Astragaloside IV and Statin Increased the Islet Function and Proliferation for Beta Cells in STZ-Induced Diabetic Mice

Mincai Li, Tonghui She, Yapin Gan, Juan Shao, Suqin Li, Wenli Liao, Hongli Xia, Liangzu Yu, and Zhengwu Hu*

1. Hubei Province Key Laboratory on Cardiovascular, Cerebrovascular and Metabolic Disorders, HuBei University of Science and Technology, Xianning 437100, P.R. China
2. The Medical School, HuBei University of Science and Technology, Xianning 437100, P.R. China
3. Department of Abdominal Surgery, the Central Hospital of Xianning, Xianning 437100, P.R. China

Abstract

Objective: Diabetic hyperglycemia causes a variety of pathological process involved in metabolic changes. Astragaloside IV (AS-IV) was widely used for the treatment of diabetes and cardiovascular diseases in China. The aim of this study was to determine the effect of AS-IV on the function and the proliferation of beta cells in diabetic mice.

Methods: We used the immunostaining and reverse transcription polymerase chain reaction to detect the expression of insulin, Ki-67 and PDX-1 in pancreatic islets under AS-IV with or without pretreatment with statin in STZ-induced diabetic mice. The inflammation cytokine for TNF-α and MCP-1 expression were detected by ELISA in pancreases in all group mice.

Results: AS-IV and statin enhanced the secreted insulin and the proliferation in beta cells and attenuated the increased expression of TNF-α and MCP-1 in diabetic mice. The expression of PDX-1 increased in diabetic mice with AS-IV treatment.

Conclusions: Our data suggested that AS-IV enhanced the insulin expression and the islets function through the anti-inflammation and the proliferation pathway in beta cells in diabetes and provided a new effect for Chinese traditional drug of AS-IV.

Keywords: Astragaloside IV; Beta cells; Insulin; Anti-inflammation; Proliferation; Diabetes

Introduction

Diabetes mellitus (DM) is a chronic and progressive disease which is becoming one of the increasing health problems in the worldwide, according to the increasing prevalence and incidence. The increasing incidence of DM is the prevalence of cardiovascular disease and chronic kidney disease resulting from diabetic complications. The relative deficiencies or the absolute deficiencies in beta-cell mass induce to decreasing insulin secretion, the increasing insulin resistance, and the elevating hepatic glucose production. The abnormal insulin secretion was caused by the impaired beta-cell function as well as insulin resistance in target tissues in DM [1]. Therefore it is very important for the treatment with DM to find the way to preventive intervention against the progression of pancreatic beta-cell dysfunction.

The prevalence of metabolic syndrome has increased in modern society and the condition is proving to be a common precursor of cardiovascular disease and Type 2 diabetes mellitus. The prevention and treatment of metabolic syndrome are important in reducing cardiovascular morbidity and mortality. Astragaloside IV (AS-IV) is commonly used in the treatment of many disorders, including cardiovascular diseases. AS-IV is the major active component extracted from the traditional Chinese medicine Astragalus membranaceus (Fisch) Bge. Previous studies have shown that AS-IV can protect the myocardium and central nervous system against ischaemic injury [2,3]. Rat fed fructose in diet is a suitable model for non-obese rats with some aspects of the metabolic syndrome such as hypertension, hyperinsulinemia and hypertriglyceridemia [4,5]. It has been reported recently that AS-IV can increase insulin-induced preadipocyte differentiation, improve high glucose-induced insulin resistance in adipocytes and prevent tumor necrosis factor (TNF)-α-induced apoptosis in endothelial cells in-vitro [3,4,6]. However, it is not clear whether AS-IV can exert any beneficial effects on DM and, if so, the mechanisms involved. However, the protective effects of ASIV on DM have not been investigated yet.

Simvastatin belongs to the statin family, a class of drugs that competitively inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. This enzyme catalyzes the conversion of HMG-CoA to mevalonate, the rate-limiting step of the cholesterol synthesis pathway. Statins are frequently prescribed to prevent coronary heart disease and have been shown to exert this action even without a significant drop in blood cholesterol levels, suggesting anti-inflammatory properties independent of its cholesterol-lowering effects. The possible beneficial effect of simvastatin therapy on the beta cells destructive process in pancreatic islets has been analyzed in animal models, with inconsistent results. Most studies of simvastatin treatment in animal models of immune destruction of beta cells also observed some protection of beta cells or improved regeneration [7-9].

The aim of the present study was to examine the insulin expression on pancreatic islets under the treatment with AS-IV or statin. We...
extensively detected the expression levels of the proinflammatory cytokines for TNF-α and MCP-1 and examined the proliferation expression levels to explain the underlying mechanism of AS-IV or statin which are responsible for improving the diabetes-associated functional impairment on DM.

Materials and Methods

Materials and subjects

AS-IV, Simvastatin and STZ were purchased from Sigma (Germany); anti-insulin, anti-kI67, anti-PDX-1 and anti-CD34 antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The TNF-α ELISA kit and MCP-1 ELISA kit were purchased from R&D Systems (USA). All secondary antibodies were from Proteintech Group (USA). All animals’ procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, revised 1996) and approved by the institutional animal care and use committee at the Hubei University of Science and Technology, China.

Experimental animals and induction of experimental diabetes on rats

Forty-eight male 4-week C57/BL6 mice were purchased from Laboratory Animal Center. Before experiments, all animals were fed with basal diet for one week. Experimental diabetic mice were intraperitoneally injected with STZ at the dose of 60 mg/kg dissolved in 100 mmol/L citrate buffer pH 4.5. Blood glucose levels were measured 72h after STZ injection by tail vein puncture blood sampling. Mice which had blood sugar values under 11.6 mmol/L were re-injected with STZ. Diabetic mice were randomly separated into four groups and were treated with saline, simvastatin (3 mg/kg/day), AS-IV (20 mg/kg/day) or the combination of simvastatin and AS-IV for 8 weeks. All the animals were sacrificed for the following detection.

Immunohistochemistry and immunofluorescence

The pancreas was cut into 4-µm sections for immunostaining. To reduce the background staining intensity, sections were incubated with Tris-buffered saline (TBS, pH 7.6) containing 3% (wt/vol) BSA for 1h and were incubated with a mixture of primary antibodies at 4°C for overnight. For immunohistochemistry staining,, an adequate amount of second antibody was added, and the reaction was allowed to proceed at 37°C for 30 min after washing with TBS. After another washing in TBS, they were visualized by reacting with a simple stain dianaminobenzidine solution (Nichirei). The sections were counterstained with haematoxylin. Digital images of sections at a magnification of x40 were obtained and stitched using NIS-Elements software (NIKON), and The densities of positive cells for insulin, PDX-1 and CD34 staining in 10 random fields of each sample were quantified at 200-fold magnification by a digital microscope (BZ- 9000, KEYENCE, Japan). For immunofluorescence staining, a mixture of the second antibodies (FITC donkey anti-mouse IgG and TRITC donkey anti-rabbit IgG) was added and the reaction was allowed to proceed at 37°C for 30 min and was observed under the fluorescence microscopy. Nuclei counter-stained with Hoechst.

ELISA

The amounts of plasma TNF-α and MCP-1 in serum of diabetic mice were determined using commercially available ELISA-based assay systems. Assays were performed using the protocols recommended by the manufacturer.

RT-PCR

We performed RT-PCR as previously described [10,11]. Total RNA was isolated using Trizol-reagent (Molecular Research Center, USA) according to manufacturer’s instructions. Reverse transcription reactions were carried out by boiling 1 µg RNA with 1 µM oligod(T) [12-18] (Amersham Pharmacia, USA) and 20 U MMLV-RT (Epicentre, USA). PCR analysis was performed using 100 ng of the resulting cDNA for specific primers: PDX-1, forward 5’- CCGCGTACTAGTTTCTATCTTC-3’, reverse 5’- GGTGAAATCCCAAAAGCTCAC-3’, GADPH, forward 5’-CCACAGTCCATGCCATCCTG-3’, reverse 5’-CGCTGTGAAAGTGCAGGAGA-3’. PCR samples were analyzed by electrophoresis on 2% agarose-TAE gels and photographed using an Alphalnotech Chemilimager™ 4400 alpha imager (Imgen Technology, USA). The density of each band was quantified with the application Image J.59 program (Vilber Lourmat, France). PDX-1 mrRNA was expressed as a ratio to GAPDH. All results represented the average density of positive bands obtained from 3 independent experiments.

Statistical analysis

All results were presented as mean ± SEM of at least three independent experiments. Statistical significance was evaluated using the unpaired Student’s t test for comparisons between two means and was analyzed by ANOVA and post hoc Tukey’s test for multiple comparisons between groups. A probability value of P<0.05 was considered significant.

Results

The effect of AS-IV on the islets morphology and secreted insulin

The insulin staining in beta cells was used to evaluate the islets morphology and insulin content in pancreas. In the statin group and the AS-IV group, most islets were significantly enlarged and the insulin-positive cell for immunohistochemistry increased 8 fold and 20 fold, compared with the NC group (P<0.05), which indicated the increased insulin reserve in the satin group and the AS-IV group. Compared to the control group, as shown in Figure 1, the insulin-positive cells enhanced higher in the AS-IV and statin group (P<0.01).

The TNF-α and MCP-1 expression analyses in pancreatic tissues

TNF-α is a potent cytokine involved in inflammatory reactions and the elevated levels of TNF-α are seen in atherosclerotic plaques of diabetics [11]. The TNF-α protein analysis was examined by ELISA and decreased significantly in diabetic mice treated with either AS-IV or AS-IV and statin than in NC group (p<0.05) (Figure 2A). MCP-1 plays an important role in leukocyte recruitment to sites of infection and inflammation [12]. The MCP-1 serum level was reduced in mice treated with either AS-IV or AS-IV and statin (Figure 2B). These observations suggested that AS-IV might play the effect of anti-inflammation.

Cell proliferations of pancreatic islet cells

Proliferative cells in pancreatic tissue were determined by Ki67 immunostaining. Histological sections of the pancreatic islet were stained by antibodies specific for Ki67 and the proportion of positive cells was used to evaluate the proliferative effect of AS-IV upon pancreatic islets. As shown in Figure 3, the insulin-positive cells in the control group or the statin group were most and the Ki-67-positive cells were less. However, the Ki-67-positive cell for immunohistochemistry increased 8 fold and 20 fold, compared with the NC group (P<0.05), which indicated the increased proliferative effect of AS-IV upon pancreatic islets.

Citation: Li M, She T, Gan Y, Shao J, Li S, et al. (2015) Astragaloside IV and Statin Increased the Islet Function and Proliferation for Beta Cells in STZ-Induced Diabetic Mice. J Diabetes Metab 6: 611. doi:10.4172/2155-6156.1000611
The molecule PDX-1 is a key regulator in differentiation of beta-cell [13]. The PDX-1 mRNA expression was examined by RT-PCR and increased significantly in diabetic mice treated with either AS-IV or AS-IV and statin group (1.6 fold and 2.4 fold, respectively) than in the control group (Figure 4A). The PDX-1 protein expression of was detected by immunohistochemistry. As shown in Figure 4B, the expression of PDX-1 increased in AS-IV group also showed the significantly change compared with the NC group.

The CD34+ cell in the pancreatic islet cells

The transplantation of bone marrow-derived stem cell can restore beta-cell function in type 1 diabetes [14]. The bone marrow-derived stem cell retains its potential to induce pancreatic regeneration [15]. We examined the CD34 positive cells in diabetes to represent the regeneration. The CD34 positive cells in the pancreatic islet cells were analyzed by staining for CD34 (Figure 5A). The ratio of CD34 positive cells increased in the mice treated with AS-IV or AV-IV and statin AS-IV group. The Proliferating cell further increased after combined treatment with in the AS-IV and statin group.

The PDX-1 expressions in pancreatic tissues

The PDX-1 expressions in pancreatic tissues were examined by RT-PCR (Figure 4A). The PDX-1 mRNA expression was increased significantly in diabetic mice treated with either AS-IV or AS-IV and statin group (1.6 fold and 2.4 fold, respectively) than in the control group. The PDX-1 protein expression was detected by immunohistochemistry. As shown in Figure 4B, the expression of PDX-1 increased in AS-IV group also showed the significantly change compared with the NC group.

The CD34+ cell in the pancreatic islet cells

The transplantation of bone marrow-derived stem cell can restore beta-cell function in type 1 diabetes [14]. The bone marrow-derived stem cell retains its potential to induce pancreatic regeneration [15]. We examined the CD34 positive cells in diabetes to represent the regeneration. The CD34 positive cells in the pancreatic islet cells were analyzed by staining for CD34 (Figure 5A). The ratio of CD34 positive cells increased in the mice treated with AS-IV or AV-IV and statin AS-IV group. The Proliferating cell further increased after combined treatment with in the AS-IV and statin group.
Figure 4: The expression level of PDX-1 mRNA and protein in diabetes mice. (A): mice were treated with AS-IV or statin and the total RNA was extracted and RT-PCR was used to detect the PDX-1 and GAPDH mRNA expression. The PDX-1/GAPDH ratios were expressed relative to the mean value of control. (B): Beta-cells were detected by PDX-1 staining in pancreatic islets obtained from the mice after STZ-administered and treated with saline or statin, or AS-IV, or statin and AS-IV. The quantification of insulin positive cells ratio were expressed relative to the mean value of control. Data are expressed as mean±S.E.M. *p < 0.05 versus saline group. The experiment was repeated three times.

Figure 5: CD34 positive cell in pancreatic islets. (A): CD34 positive cells were detected by immunohistochemistry staining in pancreatic islets obtained from the mice after STZ-administered and treated with saline or statin, or AS-IV, or statin and AS-IV. (B): Quantification of CD34 positive cells. Data are expressed as mean±S.E.M. *p < 0.05 versus saline group, **p <0.05 versus other groups (n = 8). Abbreviations: Ctrl, Control; AS-IV, astragaloside IV.
(1.7 fold and 2.3 fold, respectively) in comparison with the NC group (p <0.05). The combined administration of AS-IV and statin group resulted in more CD34 positive cells compared with the other groups (p<0.05) (Figure 5B).

**Discussions**

In this study, we found that AS-IV and statin increased the secret of insulin and improved beta-cell function in STZ-induced diabetic mice. The present results demonstrated that AS-IV and statin preserved beta-cell function though anti-inflammation and proliferations of pancreatic islet cells. This increased the PDX-1 gene expression in STZ-induced diabetic mice.

Pancreatic beta-cell dysfunction and insulin resistance are the keys of type 2 diabetes [12]. We examined the insulin expression in diabetic mice by the immunohistological staining of pancreatic islets. Our results showed that AS-IV and statin increased the expression of insulin and enlarged the pancreatic islet which contained the secret of beta-cell and indicated that AS-IV and statin directly affected beta-cell proliferation and secret. The effect of AS-IV or statin was also reported previously showing that AS-IV or statin enhanced pancreatic beta-cell secreted through increased beta-cell replication [16].

Inflammation was a key pathophysiological mechanism in the development of diabetes and the important etiological factor in the development of insulin resistance [17]. The content of the inflammatory cytokines TNF-α and MCP-1 showed in islets of insulin resistance rats, which was increased after STZ treatment and indicated obvious inflammation damage islets. Many reports have shown that AS-IV has anti-inflammatory effect. In this study, the expression of TNF-α in AS-IV and statin group was significantly lower than that in the control group, suggesting that AS-IV may affect the resistance to STZ-induced diabetes partially through the anti-inflammatory effect [18].

Cell proliferations could resistance to the apoptosis cells which was one of the main reasons for the loss of islet function in diabetic mice [19]. STZ increased the apoptosis of islet beta cells in the previous reports. In our study we found that the cell proliferation signal increased in the AS-IV or statin group which indicated the cell proliferation could be induced by AS-IV or statin. Our results also suggested that AS-IV and statin may inhibit the apoptosis of beta cells, which may explain partly the insulin secret and the pancreatic islet function in the AS-IV or statin group.

Pancreatic duodenal homeobox 1 (PDX-1), a homeodomain-containing transcription factor, is a crucial regulator for pancreatic development [13]. Previous studies showed that PDX-1 is a direct activator of several β-cell-specific genes, such as insulin, glucokinase, Glut2 and Nkx6.1 [20]. Hyperglycemia has previously been associated with decreased expression of PDX-1 in β-cells. In our study we found that the expression of PDX-1mRNA and protein increased in the AS-IV group which indicated that AS-IV might stimulate the PDX-1 expression. These results might explain the increased insulin expression in beta cells and the enhanced pancreatic islet function in the AS-IV or statin group.

Simvastatin treatment lowered the incidence of diabetes in the autoimmune diabetic NOD mouse model in one out of three studies [21]. Treatment with simvastatin prolonged survival of islets transplanted to NOD mice [22]. Our results showed that treatments with statins increased the islets function and decreased inflammatory reactions and immune activation.

**Conclusion**

In summary, our research revealed that AS-IV and statin enhanced the survival and function of beta-cell in diabetic mice. AS-IV improved the islet morphology and function through the proliferation and anti-inflammation. So we suggested that AS-IV may offer the protective effect on islets morphology and function in diabetes, and providing the potential drugs discovery for diabetes patients.

**Acknowledgments**

This work was supported by the grants from the Fund of Hubei Province Educational Department (Q20122805) and the Fund of Hubei University of Science and Technology (ZX1125 and ZX1306).

**References**

17. Evans JL, Goldfine ID, Maddux BA, Grodsky GM (2003) Are oxidative stress-


