDX6 isolation and purification

Constructs encoding a human PRDX6 protein were obtained and expressed in *E. coli* cells, strain BL21(DE3), as described earlier.[18] The obtained recombinant proteins, harboring His-tag, were purified by affinity chromatography on the Ni-NTA-agarose column (Thermo Fisher Scientific, USA), according to the column manufacturer's instructions. Protein isolation was performed as described earlier.[18] The purity of the obtained proteins was at least 98%, based on SDS-PAGE method. PRDX6 was diluted at a concentration of 10 mg/ml in phosphate buffer (1.7 mM KH_2PO_4, 5.2 mM Na_2HPO_4, 150 mM NaCl, pH 7.4) and stored in the freezer at –20°C. After 2 month of storage, no reduction of enzymatic activity was observed. To evaluate functional activity of PRDX6, its ability to reduce hydrogen peroxide (H_2O_2) and tert-butyl-hydroperoxide (t-BOOH) was determined by a slightly modified Kang's method.[19] The results showed that activity of recombinant PRDX6 was 230 nmol/min/mg of protein (measured with H_2O_2) or 100 nmol/min/mg of protein (measured with t-BOOH).

Blood plasma and splenocyte samples

Plasma samples was obtained from blood collected during decapitation of the animals. Blood was left for 3–5 h at 4°C and then centrifuged at 200 × *g*; the supernatants were used for cytokine assays. Lymphocytes from mice spleens were isolated in Dulbecco's modified Eagle's medium (DMEM; Sigma,USA)containing10 mM4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid solution, 100 μg/mL streptomycin, and 10% fetal bovine serum. Erythrocytes were lysed, using Tris-buffered ammonium chloride (0.01 M Tris-HCl, with 0.15 M NaCl and 0.83% NH_4Cl at 9:1, pH 7.2). After being washed with RPMI 1640 medium, the samples were stored at a concentration of 1×10^8 cells/mL in RPMI 1640 medium in freezer at −20°C until use.

Cytokine assay

Concentrations of cytokines in blood plasma were determined using enzyme-linked immunosorbent assay (ELISA) kits for mouse TNF-α, interleukin (IL)-5, IL-17, and interferon-γ (IFN-γ) (Peprotech, USA). Binding, visualizing and absorbance measuring were performed as reported earlier.[16]

Western blot analysis

Splenic cells (10^8 cells per sample) were lysed, and the total protein measurement using the Bradford solution (Sigma, USA), the PAGE electrophoresis using protein molecular weight (MW) marker (Thermo Scientific, USA), the protein transfer from the gel onto a nitrocellulose membrane (GE Healthcare, Amersham, UK), and the blockade with 5% w/v nonfat dry milk in TBS/Tween 20 were performed as reported earlier.[14] After blocking, membranes were incubated for 2 h with antibodies against the following murine proteins: anti-phospho-NF-κB p65 (at Ser 536) antibody (cat. number 3031, Cell Signaling Technology, Danvers, MA, USA), rabbit phospho-stress-activated protein kinase/JNK (SAPK/JNK) antibody (Cell Signaling Technology), and rabbit polyclonal antibody to HSP90a (Enzo Life Sciences, USA) and washed. Then, the nitrocellulose membranes were incubated with a secondary anti-rabbit biotinylated antibody (Jackson ImmunoResearch, West Grove, PA, USA) for 1 h, followed by incubation with peroxidase-conjugated streptavidin for 1 h. As a protein loading control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used, and the detection was performed using a rabbit monoclonal antibody against a synthetic peptide corresponding to C-terminal residues of human GAPDH

(Cell Signaling Technology). To develop blots, the ECL Plus chemiluminescent cocktail (Amersham/GE) was used according to the manufacturer's protocol. The developed blots were photographed using a TFX-35 WL transilluminator (Vilber Lourmat, France), and the protein bands were assessed densitometrically using Image Studio Software ver. 5.2.5 (Li-COR, USA). The provided values are means from three independent experiments performed (three mice). The obtained data were normalized to the corresponding loading control (GAPDH) and expressed in relative units.

Histology and immunohistochemistry

Histology and immunohistochemistry assays were performed as reported.[16]

Statistical analysis

Statistical estimations were obtained using the Statistica/Win 6.0 software (Tulsa, OK, USA). To determine a sample size, a following well-established formula was used, considering power of 80%, and a confidence level of 95%:

$$n = \left(Z_{\alpha/2} + Z_{\beta} \right)^2 * 2 * \sigma^2 / d^2,$$

where $Z_{\alpha/2}$ is the critical value of the normal distribution at $\alpha/2$ (e.g. for a confidence level of 95%, α is 0.05), Z_{β} is the critical value of the normal distribution at β (e.g. for a power of 80%, β is 0.2), σ^2 is the population variance determined in our pilot studies on BALB/c mice, and d is the difference that should be detected. We calculated a sufficient sample size of 6 for the above mentioned power and confidence level values.

One-way analysis of variance (ANOVA), followed by Tukey's post-hoc tests, was performed to determine the significance of differences. Values of $P \leq 0.05$ were considered significant.

Results

Thymulin and PRDX6 improve the physiological status of mice with streptozotocin-induced diabetes

As blood glucose measurements indicated, administration of STZ caused sustained hyperglycemia that remained throughout the observation period

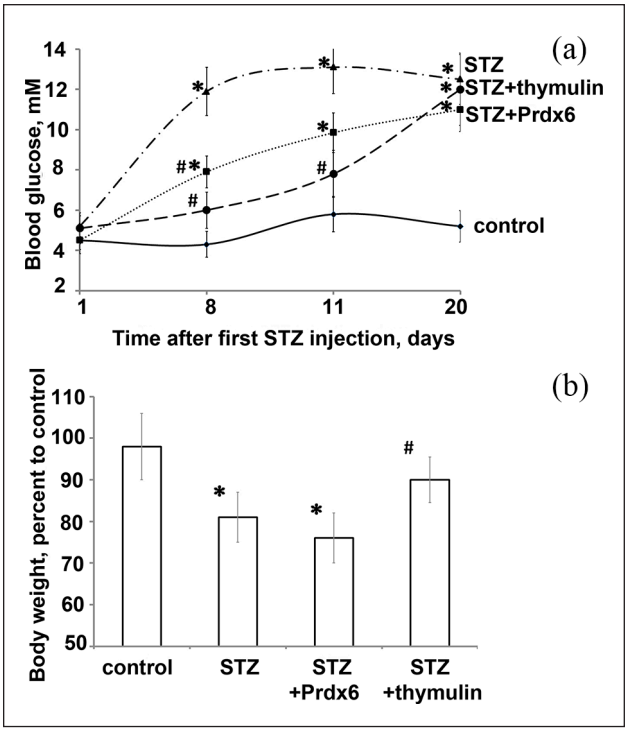

Figure 1. Effects of thymulin and PRDX6 on fasting blood glucose levels (a) and body masses (b) in diabetic mice. Four groups were used: streptozotocin-treated mice (STZ), STZ mice treated with PRDX6, STZ mice treated with thymulin, and controls (intact mice). Each group consisted of seven mice; the value showed is an average mean ± std. error (a). Body weights were measured on 21st days after the first STZ injection, and average means ± std. errors are shown as a percent of the body weight at first day (b).
*Significantly different from the control group, $P < 0.05$.
#Significantly different from the STZ group, $P < 0.05$.

(Figure 1). Both PRDX6 and thymulin significantly reduced the severity of hyperglycemia, although not to normal levels. Body weight measurements at the end of the observation period showed that the diabetes led to a statistically significant decrease in the body weight, and only thymulin, but not PRDX6, partially compensated the weight loss (Figure 1).

Then, cell counts were measured in two organs of the immune system (thymus and spleen) as well as in the pancreas, and normalized to the mass of the corresponding organ. The relative cell counts showed a significant loss in the thymus and pancreas, but not in the spleen (Figure 2). PRDX6 administration significantly increased the cell count in the pancreas, while thymulin partially restored the cell count in the thymus.

To elucidate the effects of PRDX6 and thymulin on pancreas morphology in mice with diabetes,

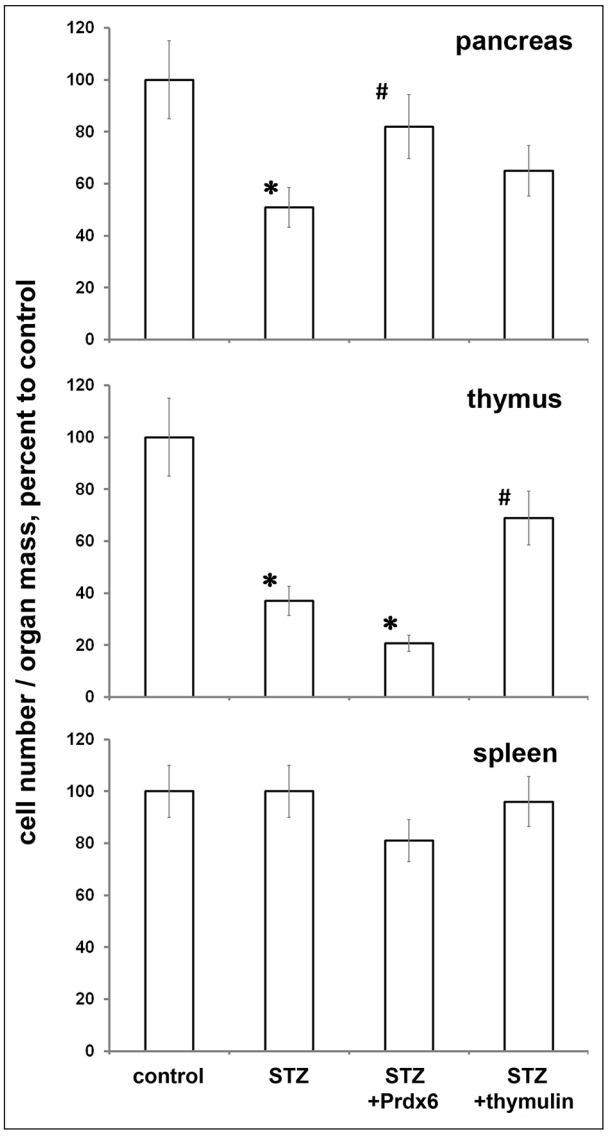

Figure 2. Effects of thymulin and PRDX6 on relative cell count in murine organs. The groups are indicated in Figure 1; the relative cell count is a cell count/organ mass ratio (average mean ± std. error) on 21st day, expressed as a percent of that in the control group.
*Significantly different from the control group, $P < 0.05$.
#Significantly different from the STZ group, $P < 0.05$.

immunostaining for insulin was performed. The results demonstrated a reduction in the islet density in diabetic mice. The remained β-cells were severely disorganized (Figure 3), demonstrating the expected damage of pancreatic β-cells in advanced diabetes. Injections of PRDX6 or thymulin somewhat restored the islet density in diabetic mice, but these effects were not significant. However, both thymulin and PRDX6 increased the insulin-positive regions in the pancreas sections.

Thymulin and PRDX6 reduce the inflammatory response and ameliorate the immune status of mice with diabetes mellitus

To evaluate the effect of thymulin and PRDX6 on the immune status of mice with induced diabetes, we measured the concentrations of several pro-inflammatory cytokines in blood plasma using ELISA. Diabetic mice demonstrated a significant cytokine response, with increase in IL-1β, IL-5, IFN-γ, and TNF-α levels (Figure 4). Administration of thymulin reduced the levels of all cytokines analyzed to normal levels, whereas PRDX6 led to significantly decreased levels only of TNF-α.

In mice with STZ-induced diabetes, a significant activation of the NF-κB signaling cascade was observed in splenocytes, as determined by RelA (p65) protein phosphorylation (Figure 5). Thymulin significantly inhibited the activation of NF-κB, while PRDX6 was even more effective, lowering the activity of the NF-κB cascade to background values. Different patterns were observed when analyzing the activation of JNK pathway. In mice with diabetes mellitus, JNK cascade was also significantly activated, and only thymulin, but not PRDX6, normalized JNK activity in the spleen cells of mice with diabetes.

It is known that the cellular response to type 1 diabetes involve heat shock proteins, for example, Hsp90α protein, for which signaling proteins are clients.[20] Next, we evaluated the expression of Hsp90α (Figure 5). In mice with STZ-induced diabetes, a sharp increase in Hsp90α protein expression was observed. PRDX6 completely normalized the levels of Hsp90α in spleen cells, whereas thymulin was less effective and only caused some decrease of Hsp90α content in the cells of the diabetic mice.

Discussion

Clinical application of STZ has recently revealed mechanisms of its action.[5] Due to glucose-like structure, it may enter β-cells similarly to glucose or alloxan. However, unlike alloxan, STZ is relatively stable[9] and the main mechanism of streptozotocin-induced β-cell death is alkylation of DNA.[10,21]

NF-κB activation is a key event in the pathophysiology of autoimmune diabetes.[22,23] Excessive activation of NF-κB lead to undesirable consequences.

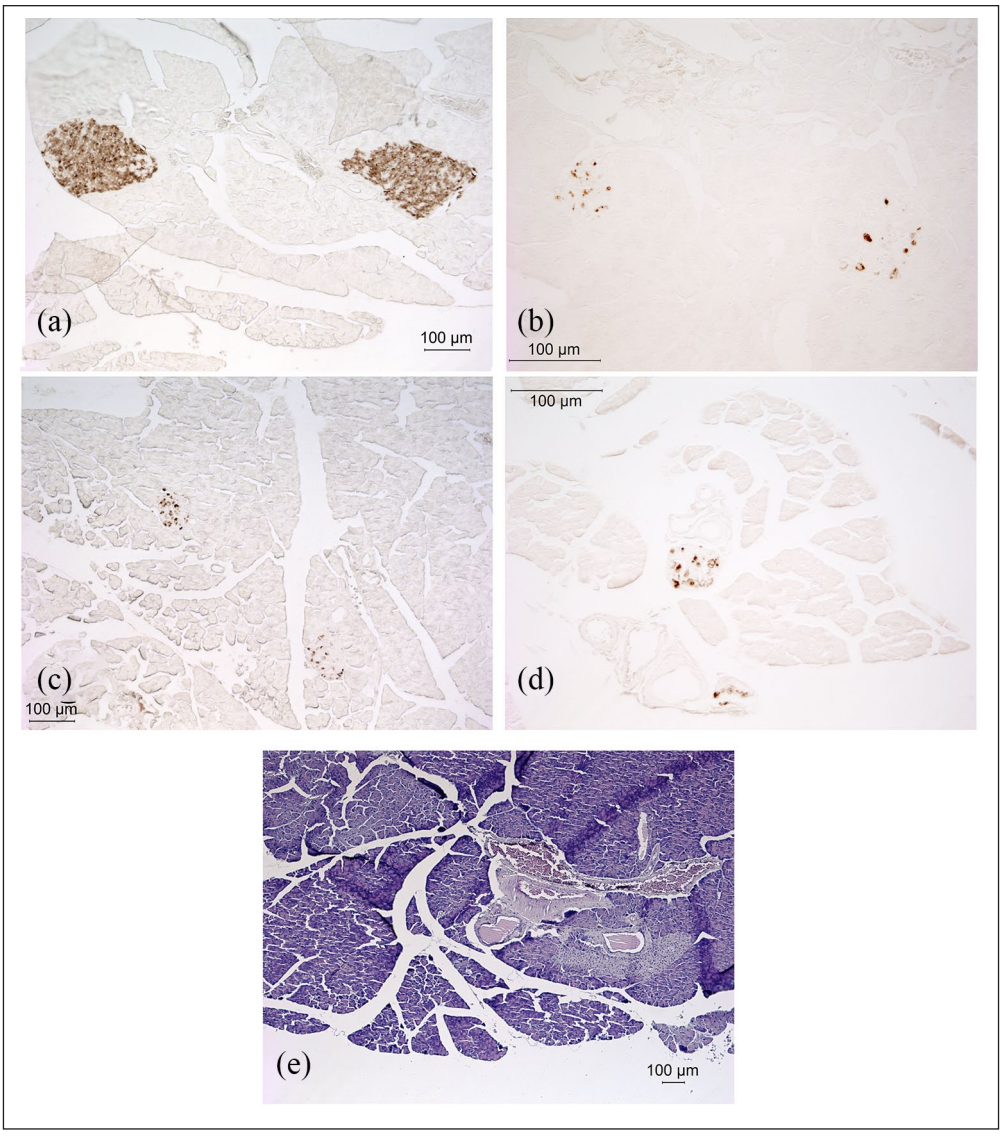

Figure 3. Effects of PRDX6 and thymulin on the pancreas structure in diabetic mice. Representative images of islet immunostaining with insulin: the pancreas of control mice (a), diabetic mice (b), diabetic mice treated with PRDX6 (c), diabetic mice treated with thymulin (d). Panel (e) shows H&E staining of the control pancreas.

Therefore, one of the goals in diabetes may be reducing the excessive activation of NF-κB signaling.[22] Our results demonstrated that administration of thymulin and PRDX6 decreased the activation of NF-κB and TNF-α level in the plasma. Interestingly, the anti-angiogenic effect of tetrandrine on blood vessels in STZ-induced diabetic rats was also associated with lowered levels of TNF-α and NF-κB.[24] Furthermore, our recent study showed a role of thymulin in developing a fast response against oxidative stress in a damage-associated molecular pattern (DAMP)-like manner, restraining the inflammatory reaction.[25]

Administration of STZ causes a sharp increase in the expression of JNK, which is considered a crucial factor in the STZ-induced β-cells death.[26] The JNK pathway is also involved in pathogenesis of diabetes.[27] Here, we demonstrated that thymulin reduced JNK activation. It was previously shown that similar effects are caused by JNK inhibitors, including PARP inhibitors.[26]

Type 1 diabetes is a heterogeneous disease, both in the phenotype and in the response to therapies.[28] Reliable biomarkers of β-cell dysfunction may improve predictions of responses to therapies and overall disease prognosis.[29] Hsp90 may be

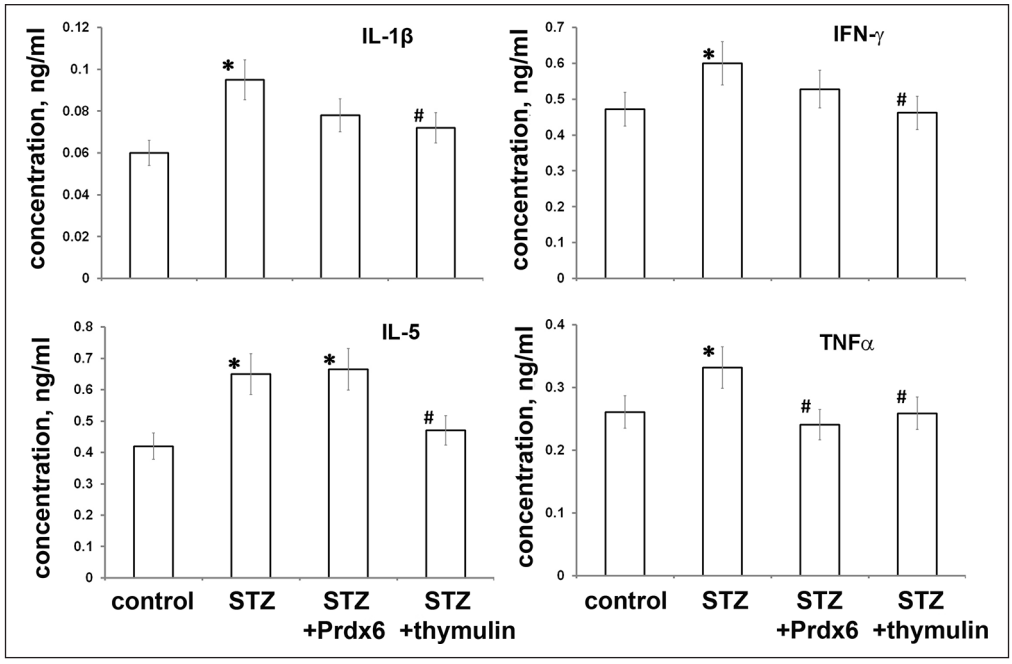

Figure 4. Effects of PRDX6 and thymulin on plasma cytokine concentrations in diabetic mice. The groups are indicated in Figure 1; each value is an average mean ± std. error for three mice; six replicate measurements were performed for each individual mouse.
*Significantly different from the control group, $P < 0.05$.
#Significantly different from the STZ group, $P < 0.05$.

considered one of the markers of β-cell stress in type 1 diabetes.[30] The authors showed that β-cells from pre-diabetic mice had four times higher Hsp90 content than in control mice. Here, we evaluated extra-islet cells; however, we also observed a four-fold increase in Hsp90 in the spleenocytes from mice with STZ-induced diabetes. It may be assumed that Hsp90 is a universal type 1 diabetes marker. PRDX6 completely prevented the increase in Hsp90 expression in spleen cells, whereas thymulin only slightly reduced Hsp90 levels.

Pancreatic β-cells are known to be constitutively vulnerable to damages. This vulnerability is primarily due to reduced levels of antioxidant enzymes.[31,32] It was shown that glutathione peroxidase activity and resistance to peroxide exposure are about 20 times higher in the liver and kidney than in the pancreas.[33] Our results demonstrated the protective effects of the antioxidant PRDX6. Indeed, PRDX6 reduced the severity of pancreatic cell loss, reduced hyperglycemia, normalized plasma TNF-α levels, NF-κB cascade activity, and Hsp90 levels in diabetic mice. We previously showed that NF-κB is involved in the protection of β-cells in mice with alloxan-induced diabetes.[16] In addition, cells with PRDX6 deficiency display increased susceptibility to the harmful effects of cytokines and oxidative stress.[34] We believe that the main function of PRDX6 is to reduce the level of oxidative stress. Peroxides, formed intracellularly due to oxidative stress in diabetes, released into the extracellular space through aquaporins and then neutralized by the PRDX6 as long as there were enough reducing agents in the extracellular fluid.[18]

We previously showed that at least 30% of exogenous PRDX6 remained in the blood circulation 360 min after intravenous injection.[35]

Another approach to a therapy of immune system-mediated diseases, such as diabetes, may include immunomodulators, as immune cells directly participate in beta-cells elimination, even in chemically induced diabetes. Thymulin was earlier shown to produce beneficial effects in experimental autoimmune encephalomyelitis (EAE),[13] reducing a cytokine response and signal cascades activation in immune cells. Thymulin attenuated some of consequences of diabetes; it normalized the physiological status of mice, reduced hyperglycemia and weight loss, normalized cytokines plasma levels. However, the most striking effect of thymulin was the normalization of JNK signaling.

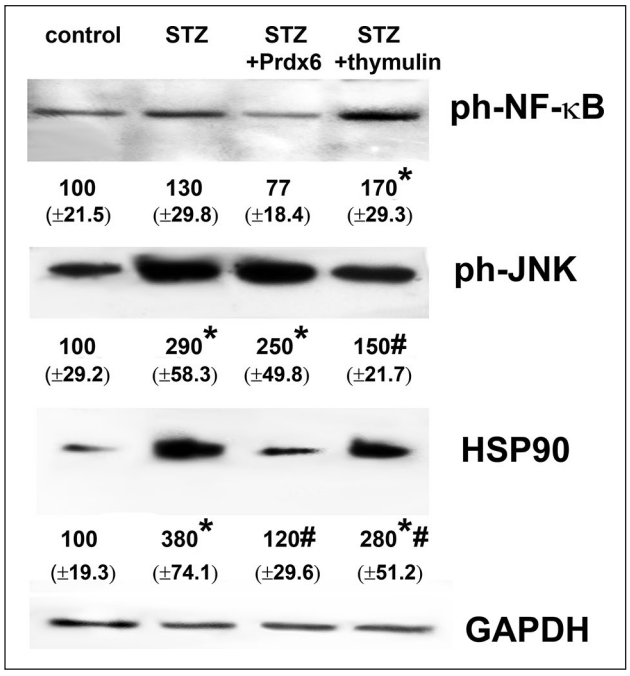

Figure 5. Effects of PRDX6 and thymulin on the activity of NF-κB and JNK pathways and expression of heat shock protein Hsp90α in splenic cells. The groups are indicated in Figure 1; equal amounts of total protein were analyzed in spleen cells using Western blot with the corresponding antibodies with normalization to a GAPDH loading control (bottom). Blot images show a single representative experiment, while values below the protein bands show signal protein levels in relative units corresponding to the internal GAPDH control and represent band densitometry values from four individual mice (average means ± std. error).
*Significantly different from the control group, $P < 0.05$.
#Significantly different from the STZ group, $P < 0.05$.

These results are consistent with data that thymulin is an important modulator in inflammations of various etiology, such as septic-type inflammations[10] or autoimmune diseases,[13,14] and with the findings on the possible role of thymulin in response to oxidative damage.[25] The study have some limitations. The chemically-induced diabetes in animals as a model of type I diabetes mellitus have certain differences as compared to type I diabetes in humans. Moreover, STZ-induced diabetes is related not only to oxidative stress, but also to DNA fragmentation.

The results suggest both thymulin and PRDX6 improved the physiological status, inhibited inflammation, and ameliorated the immune response of STZ-induced diabetic mice. The results indicate that thymulin and PRDX6 may be promising therapeutic agents against type 1 diabetes mellitus, and may serve as a supporting treatment in the therapy of COVID-19 in patients with diabetes. Diabetes, older age and other comorbidities are reported as significant predictors of morbidity and mortality in COVID-19 patients.[36,37] To date, there is a lack of sufficient evidences that diabetes is a risk factor in patients with COVID-19. However, we believe that future studies of PRDX6 and thymulin should be performed to develop new drugs for treatment of patients with COVID-19 complicated by diabetes mellitus.

Conclusion

This study demonstrated that streptozotocin-induced type 1 diabetes led to multifactorial disturbances in physiological and immune status in mice, such as hyperglycemia, the weight loss, the cytokine storm, the activation of NF-κB and JNK signaling cascades, and the rise in HSP90 stress protein expression. The PRDX6 antioxidant protein or thymulin administration reduced the pathophysiological and immunopathological consequences in the mouse model of type 1 diabetes to varying degrees. The main difference between the protective effects of PRDX6 and thymulin was the involvement in different signaling pathways. Thus, PRDX6 prevented excessive activation of NF-κB, and thymulin regulated the activity of the JNK pathway.

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Declaration of conflicting interests

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Ethical approval

Ethical approval for this study was obtained from the Institutional Ethical Committee (approval #57, 30/12/2011).

Animal welfare

The present study followed international, national, and/or institutional guidelines for humane animal treatment and complied with relevant legislation.

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