

Expression of heat shock protein 90 in the kidneys of diabetic db/db mice

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Abstract. – OBJECTIVE: To identify novel genes regulated in diabetic nephropathy.

MATERIALS AND METHODS: Total RNA from the renal cortex of db/+ and db/db mice was isolated and DNA microarrays specific for diabetes signaling pathways were used for expression profiling. Expression of mRNA and protein was determined by RT-PCR and western blotting. The terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick-end labeling (TUNEL) assay and immunohistochemical staining were assessed in renal cortex of db/db mice.

RESULTS: Microarray data revealed that 7 genes show up- or down-regulated pattern and diabetic mice specifically decreased heat shock protein (Hsp) 90 α expression of genes compared to control mice (diabetic mice 0.68 vs. control mice 1 relative density). Expression of Hsp90 α mRNA and Hsp90 protein was significantly decreased in the renal cortex of diabetic mice. However, Hsp70 mRNA and protein expression was not changed. Apoptosis was increased in glomeruli of diabetic mice due to increased expression of cleaved caspase-3 and Bax.

CONCLUSIONS: Our results suggest that Hsp 90 expression was decreased in diabetic glomeruli and decreased Hsp90 expression may mediate podocyte apoptosis in type 2 diabetic kidneys.

Key Words:

Hsp90 (heat-shock proteins), Renal cortex, Apoptosis, db/db mice.

basement membrane thickening, mesangial expansion and hypertrophy, and the accumulation of extracellular matrix (ECM) components¹.

A variety of experimental animal models of diabetic kidney disease induced hyperglycemia are used as models of human diabetes. The db/db mouse, a type 2 diabetic model, develops progressive hyperglycemia and glomerular hypertrophy. In renal cortical species and glomeruli, there is increased gene expression and protein synthesis of several ECM components, such as collagen, laminin, and fibronectin²⁻⁴. The pathogenesis of diabetic nephropathy still remains to be ascertained. In this article, we demonstrate novel mRNA changes in the renal cortex of type 2 diabetic animals using microarray technology, a tool that may reveal new therapeutic targets for treatment of diabetes and diabetic microvascular complications. During microarray profiling, we observed a decrease in heat shock protein (Hsp) 90 α in the renal cortex from kidneys of db/db mice.

Hsps are molecular chaperones that account for more than 1-2% of all cellular protein and play a key role in cytoprotection. Specifically, Hsps help to prevent denatured protein refolding, nonspecific protein assembly, and interfere with pro-apoptotic pathways. Hsps are grouped according to their molecular weight: 110, 90, 70, 60, 40 kDa, and low molecular weight families⁵. The Hsp90 family consists of 17 genes classified into three classes: Hsp90A, Hsp90B, and tumor necrosis factor receptor-associated protein. The Hsp90A family is localized in the cytoplasm and can be further sub-divided into Hsp90AA (inducible) and Hsp90AB (constitutive), yielding Hsp90 α and Hsp90 β proteins, respectively⁶. Hsp90 forms either homo or heterodimers of the α and β isoforms which then bind protein substrates that are unfolded and/or misfolded to assist in folding and prevent aggregation⁷. Hsp90 α plays a role in modulating signal transduction, es-

Introduction

Diabetes is a metabolic disorder of multiple causes characterized by chronic hyperglycemia and is responsible for 30-40% of all end-stage renal disease. Diabetic nephropathy is the leading cause of chronic renal failure and one of the most common long-term complications in patients with type 1 or type 2 diabetes mellitus. It is characterized by persistent albuminuria, glomerular

pecially in tumor cells, and has become a novel therapeutic target in cancer therapy⁸. Little is known about the expression of Hsp90 in diabetic nephropathy in the type 2 animal model. In this study, we investigated that Hsp90 and apoptotic factor expressions in the glomeruli of type 2 animal model.

Materials and Methods

Animals

Male C57BL/KSJ-db/db mice and C57BL/KSJ-db/+ age-matched control mice were used (SLC, Japan) at 5 weeks of age. The experiments conformed to standard environmental conditions (room temperature ~23°C and humidity ~60%). Mice were divided into two groups of 8-10 individuals: (1) db/+ mice and (2) db/db mice. After 13 weeks, mice were anesthetized with diethyl ether and whole kidney samples were taken for RNA isolation and histological examination. All animal procedures were approved by the Committee on Animal Care at our Institute and were carried out according to institutional guidelines.

Total RNA Preparation and Microarray Experiments

Total RNA from the renal cortex of db/+ and db/db mice was isolated and purified (RNeasy Mini kit; Qiagen, Valencia, CA, USA). RNA quality was determined by the $A_{260}:A_{280}$ absorbance ratio (> 2.0) and the $A_{260}:A_{230}$ absorbance ratio (> 1.7). Diabetes Oligo-GEarray[®] (OMM-023) was used for expression profiling (SuperArray Bioscience Corporation, Frederick, MD, USA) in conjunction with the TrueLabeling-AMP linear RNA amplification kit, according to the manufacturer's instructions. Expression profiles from array experiments were analyzed using the GEArray expression analysis program.

Semi-quantitative RT-PCR for Hsp90 α and 70 Gene Expressions

For RT-PCR, cDNA was synthesized with 1 μ g RNA using RT-premix (Bioneer, Daejeon, Korea). PCR primer sequences were: mouse Hsp90 α , 5-ATG ACA GCG GCA AAG ACA AG-3 and 5-AGG TCC TCG GAG TCA ACC AC-3; mouse Hsp70, 5-GCG ACC TGA ACA AGA GCA TC-3 and 5-GAG CTT GCC CTT GAG ACC C-3; and mouse β -actin, 5-ATG GAT GAT ATC ATC GCC GC-3 and 5-TGA CCA

CGG ACC CCG CGG-3. PCR products were analyzed by agarose (1.2%) gel electrophoresis along with DNA molecular markers, stained with ethidium bromide, and visualized under UV light. RT-PCR products were separated by electrophoresis, and the intensities of the DNA bands in the agarose gels were quantified by densitometry (Las-3000, Fuji photo).

Western Blot Analysis

Western blotting was performed using a previously described method^{9,10}. Equal amounts of protein (25-50 μ g/lane) were subjected to immunoblotting with the indicated antibodies. The antibodies used were as follows: Hsp90 (1:2,000, Abcam), Hsp70 (1:2,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), caspase 3 (1:1,000, Cell Signaling Technology Inc., Beverly, CA, USA), and β -actin (1:2000). The bound horseradish peroxidase-conjugated secondary antibody was detected using an enhanced chemiluminescence detection system (iNtRON Biotechnology, SeongNam-Si, Korea). Protein expression levels were determined by analyzing the signals captured on the nitrocellulose membranes using an image analyzer (Las-3000, Fuji-film, Tokyo, Japan).

Terminal Deoxynucleotidyl Transferase-Mediated Biotinylated UTP Nick-End Labeling (TUNEL) Assay

The TUNEL assay was performed with DAPI (4', 6-diamidino-2-phenylindole) staining¹¹. The prepared tissue sections were deparaffinized in xylene for 10 min and hydrated through a graded ethanol series. The TUNEL assay was then carried out according to the manufacturer's instructions (Roche Diagnostics, Meylan, France).

Immunohistochemical Staining

Mouse kidney was preserved in 4% paraformaldehyde at room temperature for 24 hr, embedded in paraffin, and sectioned (3 μ m). Paraffin sections were deparaffinized, hydrated with water, and then stained with hematoxylin and either eosin as a counterstain for advanced glycation end (AGE) products (6D12, 1:250, TransGenic Inc., Kumamoto, Japan) or DAPI as a counterstain for caspase-3 (1:300, Cell Signaling Technology Inc.). The sections were incubated with fluorescein isothiocyanate-labeled anti-mouse immunoglobulin G (IgG; 1:200) and Texas red-labeled anti-rabbit IgG (1:200) for 30 min at room

temperature. Immunoreactivity was examined under an Olympus fluorescence microscope (BX41, Olympus, Tokyo, Japan).

Data Analysis

Data are expressed as the mean $\pm$ standard error of the mean (SEM) of multiple experiments. Unpaired Student's *t*-tests were performed to compare two groups using PRISM software (Graph Pad, San Diego, CA, USA). Values of *p* < 0.05 were considered statistically significant.

Results

Body Weight and Characteristic Parameters

Body weight and blood glucose were significantly higher in diabetic mice compared with control mice. Proteinuria, total cholesterol, low-density lipoprotein-cholesterol, and free fatty acids were also elevated in diabetic mice (Table I). However, kidney weight was not changed in diabetic mice compared with control mice.

Decreased Expression of Hsp90 α in the Renal Cortex of Diabetic db/db Mice

To reveal novel genes regulated in diabetic nephropathy, we performed gene expression profiling of the renal cortex of diabetic db/db mice using microarrays specific for diabetes signaling pathways (Figure 1). In diabetic renal cortex, Hsp90 α expression was decreased compared to control mice (yellow circle). Table II shows the up or down-regulated gene patterns. Follow-up validation of our microarray data demonstrated that diabetic mice specifically decreased Hsp90 α ex-

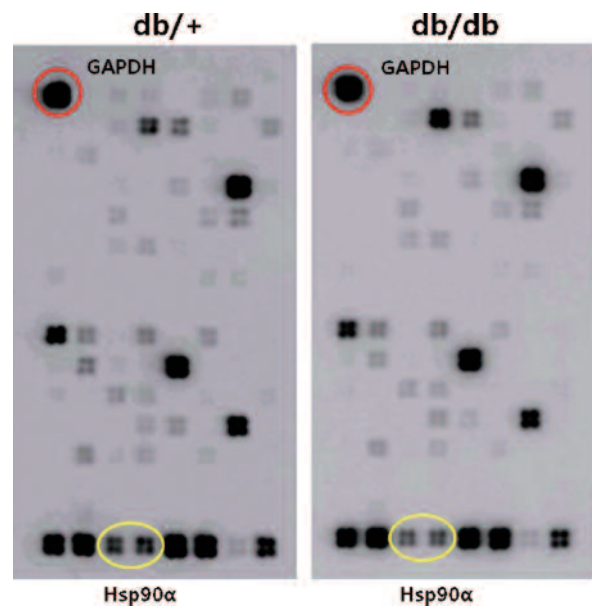


Figure 1. Profiling of genes from the renal cortex of diabetic db/db mice. DNA array profiling shows the differentially expressed genes in the renal cortex of diabetic db/db mice (right panel) and db/+ mice as a control (left panel).

pression compared to control mice (diabetic mice 0.68 vs. control mice 1, relative density). Figure 2A and B show that expression of Hsp90 α mRNA and Hsp90 protein was significantly decreased in the renal cortex of diabetic mice. Next, to assess the change in Hsp isoforms, we analyzed Hsp70 mRNA and protein expression in the renal cortex of diabetic mice using western blot and RT-PCR (Figure 3). Expression of Hsp70 mRNA was not changed and, although Hsp70 protein showed a tendency to decrease, it was not significant.

Table I. Body weight and characteristic parameters.

	db/+ (n = 8)	db/db (n = 8)
Body weight (g)	30.53 $\pm$ 0.53	41.77 $\pm$ 4.56**
Blood glucose (mg/dl)	132.4 $\pm$ 11.44	600.0 $\pm$ 69.32***
Right kidney weight (g/100 g body weight)	5.78 $\pm$ 0.24	5.13 $\pm$ 0.71
Left kidney weight (g/100 g body weight)	6.11 $\pm$ 0.72	5.19 $\pm$ 0.73
Proteinuria (mg/kg/day)	615.1 $\pm$ 76.01	1423 $\pm$ 390.2*
Total cholesterol (mg/dl)	113.7 $\pm$ 3.82	194.9 $\pm$ 19.51*
HDL-cholesterol (mg/dl)	61.96 $\pm$ 2.70	70.10 $\pm$ 7.49
LDL-cholesterol (mg/dl)	5.10 $\pm$ 0.42	8.48 $\pm$ 1.22*
FFA (μ Eq/L)	0.421 $\pm$ 0.07	2.759 $\pm$ 0.489**

HDL: high-density lipoprotein; LDL: low-density lipoprotein; FFA: free fatty acids.

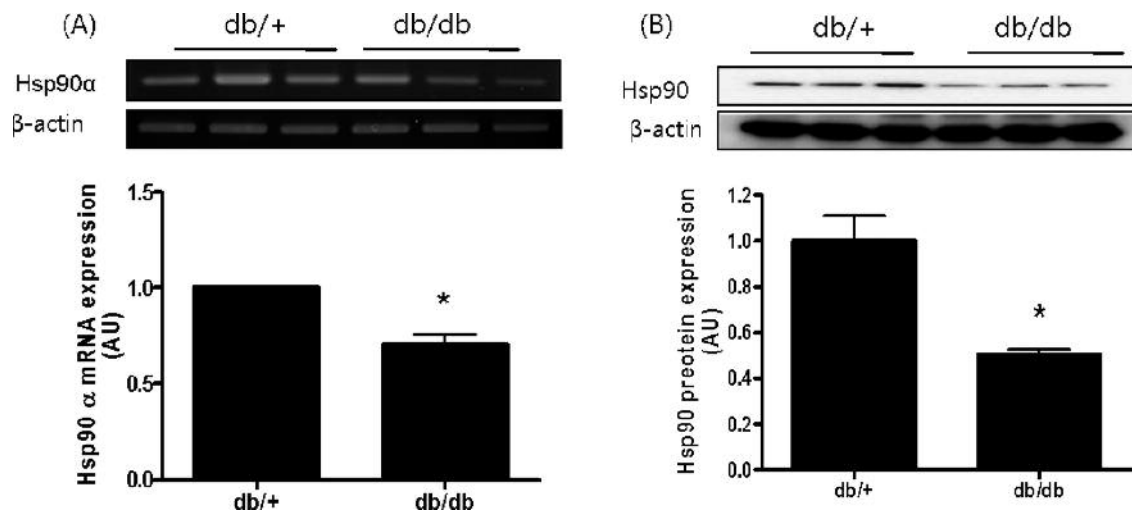


Figure 2. Decreased expression of Hsp90α in the renal cortex of diabetic db/db mice. **A**, Expression of Hsp90α mRNA was analyzed by RT-PCR. Total RNA was isolated from the renal cortex of diabetic db/db mice. **B**, Expression of Hsp90 protein was analyzed by western blot. All data are expressed as the mean ± SEM (n=3). * $p < 0.05$ vs. db/+ mice.

Increased Apoptotic Cells in the Glomeruli of Diabetic Mice

Previous studies have suggested that Hsps can prevent pro-apoptotic signalling and apoptosis¹². Therefore, to assess apoptotic factors and apoptosis in the glomeruli of diabetic mice, apoptosis was examined by the TUNEL assay. Expression of cleaved caspase-3 and Bax, which is a pro-apoptotic Bcl-2 family protein,

was analyzed by immunohistochemistry and western blot. The apoptosis detection system revealed positive staining only in the renal cortex of diabetic mice (Figure 4A, green). However, synaptopodin, podocyte marker, was decreased in the glomeruli of diabetic mice (Figure 4B). Cleaved caspase-3 and Bax were increased in the glomeruli of diabetic mice compared to controls (Figure 4C).

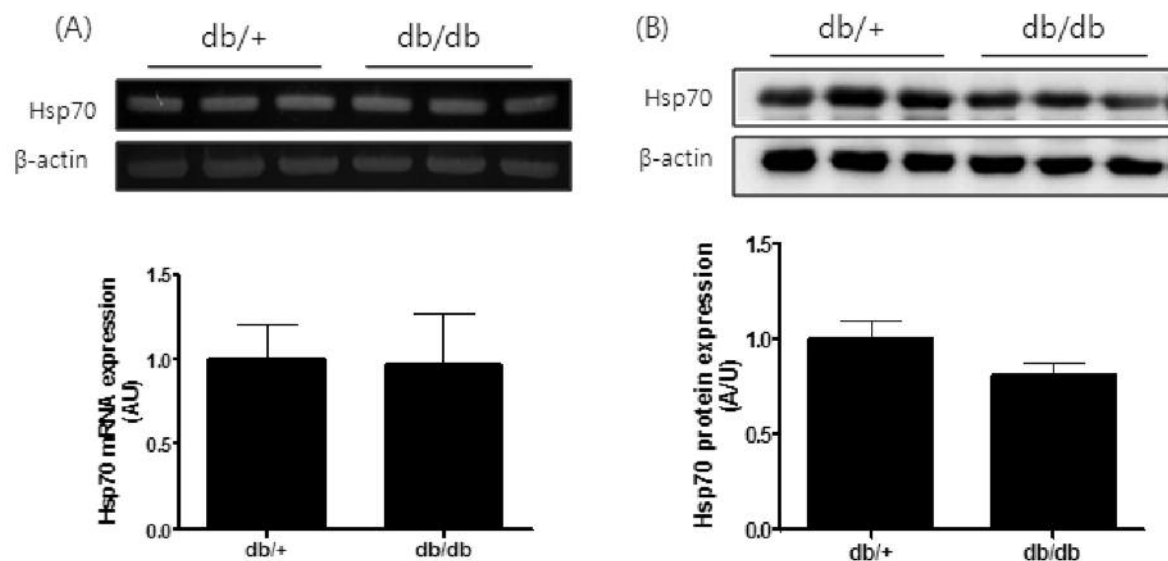


Figure 3. Expression of Hsp70 in the renal cortex of diabetic db/db mice. **A**, Expression of Hsp70 mRNA was analyzed by RT-PCR. Total RNA was isolated from the renal cortex of diabetic db/db mice. **B**, Expression of Hsp70 protein was analyzed by western blot. All data are expressed as the mean ± SEM (n = 3).

Table II. The list of genes showing up- or down-regulated pattern.

Gene accession no	Name	Ratio of expression to GAPDH 1.0
NM_009696	Apolipoprotein E	1.58
NM_010292	Glucokinase	1.26
NM_010938	Nuclear respiratory factor 1	1.12
NM_008928	MAPK kinase 3	1.08
NM_013671	Superoxide dismutase 2, mitochondrial	0.84
NM_008509	Lipoprotein lipase	0.76
NM_008302	Heat shock protein 90 kDa ab 1	0.68

MAPK: mitogen-activated protein kinase; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

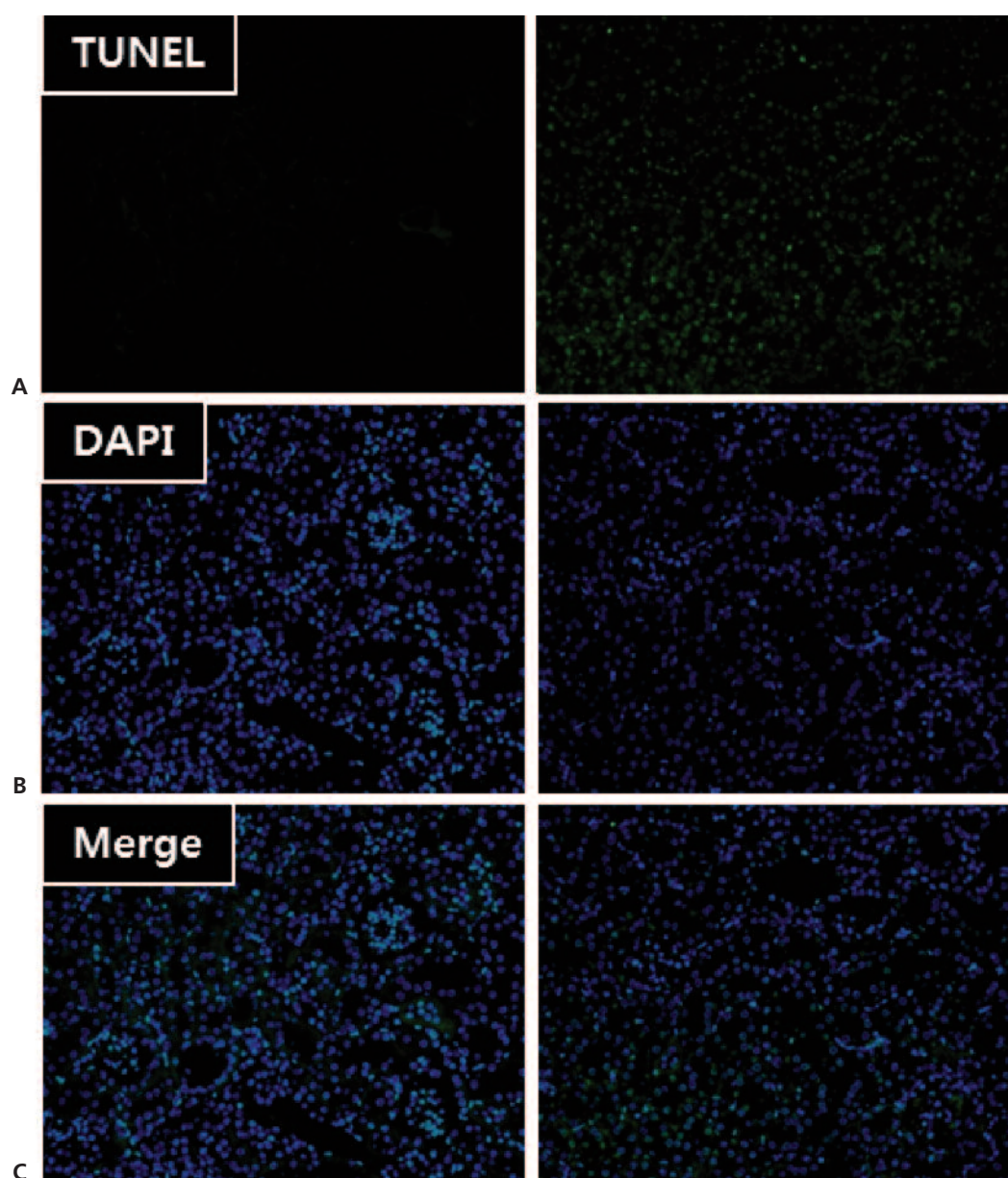


Figure 4. TUNEL assay and expression of caspase3 and Bax in the renal cortex of diabetic db/db mice. **A,** Representative photographs showing apoptotic cells by TUNEL assay in db/+ and db/db mice. Merged images of DAPI and TUNEL (lower panels). **B,** Paraffin section of kidney immunolabeled with caspase3 and synaptopodin from db/+ and db/db mice. **(C)** Expression of Bax and cleaved caspase3 proteins was analyzed by western blot.

Discussion

The microarray is a tool that can be used to elucidate new therapeutic targets for the treatment of diabetes and diabetic microvascular complications¹³. By microarray profiling, we noted that Hsp90 α mRNA expression was decreased in the renal cortex from kidneys of db/db mice (Table II). Furthermore, apolipoprotein E (apoE) mRNA, a well-known player in lipid metabolism, was increased in renal cortex of db/db mice (diabetic mice 1.58 vs. control mice 1 relative density). A previous study showed that cholesterol and apoE levels are exaggerated in the plasma of db/db mice compared with control mice¹⁴. ApoE4, one of apoE isoforms, is a predictor of diabetic nephropathy progression, as apoE is associated with the development of diabetic nephropathy¹⁵ and¹⁶. However, the association between Hsp 90 and renal damage of type 2 diabetic mice hasn't been reported. The Hsp90 family comprises of Hsp90 α and Hsp90 β , which form an inactive complex with the steroid hormone receptor before agonist binding and, therefore, participate in steroid hormone signaling¹⁷. Furthermore, Hsp90 is an abundant cytosolic molecular chaperone involved in maturation and conformational stabilization of proteins, most of which are involved in transducing proliferative and survival signals^{6,12}. Inhibition of Hsp90 has been reported to induce apoptosis in cancer cells, suggesting Hsp90 as a novel target for cancer therapy^{7,18}. Previous study showed that Hsp90 is only highly expressed in renal medulla and glomeruli, but expression levels are similar in STZ-induced type 1 diabetic rats and control rats¹⁹. They have shown the Hsp90 protein levels using immunohistochemical staining. Although the loss of glomerular cells (especially podocytes) by apoptosis has been viewed as an early phenomenon triggering the initiation of glomerular lesions²⁰, there is no Hsp90 expression difference in STZ-induced type 1 diabetic rats and control rats¹⁹. Our findings showed that Hsp90 α mRNA and Hsp90 protein in the glomeruli were decreased by RT-PCR and western blot analysis (Figures 1 and 2). The glomerular cell death shown in type 2 diabetic mice indicates that apoptosis is a central feature of experimental diabetic nephropathy, in keeping with previous reports from other researchers²⁰. Next, to assess apoptotic factors and apoptosis in the glomeruli of diabetic mice, apoptosis was examined by the TUNEL assay. As shown in Figure 4, significant apoptosis was detected in glomeruli of db/db mice as as-

sessed by TUNEL assay and caspase-3 cleavage. Expression of synaptopodin, podocyte marker, was decreased in the glomeruli of diabetic mice compared with control mice (Figure 4B). The pro-apoptotic factor Bax and cleaved caspase-3 also increased in the cortex of db/db mice, as shown by western blot (Figure 4C). Bax-induced caspase activation and apoptosis via cytochrome c release from mitochondria is inhibitable by Bcl-xL²¹. The coordinated activities of the Hsps modulate multiple events within apoptotic pathways to help sustain cell survival following damaging stimuli¹².

Conclusions

This study demonstrates for the first time that Hsp90 α mRNA and Hsp90 protein decrease in the renal cortex of db/db mice. Increased apoptosis is observed in the glomeruli of diabetic mice, mediated by an increase in expression of cleaved caspase-3 and Bax. In this study, we suggest that decreased expression of Hsp90 may mediate podocyte apoptosis in the type 2 diabetic kidney.

Acknowledgements

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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