HSP27/HSPB1 as an adaptive podocyte antiapoptotic protein activated by high glucose and angiotensin II

Maria D. Sanchez-Niño PhD1, Ana B. Sanz PhD2, Elsa Sanchez-Lopez PhD4, Marta Ruiz-Ortega PhD3, Alberto Benito-Martin1, Moin A. Saleem MD, PhD5, Peter W. Mathieson MD, PhD5, Sergio Mezzano MD7, Jesus Egido MD, PhD1,3,6, Alberto Ortiz MD, PhD1,3,6*

1 Nefrología, IIS-Fundación Jiménez Díaz, Madrid, Spain
2 Servicio de Nefrología, Fundacion para la Investigacion Biomedica del Hospital Universitario La Paz, Madrid, Spain
3 Universidad Autonoma de Madrid
5 Academic and Children's Renal Unit, University of Bristol, Bristol, U.K
6 Instituto Reina Sofia de Investigaciones Nefrológicas-IRSIN, Madrid, Spain
7 Nefrología, Universidad Austral, Valdivia, Chile

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* Correspondence should be addressed to:
Alberto Ortiz, MD, PhD
Dialysis Unit
Fundación Jiménez Díaz
Avd. Reyes Católicos 2
28040 Madrid,
Spain
E: aortiz@fjd.es
T: 34 91 5504940
F: 34 91 5442636
ABSTRACT

Apoptosis is a driving force of diabetic end organ damage, including diabetic nephropathy. However, the mechanisms that modulate diabetes-induced cell death are not fully understood. Heat-shock protein 27 (HSP27/HSPB1) is a cell stress protein that regulates apoptosis in extrarenal cells and is expressed by podocytes exposed to toxins causing nephrotic syndrome. We investigated the regulation of HSPB1 expression and its function in podocytes exposed to factors contributing to diabetic nephropathy, such as high glucose and angiotensin II. HSPB1 expression was assessed in renal biopsies from patients with diabetic nephropathy, minimal change disease or focal segmental glomerulosclerosis, in a rat model of diabetes induced by streptozotocin and in angiotensin II-infused rats. The regulation of HSPB1 was studied in cultured human podocytes and the function of HSPB1 expressed in response to pathophysiologically relevant stimuli was explored by short interfering RNA (siRNA) knock-down.

Total kidney HSPB1 mRNA and protein expression was increased in rats with streptozotocin-induced diabetes and in rats infused with angiotensin II. Upregulation of HSPB1 protein was confirmed in isolated diabetic glomeruli. Immunohistochemistry showed increased glomerular expression of HSPB1 in both models and localized glomerular HSPB1 to podocytes. HSPB1 protein was increased in glomerular podocytes from patients with diabetic nephropathy or focal segmental glomerulosclerosis.

In cultured human podocytes HSPB1 mRNA and protein expression was upregulated by high glucose concentrations and angiotensin II. High glucose, but not angiotensin II, promoted podocyte apoptosis. HSPB1 siRNA targeting increased apoptosis in a high-glucose milieu and sensitized to angiotensin II or TGFβ1-induced apoptosis by promoting caspase activation.

In conclusion, both high glucose and angiotensin II contribute to HSPB1 upregulation. HSPB1 upregulation allows podocytes to better withstand an adverse high-glucose or angiotensin II-rich environment, such as can be found in diabetic nephropathy.

Abbreviations

Ang: Angiotensin
DAPI: 4',6-Diamidino-2-phenylindole dihydrochloride
DN: Diabetic nephropathy
DNA: Deoxyribonucleic acid
ECL: Enhanced chemiluminescence
FSGS: Focal Segmental Glomerulosclerosis
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
HSPs: Heat shock proteins
ITS: Insulin, transferrin, selenite
MAPK: Mitogen-Activated Protein Kinase
MCD: Minimal change disease
PCR: Polymerase chain reaction
RAS: Renin angiotensin system
RNA: Ribonucleic acid
siRNA: Small interfering RNA
STZ: Streptozotocin
SV40: Simian virus 40

Key words: apoptosis, diabetes, HSP27, kidney, podocytes,
INTRODUCTION

Diabetic nephropathy (DN) is the most frequent cause of end-stage renal disease. The diabetic milieu induces apoptosis in various end-organ systems and contributes to the gradual loss of renal function and mass in DN (1-3). Studies in humans and DN animal models reveal podocyte injury, detachment, apoptosis, and loss (4-8). Although some stimuli induce necrosis of podocytes in culture (9), there is more in vivo evidence for a role of apoptosis in podocyte loss in DN (10-12). More specifically, podocyte apoptosis coincided with the onset of albuminuria and preceded podocytopenia in different mouse models of diabetes (13). Podocyte apoptosis has also been documented early in the course of kidney disease in patients with type 2 diabetes (14). The molecular pathways contributing to podocyte injury in DN include, but are not limited to, high glucose and activation of the local renin angiotensin system (RAS) (15). Angiotensin (Ang) II is one of the major effector molecules of the RAS and is the main target of established therapy for clinical DN (15). Intraglomerular Ang II levels are increased in DN (16), podocytes express Ang II receptors (17) and local glomerular RAS directly promotes filtration barrier injury (15). Ang II promoted apoptosis of cultured rat and mouse podocytes (18-20). However, Ang II-induced apoptosis in human podocytes is not well characterized.

Cells respond to environmental stressors by rapidly synthesizing protective proteins such as heat shock proteins (HSPs) (21;22). HSPs are ubiquitous, highly evolutionary conserved intracellular proteins categorized according to their molecular weight (23;24). HSPs have chaperone-like activity and protect proteins from damage induced by environmental factors, such as free radicals, heat, ischemia and toxins, allowing denatured proteins to adopt their native configuration. HSPB1 is a member of the small HSP family of proteins with a molecular weight of approximately 27 kDa (25). HSPB1 controls a wide range of biological activities, including actin stabilization and protection from stress and apoptosis (25-30). HSPB1 may be of particular interest in podocyte injury since its expression is induced by the podocyte toxin puromycin aminonucleoside (PAN) (31). Protective cellular responses in podocytes are poorly understood. HSPB1 regulates actin microfilament dynamics in podocyte foot processes (31). In addition, enhanced podocyte HSPB1 expression/phosphorylation has been reported in experimental nephrotic syndrome induced by the podocyte toxin PAN (31) suggesting a role for HSPB1 in the pathophysiological changes of the podocyte cytoskeleton during the development of proteinuria. However, there is an incomplete understanding of the relationship between podocyte HSPB1 and stressors relevant to DN, such as high glucose and angiotensin II.
The present study was designed to assess podocyte HSPB1 expression in vivo during kidney injury induced by diabetes or following Ang II infusion and the role of HSPB1 on apoptosis in cultured human podocytes exposed to either high glucose or Ang II. Our results indicate that HSPB1 upregulation is a compensatory protective mechanism that prevents podocyte apoptosis in the presence of Ang II or a high glucose milieu.

**MATERIAL AND METHODS**

**Cell culture and reagents**

Human podocytes are a previously described cell line (32) transfected with a temperature-sensitive SV40 gene construct and a gene encoding the catalytic domain of human telomerase. At a permissive temperature of 33°C, the cells remain in an undifferentiated proliferative state, whereas raising the temperature to 37°C results in growth arrest and differentiation to the parental podocyte phenotype. Undifferentiated podocyte cultures were maintained at 33°C in RPMI 1640 medium with penicillin, streptomycin, ITS (Insulin, transferrin, selenite), and 10% FCS. Once cells had reached 70 to 80% confluence, they were cultured at 37°C for at least 14 days before use, when full differentiation and nephron expression had taken place (5). Experiments were performed in differentiated cells. For experiments cells were cultured in serum-free media 24 hours prior to addition of stimuli and throughout the experiment. For high glucose experiments, glucose was added in the media to reach a final concentration of 700 mg/dl versus control media with 200 mg/dl glucose. The same amount of mannitol was added as an osmolarity control. AngII (10⁻⁷ mol/L) was added each day (Bachem). Human recombinant TGF-β1 (Peprotech, London, UK) was used at a concentration of 10 ng/mL.

**Animal models**

Two groups of ten 10-week-old Wistar Kyoto rats (Crifìa, Barcelona, Spain) were studied. Diabetes was induced by a single intraperitoneal 50 mg/Kg streptozotocin (STZ) (Sigma, St. Louis, MO) injection (5). Control rats received the STZ vehicle (0.01 M citrate buffer pH 4.5). Rats were sacrificed at 7 months following induction of diabetes (5). Insulin (1-4 IU s.c., Insulatard NPH, Novo Nordisk, Denmark) was administered weekly so as to prevent death, but not with the aim of totally correcting hyperglycemia. Insulin administration was initiated 7 days after STZ, having checked that glycemia was >400 mg/dl (Glucocard, Menarini, Barcelona, Spain). Systolic blood pressure was measured monthly in
conscious, restrained rats by the tail-cuff sphygmomanometer (NARCO, Biosystems, CO). Albuminuria was measured by ELISA (Celltrend, Luckenwalde, Germany). (Supplemental Table 1).

In an independent experiment, two groups of 10-week-old Wistar Kyoto rats (Crippa) were studied. Diabetes was induced by a single intraperitoneal 50 mg/Kg streptozotocin injection. Rats were sacrificed at 6 weeks following induction of diabetes. At that time albuminuria was already increased (2310 ± 400 µg/24h vs 440 ± 300 µg/24h in controls, p<0.05).

AngII 100 ng/kg/min was systemically infused by subcutaneous osmotic minipumps (Alza Corp., CA) for 24 hours to 2 weeks to 3-month-old male Wistar rats (n=4-8 animals per group). A control group of same age saline-infused rats was also studied (n=8 animals).

Rats were killed and kidneys perfused in situ with cold saline before removal. One kidney was fixed in buffered formalin, embedded in paraffin and used for immunohistochemistry, and the other kidney was snap-frozen in liquid nitrogen for RNA and protein studies of renal cortexes. All experimental procedures were approved by the Animal Care and Use Committee of our Institution, according to the guidelines for ethical care of the European Community.

Immunohistochemistry and immunofluorescence

Immunohistochemistry and immunofluorescence were carried out in paraffin-embedded rat kidney sections 5 µm thick (33). The primary antibodies were goat polyclonal anti-HSPB1 (1:60, Santa Cruz Biotechnology) and FITC-mouse anti-synaptopodin (1:10, Progen, Heidelberg, Germany). Secondary HRP- and phycoerythrin-conjugated antibodies were used for HSPB1. For immunohistochemistry sections were counterstained with Carazzi’s hematoxylin. Negative controls included incubation with a non-specific immunoglobulin of the same isotype as the primary antibody.

Western blot

Western blots were performed as described previously (34). Membranes were incubated overnight at 4°C with rabbit polyclonal anti-HSPB1 antibody (1:500; Santa Cruz Biotechnology), mouse monoclonal anti-HSP70 and anti-HSP47 (1:1000, SPA-810, Stressgen), rabbit polyclonal anti–cleaved caspase-3 (1:1,000; Cell Signaling, Hertfordshire, U.K.), or mouse anti-tubulin monoclonal antibody (1:5000, Sigma, St. Louis, MO) followed by incubation with horseradish peroxidase-conjugated secondary antibody (1:2,000, Amersham, Aylesbury, UK). Blots were developed with the enhanced chemiluminescence method (ECL) following the manufacturer’s instructions (Amersham).
Isolation of glomerular protein

Kidneys from 7-month diabetic rats and their control were harvested, decapsulated, and macerated through three sieves (150, 106, and 63 µm). Tissue trapped by the 63 µm sieve was washed with PBS and centrifuged at 4°C for five minutes. The pellet was resuspended in PBS and examined under phase contrast microscopy. The preparation contained >95% glomeruli with minimal tubular contamination. Then, Western blot was performed as described previously.

Real time reverse transcription-polymerase chain reaction

RNA was isolated by Trizol (Invitrogen, Paisley, UK). One µg of RNA was reverse transcribed with High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Real-time PCR reactions were performed on an ABI Prism 7500 sequence detection PCR system (Applied Biosystems) according to manufacturer's protocol using the DeltaDelta Ct method (35). Expression levels are given as ratios to GAPDH. Pre-developed primer and probe assays (PDAR) were obtained for human GAPDH and HSPB1 from Applied Biosystems.

Confocal microscopy

Cells plated onto Labtek™ slides were fixed in 4% paraformaldehyde and permeabilized in 0.2% Triton X-100 in PBS for 10 min each. After washing in PBS cells were incubated overnight at 4°C with goat polyclonal anti-HSPB1 antibody (1:50, Santa Cruz) followed by incubation with anti-goat Alexa Fluor 488 (1:300, Invitrogen). Cell nuclei were counterstained with DAPI (Vector Laboratories, Inc., Burlingame, CA) to observe the typical morphological changes. After washing, cells were mounted in 70% glycerol in PBS, and analysed with a DM-IRB confocal microscope (Leica DM, Bannockburn, IL) (36).

Cell death and apoptosis

Cells were cultured to subconfluence in twelve-well plates. Apoptosis was assessed by functional and morphological studies. Flow cytometry of DNA content was used to quantitate apoptosis. Adherent cells were pooled with spontaneously detached cells, and stained in 100 µg/mL propidium iodide, 0.05% NP-40, 10 µg/mL RNAse A in PBS and incubated at 4°C for >1 hour. This assay permeabilizes the cells. Permeabilization allows entry of propidium iodide in all cells, dead and alive. Apoptotic cells are characterized by a lower DNA content (hypodiploid cells) because of nuclear fragmentation. Thus, this assay is not based on the known ability of propidium iodide to enter dead cells. The percentage of apoptotic cells with decreased DNA content (A0) was counted (37,38). Cells were exposed to high (700 mg/dL) glucose, Ang II (10-7 M) or TGFβ1 (10 ng/mL).
To assess the typical apoptotic nuclear changes cells were cultured in chamber slides (Labtek, Nunc, Naperville, IL), fixed with methanol:acetone (1:1), and stained with DAPI (Sigma) and anti-HSPB1. The typical condensed, shrunk and fragmented nuclei of apoptotic cells were identified by a laser scanning confocal microscope (Leika).

The ApoScreen Annexin V-FITC kit (Southern Biotechnology Associates, Birmingham, Alabama, USA) was used to assess both apoptosis and necrosis by flow cytometry. This method discriminates between early apoptotic (propidium iodide negative, annexinV positive), and late apoptotic or necrotic cells (propidium iodide positive, annexinV positive) (39).

Caspase-9 activity was assessed in cells transfected with HSPB1 siRNA and stimulated with Ang II or high glucose. Caspase-9 activity (R&D Systems, Minneapolis, MN) was measured following the manufacturer’s instructions. In brief, cell extracts (100 µg protein) were incubated in half-area 96-well plates at 37°C with 200 µM LEDH-pNA peptide in a total volume of 100 µl. The pNA light emission was quantified using a spectrophotometer plate reader at 405 nm. Comparison of the absorbance of pNA from an apoptotic sample with an uninduced control allows determination of the fold increase in caspase activity.

**Transfection of Small Interfering RNA**

Cells were grown in six-well plates (Costar, Cambridge, MA) and transfected with a mixture of 25 nmol/mL HSPB1 siRNA (Santa Cruz), Opti-MEM I Reduced Serum Medium and Lipofectamine 2000 (Invitrogen) (5). Two independent siRNAs were used and similar results were obtained. After 18 hours, cells were washed and cultured for 24 hours in complete medium, and serum-depleted for 24 h before addition of high glucose or Ang II. This time point was selected from a time-course of decreasing HSPB1 protein expression in response to siRNA. A negative control scrambled siRNA provided by the manufacturer did not reduce HSPB1 protein.

**Human renal biopsies**

Kidney samples were obtained from type 2 diabetes mellitus (n=8) patients biopsied because of nephrotic proteinuria. Clinical data at biopsy were: mean age 54 years (range 28 to 78 years); mean serum creatinine 2.5 mg/dl (range 1.1 to 3.6 mg/dl); mean proteinuria 6.1 g/d (range 1.0 to 14.5 g/d). Biopsies showed a clear picture of diffuse or nodular glomerulosclerosis with a marked tubulointerstitial involvement. Normal kidneys were also assessed. Minimal change disease (MCD) was used as a non-progressive proteinuric control (n=9: mean age 18 years (range 2 to 56 years); mean serum creatinine
0.78 mg/dl (range 0.34 to 2.5 mg/dl); mean proteinuria 4.61 g/d (range 0.3 to 14 g/d). Focal segmental
glomerulosclerosis (FSGS) was studied as a progressive proteinuric disease (n=9: mean age 34 years
(range 13 to 57 years); mean serum creatinine 1.0 mg/dl (range 0.51 to 1.7 mg/dl), mean proteinuria 5.2
g/d (range 0.75 to 9.6 g/d). The study protocol was approved by the local Ethics Committee and informed
consent was obtained.

Immunohistochemistry was carried out in paraffin-embedded human kidney, fixed in 4%
buffered formalin, or Bouin. The primary antibody was goat polyclonal anti-HSPB1 (1:5000, Santa Cruz
biotechnology, CA, USA). Briefly, 5 µm thick renal sections were adhered to polylysine-coated glass
slides and fixed overnight at 56°C. After deparaffinizing through xylene, alcohol and distilled water,
endogenous peroxidase was blocked by 3% H2O2 for 10 min. Sections were microwaved in 0.1mM
citrate buffer pH 6.0 for 20min, and transferred to distilled water. After rinsing in Tris–HCl–phosphate-
buffered saline (TPS), sections were incubated with 1:10 normal rabbit serum in TPS/1% BSA and then
incubated overnight at 4°C with unlabelled goat anti-human HSPB1. Next, they were incubated with
biotinylated rabbit anti-goat antibody (1:500) in TPS–1%-BSA for 30 min at 22°C. After three rinses in
TPS, they were incubated with streptavidin–peroxidase (Dako) 1:500 for 30 min. Color was developed
with diaminobenzidine and then counterstained with haematoxylin, dehydrated and mounted with
Canadian balsam (Polysciences, Inc.). Specificity was checked by omission of primary antibodies and use
of non-immune sera.

Statistics

Data are given as mean ± standard deviation. Mann-Whitney, 2-sided t-test or one-way ANOVA
were applied to indicate significantly different mean values in comparison to the control group. A p value
< 0.05 was considered statistically significant.
RESULTS

Increased HSPB1 expression in experimental diabetic nephropathy

Increased podocyte HSPB1 had been observed in rats with nephrotic syndrome induced by PAN (25). However, this toxic model has no obvious human counterpart and it was unknown whether similar changes occur in subnephrotic proteinuria or podocyte injury induced by endogenous pathophysiological stimuli. We studied a chronic rat model of DN induced by a single STZ injection and characterized by the development of albuminuria (1071±247 vs 390±70 μg/24h, p<0.02, at 7 months, supplemental table 1). At this time point whole kidney HSPB1 mRNA (Figure 1.A) and protein (Figure 1.B) levels were increased. The glomerular HSPB1 mRNA expression was significantly increased in DN compared with control rats (Figure 1.C). Immunohistochemistry also localized increased HSPB1 expression to glomeruli of DN rats (Figure 1.D), where HSPB1 co-localized with anti-synaptopodin positive podocytes (Figure 1.E). In an early rat model of DN (6 weeks) in which albuminuria was starting to increase, immunohistochemistry showed an increased glomerular HSPB1 expression (Figure 2.A).

Chronic AngII infusion in rats increased kidney HSPB1 expression

AngII is a key determinant of DN. AngII infusion in rodents causes renal damage, characterized by glomerular and interstitial inflammatory cell infiltration, observed at 3 days, and tubular atrophy and interstitial fibrosis found after 2 weeks (40-42). At this time point, Ang II increased total kidney HSPB1mRNA and protein levels, as assessed by real time PCR (Figure 2.B) and Western blot (Figure 2.C), respectively. Immunohistochemistry localized HSPB1 expression to glomeruli, mainly in podocytes (Figure 2.D).

Increased expression of HSPB1 in human diabetic nephropathy

HSPB1 was studied in normal human kidneys, MCD, DN and FSGS. MCD is a non-progressive proteinuric kidney diseases, while both DN and FSGS are characterized by progressive glomerular injury and podocyte loss. HSPB1 expression was absent from normal glomeruli or MCD podocytes (Figure 3.A, B). Increased glomerular HSPB1 expression was noted by immunohistochemistry in all human DN and FSGS biopsies and morphologically localised to podocytes (Figure 3.C,D). HSPB1 tubular staining was observed in FSGS and DN and in occasional tubules in MCD.

Regulation of HSPB1 in cultured human podocytes

HSPB1 is upregulated in response to stress (43). Since HSPB1 was upregulated in human DN we tested the hypothesis that stressed human podocytes could synthesize HSPB1 as a defense mechanism.
Based on experimental model results, we tested the regulation of HSPB1 expression by high glucose or Ang II, two of the main contributors to the pathogenesis of human DN. Either a high glucose concentration or Ang II increased HSPB1 mRNA (Figure 4.A,C) and protein expression in human podocytes (Figure 4.B,D). Mannitol, used as an osmolarity control, did not change HSPB1 expression (Figure 4.A).

**HSPB1 expression is a compensatory mechanism that protects human podocytes from high glucose or angiotensin II-induced apoptosis**

Once demonstrated that HSPB1 expression is upregulated by high glucose and Ang II in vivo and in cell culture, we approached the function of HSPB1 under these conditions. That is, we assessed the function of the increased HSPB1 levels observed in response to an environment found in DN in vivo. High glucose induces human podocyte stress and apoptosis (5). To investigate the function of HSPB1 in human podocytes exposed to high glucose, HSPB1 was knocked down by using specific siRNAs. Two different siRNAs (siRNA1 and siRNA2) were used. Results using siRNA1 are shown in figures 5.B, 5.D, 5.E, 6.B and 6.C. Results using siRNA2 are shown in figures 5.A, 5.C and 6.A. Using siRNA1 we obtained similar results to those with siRNA2 shown in figures 5.A, 5.C and 6.A (not shown). Western blot confirmed efficient gene silencing of HSPB1 in the absence of changes in the levels of other HSP proteins (Figure 5.A). HSPB1 targeting did not modulate cell death in non-stressed cells (Figure 5.B). However, HSPB1 knock-down increased the apoptosis rate, assessed as hypodiploid cells, of podocytes stressed by a high glucose environment (Figure 5.B). Ang II had a negligible effect on human podocyte apoptosis. However, preventing HSPB1 upregulation sensitized to Ang II-induced apoptosis (Figure 5.B). Western blot and confocal microscopy confirmed HSPB1 upregulation by high glucose and Ang II at the individual cell level, as well as effective HSPB1 downregulation by specific siRNAs (Figure 5.C, D). Assessment and quantification of morphological features of apoptosis confirmed the apoptosis sensitizing effect of HSPB1 targeting in the presence of high glucose or AngII (Figure 5.E). The results were confirmed using a different siRNA and method to assess apoptosis. In this regard, the percentage of apoptotic cells assessed as propidium iodide negative, annexin V positive cells was higher after HSPB1 knock-down with HSPB1 siRNA (Figure 6.A). HSPB1 targeting also increased TGFβ1-induced apoptosis (Figure 6.A). These results suggest that HSPB1 upregulation in the presence of DN environmental stressors protects human podocytes from stress induced by stimuli present in the DN environment.
**HSPB1 targeting promotes caspase activation in podocytes**

In the mitochondrial pathway for apoptosis release of cytochrome c from mitochondria promotes activation of caspase-9 (44). Caspase-9 promotes activation of downstream caspases, such as caspase-3, the central executioner caspase, common to several apoptotic pathways. HSPB1 targeting using specific siRNAs promoted caspase-3 activation in podocytes stressed by AngII or by a high glucose environment (Figure 6.B). By contrast, caspase-9 activity was increased only in podocytes stressed by AngII (Figure 6.C) but not in those stressed by a high glucose environment, suggesting that these stressors activate different apoptotic pathways that have in common the regulation by HSPB1.

**DISCUSSION**

The role of HSPB1 on podocytes exposed to stressors relevant for DN has not been well characterized. We now show that in podocytes HSPB1 is upregulated in vivo and in culture by stimuli relevant for the pathogenesis of DN, i.e. high glucose and AngII. Indeed, podocyte HSPB1 is increased in human DN. HSPB1 upregulation appears to be a compensatory mechanism that prevents or reduces podocyte apoptosis induced by high glucose or AngII. In this regard, HSPB1 targeting, effectively preventing HSPB1 upregulation by these stimuli, sensitized to apoptosis podocytes stressed by stimuli relevant to DN such as high glucose, AngII and TGFβ1.

HSPB1 is not a classic chaperone as it does not actively refold other proteins. It instead has a chaperone-like activity, preventing aggregation of other partially denatured proteins. Thus, HSPB1 interacts with a large number of different proteins and prevents cell death caused by hyperthermia, oxidative stress, staurosporine, Fas ligand and cytotoxic drugs (24;28;45-49). HSPB1 is an antioxidant and inhibits multiple steps in the extrinsic and intrinsic mitochondrial apoptotic pathways. However, HSPB1 target molecules differ for different lethal stimuli and cell types (50;51). In certain cells HSPB1 binds to cytochrome c released from mitochondria and HSPB1 overexpression prevents cytochrome-c-mediated interaction of Apaf-1 with procaspase-9 and procaspase-9 activation (28;30). In this regard, in human podocytes we observed that HSPB1 targeting promoted caspase-9 and caspase-3 activity in the presence of Ang II. Caspase-3 may be a downstream target of caspase-9 or other activator caspases (44). However, despite no change in caspase-9 activity, HSPB1 targeting increased caspase-3 activation in podocytes exposed to high glucose. This suggests caspase-9 independent effects of HSPB1. In this sense,
in oocytes HSPB1 targeting promoted caspase-3 activation in the absence of caspase-9 activation, similar to the observation in podocytes exposed to high glucose conditions (52).

Podocyte injury results in proteinuric renal disease (53). Contrary to MCD, both DN and FSGS are progressive glomerular diseases characterized by progressive loss of podocytes in which podocyte apoptosis has been observed (13,14). Podocyte HSP27 expression was found in DN or FSGS, but not in normal kidney or MCD. This suggests that DN and FSGS may share podocyte stressors that result in podocyte loss and that engage podocyte adaptive responses and that these adaptive responses are not engaged in MCD. The precise factors contributing to podocyte HSPB1 expression in FSGS may include AngII itself, since overexpression of the AT1 receptor in podocytes results in FSGS (54). It is interesting to note that in experimental nephrotic syndrome caused by toxin-mediated podocyte injury, HSP25 (the mouse homolog of human HSPB1) levels increased (31), and enforced overexpression of HSP25 in cultured murine podocytes protected the actin cytoskeleton against toxin-induced disruption (55). Previous studies had observed a transient increase in glomerular HSPB1 phosphorylation in short-term diabetes and p38MAPK-dependent HSPB1 phosphorylation in murine podocytes exposed to high glucose, but the role of HSPB1 in glucose-induced apoptosis was not studied (55). HSPB1 may become phosphorylated at different serines in response to heat shock and various stimuli (reviewed in 56). The p38 MAPK pathway plays a key role in HSPB1 phosphorylation, also in podocytes (57). Indeed high glucose promotes HSPB1 phosphorylation in renal cells in an MK(MAPKAP)-2-dependent manner (58). The phosphorylation state of HSPB1 can influence its function. Thus, it modulates the size of HSPB1 oligomers and facilitates the nuclear migration of the protein (59). HSPB1 phosphorylation may influence the fate of the cell in response to an apoptotic stimulus, although the requirement for phosphorylation may vary in different systems (60). A phosphorylation-defective mutant HSPB1 accelerated toxin-induced apoptosis in renal epithelial cells (61). Overexpression of the wild-type HSPB1, but not a non-phosphorylatable mutant, markedly reduced complement-mediated glomerular epithelial cell injury (62). Indeed increased glomerular HSPB1 phosphorylation was noted in experimental toxin-induced nephrotic syndrome and diabetes, but decreased in ischemia (58;63;64). However, prevention of renal HSPB1 phosphorylation did not interfere with the development of early diabetic nephropathy in diabetic mice (58).

In renal tubular cells, HSPB1 overexpression inhibited apoptosis and HSPB1 knockdown promoted apoptosis in response to culture in glucose-free medium containing sodium cyanide and 2-
deoxy-d-glucose (51). However, this kind of metabolic stress is of dubious clinical significance in the particular setting of DN. We now show that glomerular HSPB1 is upregulated in humans and in rats with short- (6 weeks) and long-standing (7 months) DN and that podocytes are key sources of glomerular HSPB1 in both clinical and experimental DN. This appears to be the consequence, at least in part, of high glucose levels, since high glucose upregulated HSPB1 in cultured human podocytes. High glucose is known to promote renal cell apoptosis, including podocyte apoptosis (3;5). We have observed that impairing the HSPB1 upregulation adaptive response by siRNA targeting sensitizes to high glucose-induced apoptosis, while having a negligible effect on spontaneous apoptosis. Thus, these studies expand previous data by showing that stimuli pathophysiologically relevant to DN, such as high glucose, increase podocyte HSPB1 and also by showing that prevention of this upregulation promotes apoptosis. Thus, even when not reaching very high HSPB1 levels as a consequence of enforced expression in transiently transfected cells, HSPB1 is also protective.

In DN high levels of glucose recruit secondary mediators of injury that aggravate and perpetuate renal injury. Ang II is the secondary mediator of DN with most clinical relevance. Ang II has hormonal as well as paracrine/autocrine functions that modulate renal injury. Overexpression of podocyte Ang II type 1 receptor in rats results in proteinuria, nephron loss, and glomerulosclerosis (54). Inhibition of RAS activity lowers proteinuria and slows progression to end-stage renal disease, and is the keystone of human DN therapy (65;66). HSPB1 had previously been shown to be upregulated in the kidney by short-term (<1 week) Ang II infusion (67). However, the glomerular location of HSPB1 upregulation was not described and the function of HSPB1 was not addressed (67). We now show glomerular HSPB1 upregulation in a model of chronic infusion of Ang II, in vivo localization of HSPB1 expression to podocytes, and a direct effect of Ang II on cultured podocytes promoting HSPB1 upregulation. As in the case of high glucose exposure, inhibition of HSPB1 expression promotes Ang II-induced apoptosis. Thus, upregulation of HSPB1 in response to Ang II is a podocyte adaptive response to prevent Ang II- and also TGFβ1-induced cell death. Further studies should explore whether HSPB1 might also regulate additional proinflammatory actions of AngII (68).

In conclusion, HSPB1 is upregulated in vivo and in cultured podocytes by high glucose and Ang II. This appears to be an adaptive response that allows podocytes to cope with these stressors and prevents apoptosis since depletion of this upregulated HSPB1 sensitized to these same stimuli. Increased amounts
of endogenous HSPB1 are required to prevent podocyte apoptosis in a diabetic milieu rich in glucose and Ang II. This may lead to the development of new therapeutic approaches.

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References


TITLES AND LEGENDS TO FIGURES

Figure 1. Expression of HSPB1 in experimental diabetic nephropathy. A) Whole kidney HSPB1 mRNA was increased in diabetic rats when compared to controls. Mean ±SD of 10 rats per group. *p<0.04 vs control rats. B) Quantification and representative image of Western blot of whole kidney protein. Western blot quantification expressed as percentage increase over control. Mean±SD of 10 rats per group. *p<0.006 vs control rats. C) Western blot analysis of glomerular HSPB1. Mean±SD of 10 rats per group. *p<0.03 vs control rats. D) Immunohistochemistry, representative images of control and DN kidney in which podocyte staining for HSPB1 is observed (arrows). Original magnification x200 and x400. D) Double anti-synaptopodin (green) and anti-HSPB1 (red) staining of glomeruli in rat DN. Anti-synaptopodin stains mainly the foot processes and less intensely the podocyte body and the area near the cell membrane, while anti-HSPB1 stains mainly the podocyte body. Several clearly identifiable podocytes are indicated by arrows. Original magnification x400.

Figure 2. Renal HSPB1 expression is upregulated in early diabetic nephropathy and chronic AngII infusion. A) HSPB1 immunohistochemistry, representative images of control and DN rat kidney at week 6 following injection of streptozotocin or vehicle. Podocyte staining for HSPB1 is observed in DN. Original magnification x200 and x400. B) Whole kidney HSPB1 mRNA was increased in Ang II-infused rats when compared to controls. Mean±SD of 6-8 animals per group. *p<0.008 vs control. C) Quantification and representative image of Western blot of whole kidney protein. Mean±SD of 6-8 animals per group. *p<0.01 vs control. D) Representative immunohistochemistry where HSPB1 is increased in the glomeruli in a distribution suggestive of podocyte expression. Original magnification x200 and x400.

Figure 3. Increased HSPB1 expression in human diabetic nephropathy biopsies. A) Representative images of normal kidneys. B) Minimal change disease (MCD). C) Diabetic nephropathy (DN). D) Focal segmental glomerulosclerosis (FSGS). HSPB1 immunohistochemistry. Note a marked increase in HSPB1 staining in glomerular cells with a podocyte localization (arrow) in DN and FSGS. Original magnification x200 and x400.

Figure 4. High glucose and Ang II increase HSPB1 expression in cultured human podocytes. Cells were exposed to A,B) high glucose or C,D) Ang II. High glucose (A) or Ang II (C) increases HSPB1 mRNA in a time-dependent manner. Real time RT-PCR. Mean±SD of 3 independent experiments. *p<0.03 vs control 24 hours. B and D) Quantification and representative Western blot of HSPB1. Cells
were cultured in the presence of B) high glucose and D) Ang II for 24 h. Mean±SD of 3 independent experiments. *p<0.04 vs control.

**Figure 5. Inhibition of HSPB1 expression by siRNA sensitizes to apoptosis in human podocytes. A)** HSPB1 siRNA downregulation of HSPB1. Representative Western blot of the specific siRNA. **B)** HSPB1 knock-down with HSPB1 siRNA increases high glucose-induced apoptosis and promotes Ang II-induced apoptosis. Cell death was assessed by flow cytometry of DNA content (hypodiploid cells) after culture in the presence of high glucose or Ang II (10⁻⁷ M) for 24h. Mean±SD of 3 independent experiments. *p<0.05 versus control; ** p<0.001 vs high glucose; #p<0.03 vs Ang II alone. **C)** Representative Western blot corresponding to the conditions of the experiment shown in B. **D)** HSPB1 silencing increased apoptosis in human podocytes as assessed by morphology. Representative image of apoptotic cells with typical nuclei (shrunk, bright) (DAPI staining) among cells transfected with HSPB1 siRNA (arrows). Anti-HSPB1 staining in green confirmed upregulation of HSPB1 expression by high glucose or Ang II and effective downregulation by siRNA. **E)** Quantification of apoptotic cells expressed as percentage of cells with apoptotic nuclei as assessed by DAPI staining. Mean ±SD for 3 independent experiments. *p<0.009 vs high glucose and Ang-II alone; **p<0.0001 vs control; #p<0.04 vs control.

**Figure 6. Inhibition of HSPB1 expression by siRNA sensitizes to apoptosis promoting caspase activation in human podocytes. A)** HSPB1 knock-down with HSPB1 siRNA increases high glucose- and TGFβ1-induced apoptosis and promotes Ang II-induced apoptosis. Cell death was assessed by flow cytometry following staining with propidium iodide and annexinV after culture in the presence of high glucose, Ang II (10⁻⁷ M) or TGFβ1 (10 ng/mL) for 24h. Representative experiment. **B)** Podocyte apoptosis induced by HSPB1 knocked down with HSPB1 siRNA is associated with caspase activation. Caspase-3 is processed in the course of Ang II- and high glucose-induced apoptosis when HSPB1 is knocked down. Quantification of active caspase 3 Western blot. Mean ±SD for 3 independent experiments * p<0.001 vs Angiotensin, **p<0.004 vs high glucose. **C)** HSPB1 knock-down promotes caspase-9 activity in human podocytes exposed to AngII. Mean ±SD for 3 independent experiments * p<0.02 vs control, **p<0.05 vs Angiotensin alone.
Figure 1

A) % HSPB1 mRNA levels

B) % HSPB1 protein levels

C) % glomerular HSPB1 protein levels

D) Control

E) Synaptopodin

HSPB1

Merged
Figure 2

A) HSPB1

Control

DN

B) % HSPB1 mRNA levels

WKY AngII 2 Weeks

C) % HSPB1 protein levels

WKY AngII 2 Weeks

D) Control

HSPB1

Ang II

HSPB1

HSPB1

HSPB1

HSPB1

Control AngII

HSPB1 α-tubulin

*
A) NORMAL KIDNEY

B) MCD

C) DN

D) FSGS
Figure 4

A) % HSPB1 mRNA levels

B) % HSPB1 protein levels

C) % HSPB1 mRNA levels

D) % HSPB1 protein levels
Figure 5

A) Western blot analysis of HSPB1, HSP47, HSP70, and α-tubulin in control, siRNA HSPB1, Ang II, high glucose, siRNA HSPB1 + Ang II, and siRNA HSPB1 + high glucose conditions.

B) Bar graph showing % apoptosis (hypodiploid cells) for different conditions: siRNA HSPB1, Ang II, high glucose, siRNA HSPB1 + Ang II, and siRNA HSPB1 + high glucose. Asterisks indicate statistical significance: *p < 0.05, **p < 0.01, #p < 0.001.

C) Immunofluorescence images of DAPI staining for HSPB1 and α-tubulin in control and treated conditions.

D) Table summarizing % apoptosis (DAPI staining) for different conditions:

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<th>Control</th>
<th>siRNA HSPB1</th>
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<th>siRNA HSPB1 + Ang II</th>
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E) Bar graph showing % apoptosis (DAPI staining) for different conditions: siRNA HSPB1, Ang II, high glucose, siRNA HSPB1 + Ang II, and siRNA HSPB1 + high glucose. Asterisks indicate statistical significance: *p < 0.05, **p < 0.01, #p < 0.001.
Figure 6

A)

Control         High Glucose        Angiotensin II         TGF-β1

Necrosis        Late apoptosis      Early apoptosis        -

3.32%           11.48%             4.6%                   7.1%

Propidium iodide

7.15%           18.42%             12.2%                  13.5%

Annexin-V

siRNA HSPB1

B)

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C)

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