Proteinuria and Hyperglycemia Induce Endoplasmic Reticulum Stress

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ABSTRACT

The endoplasmic reticulum (ER) is an important site for protein folding and becomes "stressed" when its capacity to fold proteins is overwhelmed. In response, "unfolded protein response" (UPR) genes are induced, increasing the capacity to fold proteins; if the response is insufficient, then apoptosis ensues. For investigation of whether proteinuria and hyperglycemia induce ER stress in renal epithelial cells, microarray data from biopsies of established diabetic nephropathy (DN) were analyzed. Expression of UPR genes was significantly different in these biopsies than in control kidneys or biopsies of patients with mild DN, suggesting an association between the degree of DN and UPR gene expression. Expression of the transcription factor XBP1 and the ER chaperones HSPA5 and HYOU1 were increased, but the proapoptotic gene DDIT3 was unchanged. These findings were replicated in an independent cohort of patients with established DN by real-time reverse transcriptase–PCR. Immunofluorescence of renal biopsies from patients with DN confirmed the upregulation for HSPA5 and HYOU1 proteins in tubular epithelia. In biopsies of minimal-change disease, the mRNA levels of some ER stress molecules were also induced, but protein expression of HSPA5 and HYOU1 remained significantly lower than that observed in DN. Exposure of renal tubular epithelial cells to albumin and high glucose *in vitro* enhanced expression of genes involved in ER stress. These observations suggest that in proteinuric diseases, tubular epithelial cells undergo ER stress, which induces an adaptive, protective UPR. Although this may protect the cells from ER stress, persistence of hyperglycemia and proteinuria may eventually lead to apoptosis.

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One of the major complications of diabetes is DN, which is a leading cause of end-stage renal disease (ESRD).[1] Chronic proteinuria and tubulointerstitial fibrosis, characteristics of established DN, correlate best with the degree of renal dysfunction and are considered reliable prognostic indicators for ESRD.[2–6] In fact, proteinuria *per se* has been postulated to contribute to progressive tubulointerstitial fibrosis in general.[7] In this context, it has been proposed that urinary protein represents a direct stress for renal tubular epithelial cells. The endoplasmic reticulum (ER) is considered a key player in the response to cellular stress and protein overload of the ER. We therefore hypothesized that proteinuria and hyperglycemia in DN may induce ER stress in tubular cells of the kidney. ER stress could be

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brought about by hyperglycemia resulting in nonenzymatic glycosylation of proteins and generation of reactive oxygen species, and by increased turnover of tubular epithelial proteins and membrane components as a consequence of the massive protein and lipid reabsorption occurring in proteinuria.[8,9]

The ER is responsible for protein folding within each cell and is highly sensitive to alterations in its homeostasis. Disruption of this homeostasis leads to accumulation of unfolded proteins. The imbalance between unfolded proteins and the capacity of the ER to handle this load is referred to as ER stress.[10,11] To cope with it, the ER has evolved a signaling network, termed the unfolded protein response (UPR). The intent of the UPR is to adapt to the changing environment and to reestablish normal ER function. This involves the reduction in protein synthesis and translocation into the ER, followed by the transcriptional activation of UPR target genes, including ER chaperones. If these adaptive responses cannot compensate for the ER stress, then apoptosis is triggered. This presumably protects the organism from cells that display misfolded proteins. UPR-induced cell death mediators such as DDIT3 and PPP1R15A are involved in this third step.[12,13] In the UPR, three classes of ER stress sensors have been identified, namely PERK, a transmembrane kinase; ATF6, a bZIP transcription factor; and IRE1, a dual-activity enzyme with a kinase and an endoribonuclease domain.[10,14] ATF6 induces the expression of ER chaperones, such as HSPA5, GRP94, HYOU1, and calreticulin (CALR), whereas IRE1 is involved in the splicing of XBP1 pre-mRNA, a bZIP transcription factor that actives ER chaperones. XBP1 is itself upregulated in chronic ER stress. Furthermore, ER stress has been reported to activate indirectly NF-κB–dependent pathways.[15,16] Overall, the UPR in ER stress is designed to protect cells, a goal that is achieved in most circumstances.

Previous studies showed a close association of ER stress with neuronal cell injury[17] and apoptosis of pancreatic cells.[17,18] There are also studies linking UPR to experimental ER stress in cultured renal cells.[19–21] For example, ER stress could be induced in podocytes *in vitro* and in a rodent model by protein accumulation,[20] by complement-induced glomerular epithelial cell injury,[19] or by exposure of renal proximal tubular cells to high albumin concentrations resulting in apoptosis.[21] On the basis of these considerations and our recent observation[22] of NF-κB activation in established DN as a potential sign of ER stress, ER stress response was evaluated in this study in the tubulointerstitium of renal biopsies from patients with high proteinuria from DN and from minimal-change disease (MCD).

RESULTS

Diabetes and proteinuria may result in ER stress in the kidney.[9,19–21,23,24] We therefore investigated the expression of genes involved in the ER stress response in patients with DN and MCD. Genes involved in ER stress were compiled from the literature (Supplemental Table S1) and belonged to one of the following categories: (*1*) Proteins of the ER protein translocation, folding, export, and degradation machinery; (*2*) genes encoding proteins that transiently attenuate protein biosynthesis; or (*3*) components of the programmed cell death.

Gene expression was analyzed by microarray analysis and quantitative reverse transcriptase–PCR (RT-PCR) in the tubulointerstitial compartment of renal biopsies of patients with established, proteinuric DN and serum creatinine concentration ≥1.4 mg/dl (124 μmol/L) and from biopsies with mild DN (*i.e.*, serum creatinine <1.4 mg/dl and only little histologic alteration of the interstitium). In addition, biopsies from patients with MCD were analyzed by quantitative RT-PCR. Pretransplantation biopsies served as controls. The genes significantly regulated in established *versus* mild DN are summarized in Table 1. The results for the expression levels of all genes in normal renal tissue and in biopsies from mild and established DN are shown in Supplemental Tables S1 and S2. Table 2 shows the clinical characteristics of the patients.

Microarray Analysis Reveals ER Stress in Renal Biopsies from Patients with Established DN

In patients with established DN—but not in patients with mild DN—a significant increase of tubular mRNA levels of the major genes involved in the UPR was observed (*i.e.*, HSPA5, HYOU1, XBP1, CANX, a lectin-like chaperone and member of the ER protein-folding machinery, and MBTPS1, an activator of ATF6; Table 1 and Supplemental Tables S1 and S2). In contrast, the mRNA levels of UPR-induced apoptosis mediators such as DDIT3 and PPP1R15A were reduced or were undetectable in biopsies from established DN as compared with mild DN (Table 1). Thus, the major genes involved in the adaptive UPR were upregulated only in established but not in mild DN, whereas those favoring apoptosis tended to be downregulated. This indicates a protective UPR as adaptation against chronic ER stress occurring in progressive, proteinuric DN.

Validation of Microarray Results by Quantitative RT-PCR

Quantitative RT-PCR for the UPR genes HSPA5, HYOU1, XBP1, CANX, MBTPS1, and DDIT3 was used on manually microdissected tubulointerstitium of fresh biopsies to validate the aforementioned results in a larger cohort of established DN ($n = 15$) and pretransplantation biopsies ($n = 10$; Table 2). HSPA5, HYOU1, and XBP1 were significantly upregulated in established DN as compared with controls (Figure 1). Determination of DDIT3, CANX, and MBTPS1 by real-time RT-PCR showed no significant regulation of these genes (DDIT3: Figure 1; CANX: control 1.00 ± 0.09, DN 0.74 ± 0.19, NS; MBTPS1: control 1.00 ± 0.21, DN 1.09 ± 0.25, NS). Overall, these results confirm that in established DN, major adaptive UPR genes are upregulated, whereas the proapoptotic DDIT3 is not.

To test the hypothesis that proteinuria *per se* may also lead to ER stress, we also analyzed the expression of the genes in

Table 1. Microarray gene expression of ER stress–related genes in mild and established DN[a]

Probeset ID	Gene Symbol	Alias	Category	Gene Title	Mild DN	Established DN
200779_at	ATF4		III	Activating transcription factor 4 (tax-responsive enhancer element B67)	↔	ND
217550_at	ATF6		I	Activating transcription factor 6	↔	ND
211833_s_at	BAX		III	BCL2-associated X protein	↔	ND
203684_s_at	BCL2		III	B-cell CLL/lymphoma 2	↔	ND
207004_at	BCL2		III	B-cell CLL/lymphoma 2	↔	ND
200068_s_at	CANX		I	Calnexin	↔	↑
208852_s_at	CANX		I	Calnexin	↔	↑
207181_s_at	CASP7		III	Caspase 7, apoptosis-related cysteine peptidase	↔	ND
209383_at	DDIT3	CHOP/GADD153	III	DNA damage–inducible transcript 3	↔	↓
203279_at	EDEM1		I	Eukaryotic translation initiation factor 2-α kinase 3	↔	ND
207061_at	ERN1	IRE1	I	ERO1-like (S. cerevisiae)	↔	ND
211936_at	HSPA5	GRP78/BiP	I; II; III	Hypoxia upregulated 1	↔	↑
200825_s_at	HYOU1	ORP150	I	Mitogen-activated protein kinase 8	↔	↑
201620_at	MBTPS1	S1P	I	Membrane-bound transcription factor peptidase, site 1	↔	↑
202014_at	PPP1R15A	GADD34	III	Protein phosphatase 1, regulatory (inhibitor) subunit 15A	↔	ND
37028_at	PPP1R15A	GADD34	III	Protein phosphatase 1, regulatory (inhibitor) subunit 15A	↔	ND
204413_at	TRAF2		III	TNF receptor–associated factor 2	↔	ND
200670_at	XBP1		I	X-box binding protein 1	↔	↑

[a]Comparison of the expression of 28 genes, known to be involved in ER stress, between the tubulointerstitial compartment from biopsies of patients with mild DN (serum creatinine <1.4 mg/dl) and those with established DN (serum creatinine ≥1.4 mg/dl). Genes with significant upregulation ($P < 0.05$) are marked by an upward arrow, and those that were significantly downregulated by a downward arrow. Genes that showed no change are marked as ↔. Genes that were expressed below the cutoff are labeled as not detectable (ND).

patients with MCD. As in established DN, we found a significant upregulation of mRNA levels for HSPA5, HYOU1, and XBP1 (Figure 1) and no regulation of DDIT3, CANX, and MBTPS1 (DDIT3: Figure 1; CANX: control 1.00 ± 0.09, MCD 0.87 ± 0.06, NS; MBTPS1: control 1.00 ± 0.21, MCD 0.87 ± 0.05, NS). These data indicate a potential influence of proteinuria *per se* on the UPR in ER stress response.

Immunofluorescence for HSPA5 and HYOU1 in DN, MCD, and Controls

To evaluate whether the mRNA levels correspond to protein levels, we performed immunofluorescence for HSPA5, HYOU1, and calreticulin, as ER localizer, on an independent set of renal biopsies (Table 3). In the interstitial compartment of control kidneys, HSPA5 was either completely absent or showed a minimal scattered positivity in the cytoplasm of tubular cells. In patients with DN, a markedly increased positivity was observed in proximal and distal tubuli (Figure 2A). Some glomerular staining also occurred, but the material was insufficient to perform exact localization studies. For HYOU1, a moderate basolateral staining in tubular cells of control kidneys could be detected, whereas the staining was negative in glomeruli. In biopsies of patients with DN, HYOU1 was expressed *de novo* in glomeruli and markedly increased in tubuli (Figure 2B). No induction of HSPA5 and HYOU1 was observed in renal interstitial cells. Calreticulin as an ER marker was constitutively expressed in tubuli and glomeruli of all biopsies and co-localized with HSPA5 as well as with HYOU1, confirming their ER localization (Figure 3).

We also evaluated the expression of these proteins in patients with MCD. As to be expected, MCD biopsies showed no histologic indications of significant tubulointerstitial damage. By immunofluorescence, HSPA5 and HYOU1 again co-localized with calreticulin in MCD (data not shown). A mild induction of HYOU1 was observed in MCD, but this was less than in DN (Figure 2). In contrast to DN, staining for HSPA5 in MCD was not different from controls (Figure 2). Computer-assisted quantification showed a significantly larger staining area for both proteins in DN compared with MCD and controls ($n = 5$ for each group; see Figure 2). Thus, the degree of staining for the ER stress proteins was somewhat increased in MCD but significantly more in DN.

Response of HSPA5, HYOU1, and XBP1 mRNA Expression to ER Stress Inducers in Cultured Renal Epithelial Cells

Our biopsy data indicate that proteinuria and DN induce ER stress, resulting in an adaptive UPR. To test whether induction of ER stress genes can be mimicked *in vitro* in renal tubular epithelial cells, we incubated human tubular cells (HK-2) with various concentrations of tunicamycin (TM) and thapsigargin (TG) and analyzed the expression of HSPA5, HYOU1, and XBP1 (Figure 4). Both agents induced the ER stress genes HSPA5 and HYOU1, albeit with different time courses (data not shown) and to differ-

Table 2. Clinical and histologic characteristics of biopsies from patients with mild and established DN or MCD and control subjects analyzed by oligonucleotide array–based gene expression profiling and real-time RT-PCR[a]

Biopsy Group	Gender	Age (yr)	Histology Major Diagnosis	Creatinine (mg/dl)	Proteinuria (g/d)	GFR (ml/min)	Hypertension	RR Systolic[b]	RR Diastolic[b]	Diabetes Type	HbA_{1c} (%)	Retinopathy
Established DN												
DN3 (A)	M	57	DN	1.6	9.7	48	Yes[1,4,5,6]	NA	NA	1	8.7	Yes
DN5 (A)	F	46	DN	2.4	0.4	23	Yes[1,5]	110	60	2	7.5	Yes
DN6 (A)	F	67	DN	4.8	2.4	10	Yes[3,4,5]	NA	NA	2	NA	Yes
DN9 (A)	M	67	DN	1.4	4.5	54	Yes[5]	NA	NA	2	NA	NA
DN10 (A + P)	M	62	DN	2.3	5.0	39	Yes[3,4,6]	150	80	2	7.8	No
DN11 (A + P)	M	68	DN	3.5	NA	34	Yes[3,4]	160	80	2	7.2	No
DN16 (P)	M	58	DN	1.7	0.7	44	Yes[1]	160	90	2	7.4	No
DN18 (P)	F	59	DN	3.2	21.4	16	Yes[1,3,5]	162	85	2	NA	NA
DN20 (P)	F	63	DN	3.3	7.0	31	Yes[1,3,5,6]	150	70	2	7.9	No
DN21 (P)	M	63	DN	2.1	8.6	51	Yes[1,2,3,4,5,6]	130	75	2	7.1	No
DN22 (P)	F	47	DN	7	NA	25	Yes[5]	125	70	2	13.8	Yes
DN23 (P)	M	74	DN	2.5	0.3	39	Yes[1,3]	110	55	2	4.6	No
DN24 (P)	F	63	DN	2.9	6.5	35	Yes[1,3,4,5]	130	70	2	6.4	No
DN25 (P)	M	55	DN	2.8	7.5	34	Yes[1,3,4,5]	150	80	2	7.8	Yes
DN26 (P)	M	57	DN	1.6	1.4	66	Yes[1,2,4,5,6]	NA	NA	2	8.8	NA
DN29 (P)	M	60	DN	2.2	1.0	40	Yes[1,3,4,5]	210	90	2	6.9	No
DN30 (P)	M	75	DN	1.7	6.2	40	Yes[4,5]	160	85	2	7.3	No
DN31 (P)	F	NA	DN	1.4	10.0	60	Yes[1,4,5]	NA	NA	2	NA	NA
DN32 (P)	F	63	DN	2.5	5.5	39	Yes[2,3,4,5,6]	160	100	2	6.4	NA
mean ± SEM	11/8	61.3 ± 1.8		2.7 ± 0.3	5.2 ± 1.2	38.3 ± 3.2	23/23	148 ± 7	78 ± 3		7.7 ± 0.5	
Mild DN												
DN1 (A)	M	45	DN	0.9	0.7	97	No[1]	120	80	2	8.7	Yes
DN2 (A)	F	34	DN	1.4	0.3	51	Yes[1,4,5,6]	130	90	1	7.5	Yes
DN4 (A)	M	61	DN	1.1	4.3	84	Yes[1,3]	170	90	2	NA	Yes
DN7 (A)	M	73	DN	1.1	2.4	70	Yes[2,3]	140	80	2	NA	NA
DN8 (A)	M	62	DN	1.2	2.5	54	Yes[1,2]	170	100	2	7.8	No
mean ± SEM	4/1	55 ± 6.9		1.1 ± 0.1	2.0 ± 0.7	71.2 ± 8.8	4/5	146 ± 10	88 ± 4		8.0 ± 0.4	
Control subjects												
LD1 (A)	F	66	normal tissue	<1.1	<0.2	>100	No	NA	NA	No	NA	No
LD2 (A)	M	26	normal tissue	<1.1	<0.2	>100	No	NA	NA	No	NA	No
LD3 (A)	M	49	normal tissue	<1.1	<0.2	>100	No	NA	NA	No	NA	No
LD4 (P)	F	35	normal tissue	<1.1	<0.2	>100	No	NA	NA	No	NA	No
LD5 (P)	M	39	normal tissue	<1.1	<0.2	>100	No	NA	NA	No	NA	No
LD6 (P)	F	55	normal tissue	<1.1	<0.2	>100	No	NA	NA	No	NA	No
LD7 (P)	M	41	normal tissue	<1.1	<0.2	>100	No	NA	NA	No	NA	No
LD8 (P)	M	61	normal tissue	<1.1	<0.2	>100	No	NA	NA	No	NA	No
LD9 (P)	F	58	normal tissue	<1.1	<0.2	>100	No	NA	NA	No	NA	No
LD10 (P)	M	27	normal tissue	<1.1	<0.2	>100	No	NA	NA	No	NA	No
LD11 (P)	F	54	normal tissue	<1.1	<0.2	>100	No	NA	NA	No	NA	No
LD12 (P)	F	61	normal tissue	<1.1	<0.2	>100	No	NA	NA	No	NA	No

Table 2. Continued

Biopsy Group	Gender	Age (yr)	Histology Major Diagnosis	Creatinine (mg/dl)	Proteinuria (g/d)	GFR (ml/min)	Hypertension	RR Systolic[b]	RR Diastolic[b]	Diabetes Type	HbA_{1c} (%)	Retinopathy
Control subjects (continued)												
DD5 (P)	NA	NA	normal tissue	<1.1	<0.2	>100	No	NA	NA	No	NA	No
mean ± SEM	6/6	47.7 ± 4.0		<1.1	<0.2	>100	0/13					
MCD												
MCD3 (P)	M	16	MCD	1.2	5.4	102	Yes[1,5]	135	100	No	NA	NA
MCD5 (P)	F	57	MCD	1.1	15.0	102	No[1,5]	140	80	No	NA	NA
MCD6 (P)	M	34	MCD	1.4	9.1	84	No	120	80	No	NA	NA
MCD7 (P)	M	NA	MCD	0.6	0.6		NA	100	60	No	NA	NA
mean ± SEM	5/2	35.7 ± 20.6		1.0 ± 0.3	7.5 ± 6.1	96 ± 10.4	1/4	124 ± 9	80 ± 8			

[a]A, oligonucleotide array–based gene expression profiling; DD, deceased donor; LD, living donor; NA, not available (*i.e.*, no BP values at the time of biopsy were conveyed); P, real-time RT-PCR. In column "Hypertension," figures indicate the BP-lowering medication at the time of biopsy: 1, angiotensin-converting enzyme inhibitors; 2, angiotensin II type 1 receptor blockers; 3, β blockers; 4, calcium channel blockers; 5, diuretics; 6, other antihypertensive therapy. None of the kidney donors (controls) had arterial hypertension, because this was an exclusion criterion for kidney donation.
[b]BP before biopsy (mmHg).

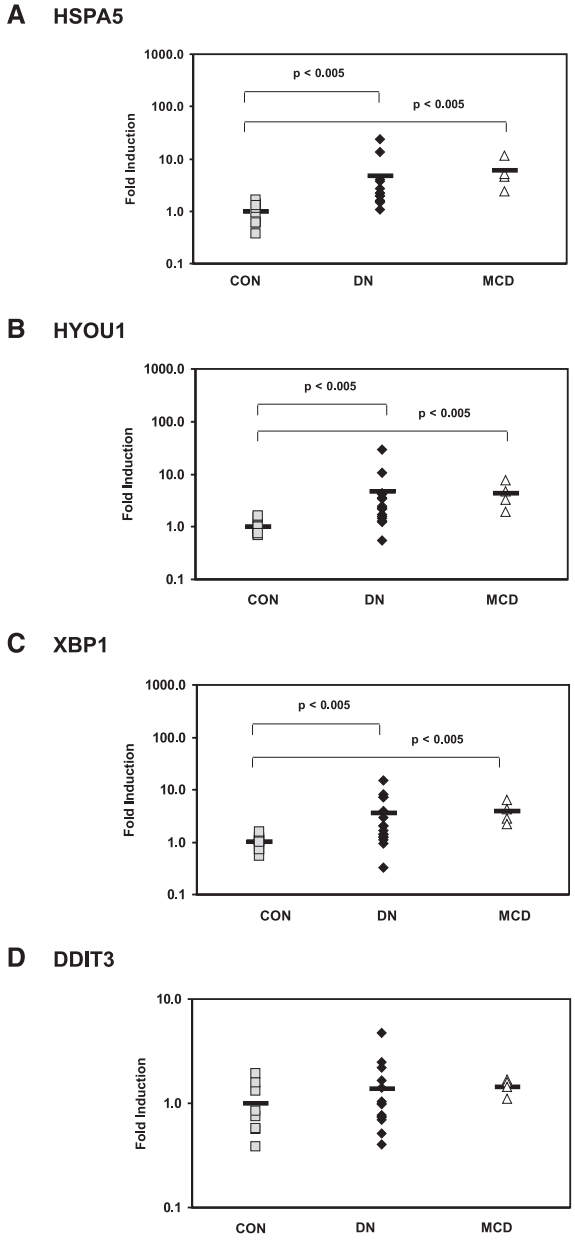

Figure 1. Evaluation of UPR genes by real-time RT-PCR. (A through D) Levels of mRNA for HSPA5 (A), HYOU1 (B), XBP1 (C), and DDIT3 (D) were quantified in microdissected tubulointerstitial compartments from control subjects (n = 10), patients with established DN (n = 15), and patients with MCD (n = 4). HSPA5, HYOU1, and XBP1 were significantly upregulated compared with control samples as indicated by the respective P values. The graphs show expression ratios of each gene normalized to all three reference genes (18S rRNA, hGAPDH, and cyclophilin A).

ent degrees. Such differences are not surprising, because both agents have distinctive modes of action: TG causes ER stress by Ca^{2+} release and TM by preventing normal glycosylation.[25,26] XBP1, induced in chronic ER stress, was not significantly induced after short-term stimulation.

Because ER stress in DN is probably a chronic process, we

Table 3. Clinical and histologic characteristics of biopsies analyzed by immunofluorescence staining for HSPA5, HYOU1, and CALR

Biopsy Group	Creatinine (mg/dl)	Proteinuria (g/d)	Glomerular No.	% Glomerular Segmental Sclerosis	% Glomerular Global Sclerosis	Mesangial Expansion (0 = Absent; 1 = Mild; 2 = Moderate-Intense)	GBM Thickness (0 = Normal; 1 = Increased)	Tubular Atrophy (0 = Absent; 1 = Present)	Interstitial Infiltration (0 = Absent; 1 = Mild; 2 = Moderate; 3 = Intense)	Interstitial Fibrosis (0 = Absent; 1 = Mild; 2 = Moderate; 3 = Severe)	Vascular Lesions
Normal kidney											
CK01	0.8	0.0	12	0	0	0	0	0	0	0	0
CK02	0.7	0.0	10	0	0	0	0	0	0	0	0
CK03	0.9	0.0	9	0	0	0	0	0	0	0	0
CK04	0.8	0.0	14	0	0	0	0	0	0	0	0
CK05	0.8	0.0	16	0	0	0	0	0	0	0	0
MCD											
MC01	0.9	6.3	8	0	0	0	0	0	0	0	0
MC02	0.8	4.8	9	0	0	0	0	0	0	0	0
MC03	0.7	8.3	10	0	0	0	0	0	1	0	0
MC04	0.8	5.2	12	0	0	0	0	0	0	0	0
MC05	1.0	4.0	13	0	0	0	0	0	0	0	0
DN											
DIA01	1.7	1.4	10	0	0	2	1	0	1	2	Hyalinosis
DIA02	1.3	2.2	12	0	0	2	1	0	0	1	0
DIA03	2.0	3.6	8	25	5	2	1	1	2	2	Hyalinosis
DIA04	1.4	1.4	10	10	0	2	1	0	1	1	Hyalinosis
DIA05	0.9	1.8	9	4	0	2	1	1	1	1	Hyalinosis

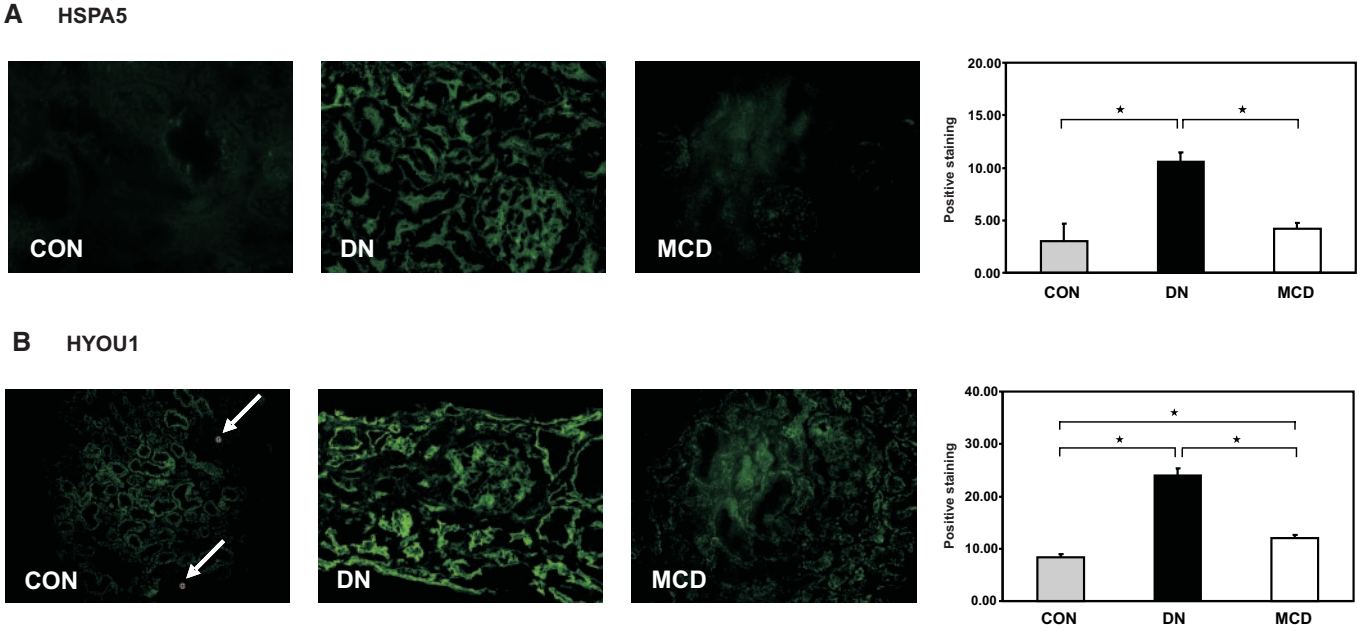

Figure 2. Immunofluorescence for HSPA5 and HYOU1 in biopsies from normal kidneys or from patients with established DN or MCD. (A) Immunofluorescence for HSPA5 is almost absent in the control kidney (CON) but is markedly increased in a biopsy from DN and displays a mild staining in MCD. (B) Immunostaining for HYOU1 shows constitutive expression in the tubulointerstitium of a control kidney (glomeruli [G, arrows] are negative) and a marked increase of intensity in DN. A mild increase of HYOU1 staining can be observed in a biopsy of MCD (indirect immunofluorescence). In addition, results from computer-assisted quantification of the stainings are shown as bar graphs. Expression of both HSPA5 and HYOU1 is higher in DN compared with controls and MCD. Staining for HYOU1 but not HSPA5 is increased in MCD compared with controls (HSPA5: CON 3.0 ± 3.7, MCD 4.2 ± 1.3 [NS *versus* CON], DN 10.6 ± 0.9 [$P <$ 0.01 *versus* CON, $P < 0.01$ *versus* MCD]; HYOU1: CON 8.4 ± 1.1, MCD 12.0 ± 1.4 [$P < 0.01$ *versus* CON], DN 24.0 ± 2.9 [$P < 0.01$ *versus* CON, $P < 0.01$ *versus* MCD]). $^{**}P < 0.01$. Magnification, ×100.

Diabetic Nephropathy

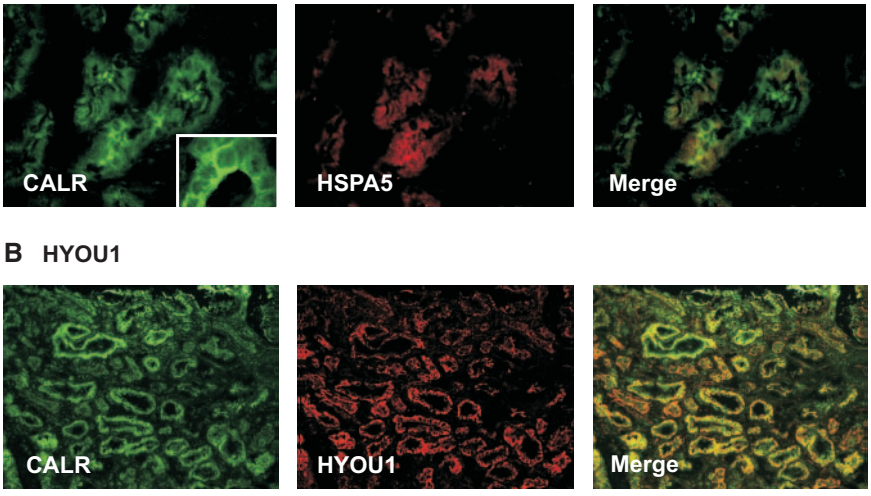

Figure 3. Immunofluorescence for CALR, HSPA5, and HYOU1 in biopsies from patients with DN. Calreticulin staining was used to localize the ER in frozen sections from kidney biopsies. (A and B) The double staining for CALR and HSPA5 (A; indirect immunofluorescence) and for CALR and HYOU1 (B; indirect immunofluorescence) shows the co-localization of the two molecules. (A insert) Granular ER staining for calreticulin is shown (DN). Magnifications: ×630 in A; ×100 in B; ×320 in A insert.

also tried to mimic a more chronic stimulation and adaptation *in vitro*; therefore, we used a long-term protocol established by Rutkowski *et al.*[27] This again resulted in stimulation of the ER stress genes HSPA5, HYOU1, and to a lesser extent XBP1. The response to TG or TM was somewhat different for the two agents and the different genes (Supplemental Figure S1), again probably because of the different modes of action. To rule out effects of TM or TG on apoptosis or cell growth, we performed apoptosis assays and counted the cells after 6 d in culture under the various conditions. Continuous treatment of HK-2 cells with low concentrations of TG (20 nM) or TM (25 ng/ml) did not result in apoptosis (Supplemental Figure S2; rate of apoptosis [$n =$ 3 per group]: Control 4.32 ± 0.23, TM 5.51 ± 0.16, TG 4.27 ± 0.40; NS). In contrast, high concentrations of TG (1 μM) or TM (2 μg/ml) resulted as expected in apoptosis already after 3 d (Supplemental Figure S2). Counting the cells after the 6-d exposure to TG (20 nM) or TM (25 ng/ml) confirmed that they survived well under

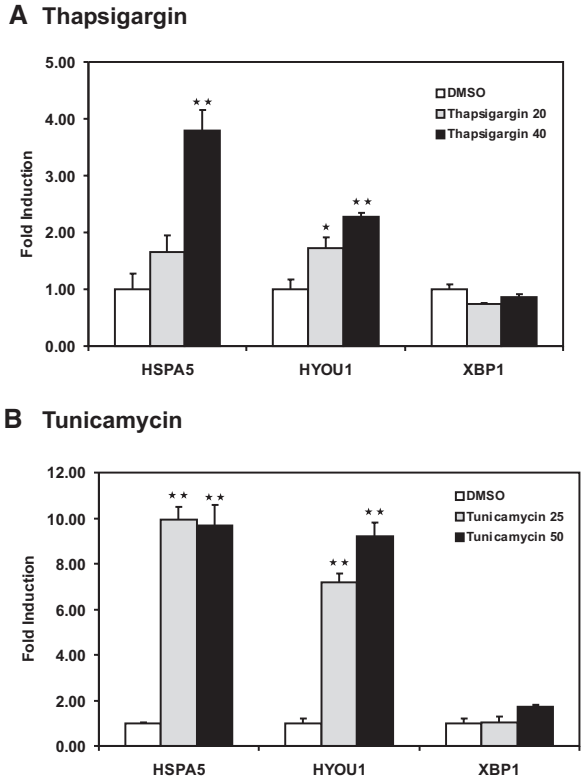

Figure 4. (A and B) Induction of ER stress in cultured HK-2 cells. Cells were treated for 24 h with 20 and 40 nM TG (A) or with 25 and 50 ng/ml TM (B) or DMSO as vehicle control (see the Concise Methods section for details). Total RNA was isolated, and the expression of HSPA5, HYOU1, and XBP1 was quantified by real-time RT-PCR. Data represent means of fold changes normalized against the respective controls ($n = 3$ for each condition). *$P < 0.05$, **$P < 0.01$ *versus* DMSO.

low concentrations of these agents (cell numbers at 6 d [$n = 3$ per group]: DMSO 2,139,167 ± 179,260, TM 1,920,833 ± 22,654, TG 1,910,000 ± 7638).

Albumin and Glucose Induce an Adaptive UPR Response In Vitro

Our biopsy results indicated a stronger ER stress response in established DN with proteinuria and hyperglycemia than in MCD. We attempted to mimic these conditions *in vitro* by culturing HK-2 cells for 6 d with various concentrations of albumin (50 and 100 μg/ml) and glucose (7.75 and 30.00 mM). As an osmotic control, we added 22.25 mM mannitol to 7.75 mM glucose. High glucose concentrations increased the mRNA levels of the three major ER genes HSPA5, HYOU1, and XBP1. Albumin alone increased only HSPA5 expression but enhanced the response of all three genes in the presence of high glucose (Figure 5). Mannitol alone or in combination with albumin did not consistently change the expression of the three genes (data not shown). Taken together, these *in vitro* data help to explain why *in vivo* the coexistence of proteinuria and hyperglycemia results in a more pronounced ER stress response in DN than in MCD.

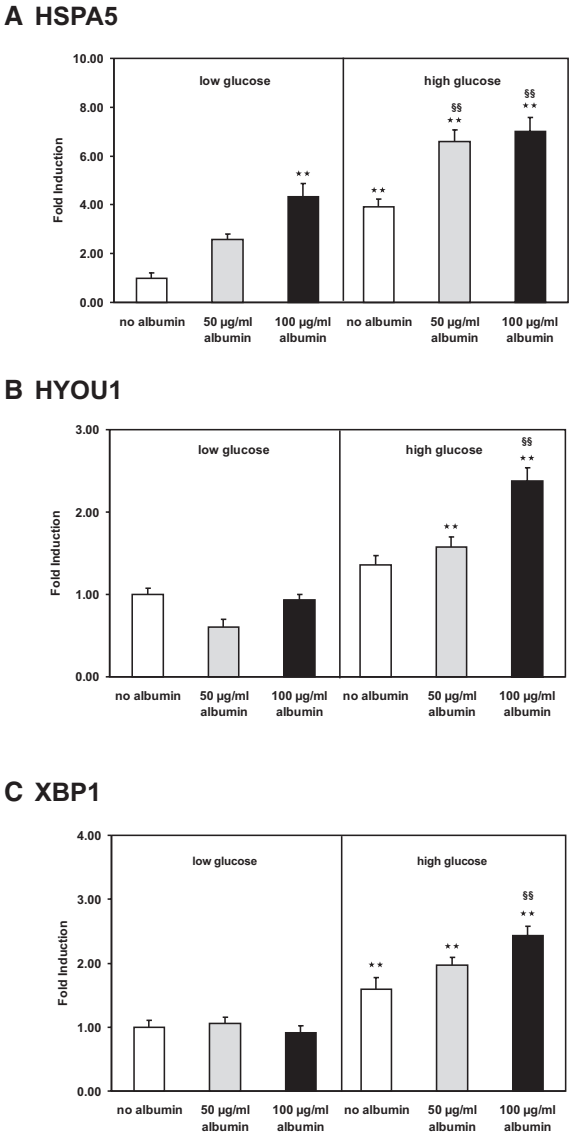

Figure 5. Effects of high glucose and albumin on the expression of ER stress genes in HK-2 cells. HK-2 cells were cultured for 6 d with 7.75 or 30.00 mM glucose alone or with addition of various concentrations of human albumin. (A through C) The mRNA expression of HSPA5 (A), HYOU1 (B), and XBP1 (C) was analyzed by real-time RT-PCR (data normalized to 18S rRNA are shown). The data represent means of fold changes normalized against control condition (0 d, 7.75 mM glucose, no albumin; $n = 13$ for each condition). **$P < 0.01$ *versus* low glucose, no albumin; §§$P < 0.01$ *versus* high glucose, no albumin.

DISCUSSION

Persistent proteinuria is considered a strong prognostic indicator for progression of renal disease in general and especially of DN. Furthermore, the degree of tubulointerstitial fibrosis correlates best with progression in any form of renal disease. On the basis of these observations, it has been proposed that proteinuria is not only a marker of prognosis but also may directly contribute to fibrosis and renal insufficiency.[5,28] In this

study, we tested the hypothesis that UPR of ER stress would be induced in the kidneys of patients with proteinuria secondary to DN. Indeed, we observed in the tubulointerstitial compartment of biopsies from patients with established DN a marked increase in mRNA expression of the UPR genes HSPA5, HYOU1, and XBP1. This was not found in patients with mild DN, so the increased expression of UPR genes was associated with the degree of both clinical and pathologic involvement of the kidneys. In contrast to the increase in adaptive UPR genes, genes involved in the proapoptotic pathway of the UPR were not induced. Supporting the mRNA results, we found increased protein levels of HSPA5 and HYOU1 by immunofluorescence in patients with established DN. In biopsies from patients with MCD, an increase in mRNA levels for HSPA5, HYOU1, and XBP1 was also noted, but on the protein level, only HYOU1 staining was increased as compared with controls. Even the staining for HYOU1 in MCD was significantly less than that observed in DN. The somewhat discrepant findings between the levels of mRNA and those for protein expression (as analyzed by immunohistology) are consistent with similar observations in other systems and are probably due to posttranscriptional changes occurring with mRNA translation into protein. Because the biologic effects are mostly dependent on the protein expression, we interpret these findings as indicating a more pronounced ER stress response occurring in renal tubules from established DN than in those from MCD. Thus, the data on the mRNA levels and the immunohistology show an overall association between the degree of ER stress response and the clinical and pathologic parameters of nephropathy.

Because proteinuria and hyperglycemia might be factors contributing to the observed ER stress response in the biopsies, we mimicked an adaptive ER stress response in HK-2 cells by exposure to low concentrations of the pharmacologic ER stress inducers TM and TG and compared the response with that by exposure for 6 d to high glucose (30 mM) and the addition of albumin (50 to 100 μg/ml). Because both glucose and albumin, especially the combination of both, enhanced a protective type of ER stress response *in vitro*, we propose that these factors may, among others, contribute to the ER stress response observed in the renal tubules of biopsies from patients with established DN and to some extent also in MCD.

On the basis of rodent experiments and cell culture, ER stress has been proposed as one of the various mechanisms contributing to cellular damage and apoptosis of podocytes and tubular epithelial cells in renal diseases.[29] In support of this hypothesis, renal cell culture experiments had been performed with TM or by addition of very high concentrations of albumin (10 to 100 mg/ml), corresponding or even exceeding plasma levels. This ER stress induction in cultured podocytes led to upregulation of HYOU1 and HSPA5.[20,21] Such increase in the ER stress proteins was also observed in transgenic rats with podocyte damage,[20] in rat tubular epithelial cells exposed to high concentrations of albumin,[21] and in the tubular epithelium of rats with proteinuria.[30] The authors concluded that tubular protein overload causes ER stress and apoptosis and thereby contributes to tubulointerstitial disease in nephrotic syndrome.[20]

Conversely, the UPR is designed to restore the balance in the ER between increased protein synthesis in the ER and its ability to handle this extra load. Only if attempts to restore the balance fail will UPR initiate apoptosis. In favor of such an interpretation are *in vitro* and *in vivo* ischemia-reperfusion experiments in MDCK cells and in mice.[31] *In vitro* overexpression of HYOU1 protected cells against hypoxia and osmotic stress. *In vivo* ischemia-reperfusion damage in mice was more profound with reduced HYOU1 and was less severe with high HYOU1.[31] Thus, upregulation of UPR genes that we observed in DN may represent a protective response. Although the microarray data showed also a slight but significant induction of the ER genes CANX and MBTPS1, this could not be confirmed by quantitative RT-PCR. These genes are not routinely evaluated in ER stress, most likely because their induction is less consistent.[10,14,20,21]

The view that persistent ER stress favors the apoptotic pathway is based on cell culture studies in which severe pharmacologic ER stress induced apoptosis.[21,31–35] Convincing experimental evidence was provided that moderate ER stress may favor an adaptive and protective rather than a proapoptotic UPR.[27] This view would conform to the slow and prolonged time course of diseases in which ER stress has been invoked as a pathophysiologic contributor (*e.g.*, chronic hepatitis, diabetes, neurodegenerative diseases). These considerations may also apply to proteinuric renal diseases in general and especially to DN. Low concentrations of the classical ER stress inducers TM and TG led to an adaptive rather than a proapoptotic response in renal tubular cells, whereas only high concentrations resulted in apoptosis. Furthermore, exposure of the cells to albumin concentrations that might be present in the ultrafiltrate of patients with nephrosis (*i.e.*, 10 to 100 μg/ml) did not affect cell viability; however, high glucose levels *per se* increased the expression of adaptive UPR genes, a response further enhanced by concomitant albumin exposure.

Hypothetically, exposure to albumin and glucose could induce ER stress by generating free radicals, by aberrant protein glycosylation, or by increased membrane and protein turnover. In patients with proteinuria and hyperglycemia, elevated glucose and tubular protein and lipid reabsorption may generate reactive oxygen species and require a marked increase in the synthesis of membrane proteins in the kidney. This may result in local ER stress. If this were not counterbalanced by an adaptive UPR with upregulation of genes such as HSPA5, HYOU1, and XBP1, then the ER would be overwhelmed and initiate apoptosis as the ultimate UPR. That progression of DN usually occurs after years may be a tribute to the effectiveness of the adaptive UPR. Nonetheless, the adaptive UPR may eventually be overcome and then lead to a proapoptotic rather than an adaptive response. Future research could be directed toward exploring potential therapeutic interventions that would

decrease ER stress and at the same time strengthen the adaptive UPR, thereby favoring renal tubular cell survival over demise.

CONCISE METHODS

Renal Biopsies for mRNA Analysis

Human renal biopsy specimens were procured in an international multicenter study, the European Renal cDNA Bank–Kröner-Fresenius Biopsy Bank (ERCB-KFB; see Acknowledgments for participating centers). Biopsies were obtained from patients when clinically indicated and were molecularly analyzed after informed consent and with approval of the local ethics committees. To identify molecules associated with adverse clinical and pathologic features, we studied four groups of patients: Established DN (serum creatinine $\geq$1.4 mg/dl), mild DN (serum creatinine $<$1.4 mg/dl), MCD, and pretransplantation biopsies. Clinical characteristics of all patients are shown in Table 2.[22] For validation of the microarray data, real-time RT-PCR on biopsies from an independent cohort of patients with DN ($n = 15$), MCD ($n = 4$), and control subjects (living donors $n = 9$; deceased donor $n = 1$) was performed (Table 2).

Microdissection and RNA Isolation

After renal biopsy, the tissue was transferred to RNase inhibitor and microdissected into glomerular and tubular fragments. Total RNA was isolated from microdissected tubulointerstitial tissue (for details, see reference[36]).

Target Preparation

A total of 300 to 800 ng of total RNA was reverse-transcribed and linearly amplified according to a protocol previously reported.[22] The fragmentation, hybridization, staining, and imaging were performed according the Affymetrix Expression Analysis Technical Manual.

For microarray analysis, Robust Multichip Analysis was performed. Subsequently, we analyzed the expression arrays with Significance Analysis of Microarrays.[37] For more details and for gene expression data of respective probe sets, see http://diabetes.diabetesjournals.org/cgi/content/full/55/11/2993.

Quantitative Real-Time PCR

Reverse transcription and real-time RT-PCR were performed as reported previously.[36] Predeveloped TaqMan reagents were used for human CANX (NM_001024649.1), HSPA5 (NM_005347.2), HYOU1 (NM_006389.2), MBTPS1 (NM_003791.2), XBP1 (NM_005080.2), and DDIT3 (NM_004083.4) as well as the reference genes (Applied Biosystems, Darmstadt, Germany). The expression of candidate genes was normalized to the mean of three reference genes, GAPDH, 18S rRNA, and cyclophilin A. The mRNA expression was analyzed by standard curve quantification.

Immunofluorescence

For immunofluorescence, the unfixed renal tissue was embedded in optimal cutting temperature compound (Tissue-Tek; Società Italiana Chimici, Rome, Italy), snap-frozen in a mixture of isopentane and dry ice, and stored at -80°C. Subsequently, 5-μm sections were placed on slides and stored at -20°C until immunostained. For a summary of clinical data, see Table 3. Cryosections were fixed in cold acetone, rinsed, and sequentially incubated with the primary antibody: Mouse anti-HSPA5 (Abnova Corp., Taipei, Taiwan), mouse anti-HYOU1 (Abnova), rabbit anti-CALR (Abcam, Cambridge, UK), followed by the proper fluorescence-tagged secondary antibody (AlexaFluor 488 goat anti-mouse or goat anti-rabbit IgG; Invitrogen, Milan, Italy). For double stainings, sections were first incubated with the first primary antibody (rabbit anti-CALR, followed by AlexaFluor 488 goat anti-rabbit); after adequate washing, the procedure was repeated with the second primary antibody (anti-HYOU1 or anti-HSPA5 followed by AlexaFluor 546 goat anti-mouse). Sections were mounted with antifading mounting medium (Vectashield; Vector Laboratories, Burlingame, CA). Specificity of labeling was demonstrated by the lack of staining after substituting PBS and proper control IgG (rabbit primary antibody isotype control and mouse primary antibody isotype control, both from Invitrogen) for the primary antibody.

Quantification of Immunofluorescence Results

Images were acquired by a Zeiss Axioscope 40FL microscope, equipped with AxioCam MRc5 digital video camera and immunofluorescence apparatus (Carl Zeiss SpA, Arese, Italy). Images were recorded using AxioVision software 4.3 and analyzed by the AxioVision analysis module (Carl Zeiss SpA). A color threshold procedure allowed selective highlighting of the stained areas in gray mode, and the software was programmed to calculate automatically the percentage of the area occupied by staining.

Cell Culture Experiments

HK-2 cells (ATCC CRL-2190), proximal tubular epithelial cells derived from normal human kidney tissue, were cultured in DMEM/F-12 supplemented with 10% FCS, 1% ITS, hydrocortisone (36 ng/ml), penicillin (100 U/ml), and streptomycin (100 μg/ml). The cells were incubated at 37°C.

For TM and TG (Calbiochem, EMD Chemicals, San Diego, CA) experiments, stock solutions were made in DMSO so that the final concentration of DMSO was 0.1%. For the vehicle controls, DMSO alone was added to a final concentration of 0.1%. Acute ER stress was induced by the addition of TG or TM in the given concentrations for 24 h (Figure 4). A more chronic model of ER stress was induced as described previously[27]: Cells were cultured for 8 d with TM (25 ng/ml), TG (20 nM), or DMSO (0.1%), with replacement of the media every 24 h. After 8 d of incubation, cells were plated at a concentration of 2×10^5 cells/well in six-well dishes and allowed to rest overnight before re-application of the stressor for the indicated time (see Supplemental Figure S1).

Apoptosis was analyzed by Annexin V/propidium iodide staining and FACS analysis (Supplemental Figure S2). After incubation of HK-2 cells for 6 d with DMSO, 25 ng/ml TM, or 20 nM TG, cells were washed twice with PBS, resuspended in binding buffer (10 mM HEPES [pH 7.4], 140 mM NaCl, and 2.5 mM $CaCl_2$) and then stained with Annexin V–FITC and propidium iodide from BD Pharmingen (Erembodegem, Belgium) according to the manufacturer. Samples were immediately analyzed by flow cytometric analysis using a BD

FACSCanto (Becton Dickinson, Franklin Lakes, NJ). Cell numbers were determined in a hemocytometer cell count chamber.

For the *in vitro* experiments with addition of glucose, mannitol, and albumin, HK-2 cells (2×10^5 cells/well) were transferred to six-well plates and grown to 80% confluence, growth-arrested (FCS deprivation), and then exposed to albumin (50 or 100 μg/ml) at 7.75 and 30.00 mM glucose or 7.75 mM glucose and 22.25 mM mannitol concentrations for various time periods (0 and 6 d) at 37°C. The media with the respective additions contained no FCS and were changed every other day. Total cellular RNA was extracted using the Qiagen RNeasy kit. The mRNA expression was analyzed by real-time RT-PCR as already described.

Statistical Analysis

Data are given as means ± SEM. Statistical analysis was performed using Kruskal-Wallis, Mann-Whitney *U* tests, and ANOVA followed by Tukey test as appropriate (SPSS 14.0; SPSS, Chicago, IL). $P < 0.05$ was considered to indicate statistically significant differences.

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DISCLOSURES

None.

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