Cell surface expression of 78 kDa glucose regulated protein (GRP78) mediates diabetic nephropathy

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Running title: csGRP78 is a mediator of diabetic nephropathy

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ABSTRACT

The 78 kDa glucose-regulated protein (GRP78) is a well-established endoplasmic reticulum (ER)-resident chaperone that maintains protein homeostasis and regulates the unfolded protein response. Under conditions of ER stress, GRP78 is also expressed at the cell surface and implicated in tumorigenesis, immunity, and cellular signaling events. The role of cell surface-associated GRP78 (csGRP78) in the pathogenesis of diabetic nephropathy has not yet been defined. Here, we explored the role of csGRP78 in regulating high glucose (HG)-induced profibrotic AKT Ser/Thr kinase (AKT) signaling and upregulation of extracellular matrix (ECM) proteins. Using primary kidney mesangial cells (MCs), we show that HG treatment, but not the osmotic control mannitol, induces csGRP78 expression through an ER stress-dependent mechanism. We found that csGRP78, known to be located on the outer membrane leaflet, interacts with the transmembrane protein integrin β1 and activates focal adhesion kinase (FAK) and downstream phosphoinositide 3-kinase (PI3K)/AKT signaling. Localization of GRP78 at the cell surface and its interaction with integrin β1 were also required for ECM protein synthesis in response to HG. Surprisingly, both the N and C termini of csGRP78 were necessary for this profibrotic response. Increased localization of GRP78 at the plasma membrane was also found in the glomerular mesangial area of type 1 diabetic mice in two different models (streptozotocin-induced and Akita). In freshly isolated glomeruli from Akita mice, csGRP78 co-localized with the mesangial cell-surface marker α8-integrin. In conclusion, our work reveals a role for csGRP78 in HG-induced profibrotic responses in MC, informing a potential approach to treating diabetic nephropathy.
Diabetic nephropathy (DN) is a major complication of diabetes and the leading cause of kidney failure in North America. It is characterized by glomerulosclerosis (1), with glomerular mesangial cells (MC) known to play a key role in its pathogenesis through their synthesis of extracellular matrix (ECM) proteins in response to high glucose (HG) (2). Recent research has indicated that the 78 kDa glucose-regulated protein 78 (GRP78) may be translocated to the cell surface and play a pathological role in the development of various diseases such as cancer, inflammatory and immune conditions and atherosclerosis (3-5). The relevance of cell surface GRP78 (csGRP78) in DN has not been previously examined.

GRP78 was originally described as an ER resident molecular chaperone that assists in the proper folding of de novo proteins in the ER (5). It is ubiquitously expressed in mammalian cells and acts to control ER stress through regulation of the unfolded protein response (5). In response to agents and/or conditions that elicit ER stress, GRP78 was also reported to translocate to the cell surface through association with the co-chaperone proteins MTJ-1 or Par-4 (6-8). At the cell surface, GRP78 acts as a signaling receptor for agonists such as α2-macroglobulin and anti-GRP78 autoantibodies. Activation of csGRP78 promotes its association with other cell surface proteins leading to intracellular signaling events, the nature of which depends on the cell type and interacting partners (9). Expression of csGRP78 has been described in a wide range of cell types, predominantly in association with various cancers, and has been suggested to drive tumorigenesis, immunity and cellular signaling events (9).

Induction of ER stress and elevated cellular expression of GRP78 are well recognized features of rodent and human DN (10,11). However, whether cell surface expression of GRP78 is increased and contributes to DN is unknown. Our results show that in response to hyperglycemia, GRP78 is translocated to the cell surface in MCs. In support of these findings, csGRP78 is also present in the glomeruli of diabetic mice. In terms of its contribution to DN, csGRP78 drives the production of ECM by HG. These data suggest that csGRP78 represents a novel potential therapeutic target for DN.

Results

High glucose induces translocation of GRP78 to the cell surface

The translocation of GRP78 to the cell surface has been noted under conditions of ER stress in carcinoma, endothelial, liver, and immune cells (9), but has not yet been assessed in diabetic kidneys or MC. Using a biotinylation/streptavidin pulldown technique, we first sought whether we could identify cell surface expression of GRP78. Figure 1A shows that HG induced persistent cell surface expression of GRP78, seen as early as 3 hours. The cell surface protein platelet-derived growth factor receptor (PDGFR) was used as a loading control. For subsequent experiments, csGRP78 was assessed after 24h of HG. Figure 1B shows that only HG, but not the osmotic control mannitol, induced cell surface expression of GRP78. We next used flow cytometry to confirm that HG induces expression of GRP78 at the cell surface (Figure 1C,D).

Cell surface translocation of GRP78 has been suggested to occur due to ER stress, although its movement to the surface can occur independently of ER stress (12). Hyperglycemia has been shown to induce ER stress in MCs (13,14), and markers of ER stress have been identified in both diabetic animals and humans (10,11). Similar to these studies, we found that HG induced ER stress in MC, characterized by the phosphorylation of eIF2α at early time points (3-12 hours), as well as the expression of CHOP and GRP78 (Figure 1E). Furthermore, we found that HG-induced cell surface expression of GRP78 was dependent on ER stress. Figure 1F shows that the ER stress inhibitors 4-PBA and salubrinal, which have both been previously shown to inhibit HG-induced ER stress (15,16), attenuate HG-induced cell surface GRP78 expression. Lastly, the protein DnaJ-like protein 1 (MTJ-1) has been shown to act as a co-chaperone for the translocation of GRP78 to the cell surface (7). We found that knockdown of MTJ-1 prevented the translocation of GRP78 to the cell surface in response to HG (Figure 1G). These data demonstrate that in MC, HG induces the cell surface expression of GRP78 in an ER-stress dependent manner through MTJ-1.

Cell surface GRP78 is required for activation of FAK and Akt in response to high glucose
FAK and Akt were shown to be downstream mediators of csGRP78 signaling in cancer cells (17-19) and we previously demonstrated that they are both important mediators of the HG-induced profibrotic response in MC (20-22). We thus used three distinct methods to inhibit csGRP78 to determine whether this would attenuate FAK and Akt activation by HG. Antibodies which bind to either the N-terminal or C-terminal portion of GRP78 are commonly used to assess effects on csGRP78 signaling (23,24). In cancer cells, C-terminal binding antibodies have been reported to inhibit Akt activation and induce apoptosis (23,25), whereas N-terminal binding antibodies promote Akt activation, survival and proliferation (26). Surprisingly, antibodies targeting either the N- or C-terminus of GRP78 attenuated FAK and Akt activation in response to HG, as assessed by their phosphorylation on Y397 and S473, respectively (Figure 2A). Of note, using an LDH assay we did not detect any apoptosis in MC treated with either antibody (not shown). The enzyme SubA is a cell-impermeable proteinase that selectively cleaves the C-terminus of cell surface GRP78, whereas the SubAA272 mutant lacks proteinase activity and serves as a control for the active enzyme (27). In line with our previous results, SubA, but not the mutant form, attenuated HG-induced FAK and Akt activation (Figure 2B). Since we and others have shown that MTJ-1 is required for the translocation of GRP78 to the cell surface (7), we tested the effects of MTJ-1 knockdown on the activation of Akt and FAK. Knockdown of MTJ-1 using siRNA also attenuated HG-induced FAK and Akt activation (Figure 2C). Together, our data suggest that csGRP78 is an upstream regulator of FAK and Akt activation in response to HG in MCs.

csGRP78 interaction with integrin β1 mediates FAK and Akt activation by HG

Integrin β1 is a cell surface receptor that mediates cell-matrix adhesion through formation of focal adhesion points (28). In colorectal tumor cells, csGRP78 interaction with integrin β1 promoted FAK activation, cell migration and invasion (19). Furthermore, HG was shown to activate integrin β1 in MC (29). We thus assessed whether integrin β1 mediated FAK and Akt activation downstream of csGRP78 in response to HG. We first determined whether csGRP78 and integrin β1 interacted. Since coimmunoprecipitation following cell surface protein biotinylation is challenging, requiring harsh conditions to release the biotin bond (30), we isolated plasma membrane proteins as described in Methods. From this, we immunoprecipitated GRP78 and assessed for interaction with integrin β1 by immunoblotting. Figure 3A shows that HG increased association between integrin β1 and GRP78 derived from the plasma membrane fraction. This was associated with an increased amount of GRP78 pulled down from plasma membrane preparations after HG treatment, consistent with increased csGRP78 in response to HG as seen in Figure 1.

To further confirm an association between csGRP78 and integrin β1, we incubated live cells with the GRP78 antibody after HG treatment to immunoprecipitate csGRP78. Figure 3B shows increased GRP78/integrin β1 association, which was also confirmed by reverse immunoprecipitation with the integrin β1 antibody in Figure 3C. Since antibodies targeting either the N- or C-terminus of GRP78 attenuated FAK and Akt activation, we next determined their effects on HG-induced integrin β1-csGRP78 association. Figure 3D shows that the C-terminal antibody C20 prevents their association, while the N-terminal antibody N20 was only partially effective. Further confirming that the C-terminus of GRP78 is required for this association, Figure 3E shows that SubA also prevents integrin β1-csGRP78 association in response to HG. These findings suggest an important role for the C-terminus of csGRP78 in mediating FAK and Akt activation through association with integrin β1, while contribution from the N-terminus of csGRP78 is independent of this integrin.

Finally, to confirm that integrin β1 is required for FAK-Akt activation in response to HG, we determined the effect of inhibiting integrin β1 with the CD29 neutralizing antibody. Figure 4A shows that this attenuated HG-mediated activation of both FAK and Akt. However, the non-neutralizing antibody to integrin β1 had no effect on either Akt or FAK activation by HG (Figure 4B), suggesting that integrin β1 activity in addition to its interaction with csGRP78 is required for downstream signaling. Figure 4C confirms that inhibition of FAK with PF573228...
csGRP78 is a mediator of diabetic nephropathy

Prevents HG-induced Akt activation. These data support an important role for csGRP78 interaction with integrin β1 in the downstream activation of FAK and Akt.

csGRP78 mediates the expression of ECM protein in response to HG

Accumulation of ECM in the glomerulus is a classic pathological hallmark of DN (1). In response to HG, MCs produce ECM proteins present in diabetic glomeruli including collagens and fibronectin (20,31). We previously showed that FAK-Akt signaling is required for this profibrotic response (20,21). We thus assessed the effects of blocking csGRP78 on HG-induced ECM upregulation. Figure 5A shows that both collagen I and fibronectin production in response to HG were blocked by pretreatment with either N- or C-terminal targeting csGRP78 antibodies, with no effect seen when using a control IgG (Figure 5A). Similarly, SubA cleavage of the C-terminus of csGRP78 or knockdown of the chaperone protein MTJ-1 prevented HG-induced collagen I and fibronectin upregulation (Figures 5B,C). These results demonstrate an important role for csGRP78 in HG-induced induction of ECM protein synthesis in MCs.

Cell surface GRP78 is upregulated in kidneys of type 1 diabetic mice

To assess the in vivo relevance of csGRP78 to DN, we sought to determine whether GRP78 was translocated to the cell surface in diabetic kidneys. We examined kidneys in two models of type 1 diabetes, that induced by streptozotocin in CD-1 mice and in the genetic model Akita mice. Our previous studies showed that induction of type 1 diabetes in CD-1 mice with streptozotocin produces robust changes of DN (32). After 12 weeks of diabetes in this model, we assessed expression of GRP78 by IHC. Figure 6A shows an increase in plasma membrane GRP78 in mesangial areas of glomeruli in diabetic kidneys. The staining pattern suggested expression of GRP78 at the plasma membrane as indicated by black arrows. To provide further evidence of expression of GRP78 at the plasma membrane in diabetic mice, we colocalized the expression of GRP78 with the plasma membrane marker WGA in non-permeabilized kidney sections by immunofluorescence. Areas showing colocalization between GRP78 and WGA are expressed as white in the mask. We found that there was increased colocalization between GRP78 and WGA in the diabetic mice, suggesting that GRP78 is upregulated at the plasma membrane during the pathogenesis of DN (Figure 6B). Of interest, we noted that WGA primarily stained the glomeruli. This has been demonstrated previously and is hypothesized to be due to binding of WGA to sialic acids that are a major component of glomerular polyanions (and thus the glomerular filtration barrier) (33).

To confirm these findings in CD-1 mice, we next assessed a second model of type 1 diabetes. Akita mice carry a mutation in the Ins2 gene leading to improper formation of insulin and pancreatic β-cell injury, resulting in type 1 diabetes (34). Similar to the CD-1 diabetic mice, Akita mice demonstrated overall increased expression of GRP78 in their glomeruli as well as surrounding tubules, as determined by IHC (Figure 7A). Plasma membrane GRP78 expression was next assessed, as above, through colocalization between GRP78 expression and WGA staining in non-permeabilized samples by immunofluorescence. Similar to the CD-1 diabetic mice, plasma membrane GRP78 expression was increased in glomeruli of diabetic mice (Figure 7B). Finally, to further confirm these findings, we stained live kidney tissue obtained from freshly isolated wild-type and Akita kidneys. Here we used α8-integrin as a cell surface marker for MCs which was also thus used to identify glomeruli (35,36). Figure 8 shows that csGRP78 was increased in the mesangium of diabetic glomeruli. Areas of co-staining between GRP78 and α8-integrin were identified as white, the quantification of which is shown in the accompanying graph. Together, our data show that csGRP78 is increased in the mesangium of diabetic kidneys.

Discussion

Presentation of GRP78 on the cell surface has been identified in a wide number of cells including various cancers, endothelial cells, and macrophages, and has been suggested to play an important role in cell signaling, viral entry, and antigen presentation (37). Our study now shows that kidney MCs express GRP78 on the cell...
surface in response to HG, as well as in diabetic kidneys. Importantly, csGRP78 mediates HG-induced matrix production through FAK/Akt activation (Figure 9). These novel findings establish a potentially highly significant role for csGRP78 in the pathogenesis of DN.

Previous studies have shown that HG induces the expression of total GRP78 in kidney cells (38), but cell surface expression had not been assessed. Our results now demonstrate that HG induces the cell surface presentation of GRP78, and that this is mediated by ER stress. ER stress induction is clearly implicated in the development of DN, and has been shown to occur due to numerous stimuli including free fatty acids, oxidative stress and advanced glycation end products (39). Although an increase in csGRP78 independently of ER stress has also been described (12), an important role for ER stress in the induction of csGRP78 has most commonly been identified (12,40). Recently, Tsai et al. discovered that ER stress activated the kinase Src, resulting in dispersion of the KDEL receptor in the Golgi and thereby leading to elevated csGRP78 (40). Whether inhibition of ER stress or Src in vivo impacts the presentation of GRP78 on the cell surface needs to be investigated.

Cell surface GRP78 regulation of Akt activation has been shown predominantly in cancer cells, but also in endothelial cells (41-43). Binding of anti-GRP78 autoantibodies or the activated antiproteinase alpha 2 macroglobulin (α2M) to the N-terminus of GRP78 promotes Akt activation, while autoantibody binding to the C-terminus is inhibitory (9,23,41). Our data also show a major role for csGRP78 in the regulation of HG-induced activation of Akt. In keeping with other studies, our results show that targeting the N-terminal domain of csGRP78 using the N20 antibody attenuates HG-induced Akt activation. Surprisingly, targeting the C-terminal domain using the C20 antibody also attenuates Akt activation to a similar extent. This suggests that in MC, the N- and C-terminal domains of csGRP78 both contribute importantly to regulate Akt activation in response to HG. This may represent a requirement for two different stimuli to activate csGRP78, such as an N-terminal autoantibody or α2M and C-terminal interaction with a transmembrane binding partner like integrin β1, discussed below. Alternatively, inhibitory antibody binding to the C-terminus or C-terminus cleavage with SubA may affect GRP78 topology at the cell surface which leads to its inability to initiate intracellular signaling in response to HG. Supporting this hypothesis, Misra et al. have shown that targeting the C-terminal domain of GRP78 attenuated α2M-mediated activation of csGRP78 (23). The detailed mechanism by which N- and C-termini of csGRP78 contribute to HG-induced signaling, however, requires further study. Furthermore, whether there is a role for csGRP78 ligands, such as α2M or autoantibodies against GRP78, in HG-induced signaling through csGRP78 also needs to be addressed.

Interaction of csGRP78 with a cell surface-anchored or transmembrane protein such as integrin β1 is likely important in promoting intracellular signaling (9,19). In colon cancer cells, csGRP78 interaction with integrin β1 led to activation of the focal adhesion protein FAK(19). Our results similarly show that HG induces interaction between csGRP78 and integrin β1, with the latter an important upstream mediator of glucose-induced FAK, as well as Akt, activation. FAK is a well-known mediator of the Akt activator phosphatidyl-inositol-3 kinase (PI3K)(44), and our data confirm that FAK is required for HG-induced Akt activation in MC. Taken together, our results support an important role for csGRP78 interaction with integrin β1 in the activation of FAK and Akt, which we have previously shown mediates downstream synthesis of matrix proteins (28,29).

Cell surface GRP78 is primarily expressed under pathologic conditions, and has thus far been demonstrated in various cancers and in atheromatous lesions (3,45-47). Although a role for cell surface GRP78 in DN has not as yet been established, several studies have suggested that csGRP78 expression may be induced by diabetes. Rondas et al. identified autoantibodies against the post-translationally citrullinated GRP78 found in pancreatic β-cells of non-obese diabetic mice (48). Although their pathogenic role in type 1 diabetes remains to be investigated, production of autoantibodies against GRP78 has been positively associated with increased expression of csGRP78 (46). Infection of endothelial cells by the fungus Rhizopusoryzae, commonly responsible for infections in patients with diabetic ketoacidosis, was found to be mediated by csGRP78. Treatment
with a GRP78 antibody (in endothelial cells challenged with glucose) and anti-GRP78 sera (in mice with diabetic ketoacidosis) reduced infection and improved survival, respectively (49). Interestingly, the adipokine and serine protease inhibitor vaspin was shown to act as a ligand for csGRP78 in obese diabetic mouse liver (50). Overexpression of vaspin improved features of the metabolic syndrome in mice fed a high fat, high sucrose diet (50). Finally, the GRP78 targeting peptide ADoPep increased weight gain and improved glycemic control in type I diabetic mice (51). These studies suggest that csGRP78 may be induced during diabetes in various tissues, although direct evidence for increase of csGRP78 in these murine models has not been provided. For the first time, our study shows that GRP78 is found at the plasma membrane in the kidneys of type I diabetic mice, and by IHC and IF these appeared to localize to mesangial cell areas. Since concerns exist using IHC and IF when determining cell surface staining due to the effects of fixation and freezing on the integrity of the cell surface in tissue, we verified our IHC and IF results with live tissue staining on freshly isolated and unfixed kidneys. Furthermore, using a mesangial cell plasma membrane marker (α8 integrin), we confirmed colocalization with csGRP78 in the mesangial cells of glomeruli.

A role for csGRP78 in matrix synthesis has not previously been demonstrated. However, in cancer cells, csGRP78 was shown to regulate signaling by TGFβ1, a profibrotic cytokine known to be a central regulator of matrix synthesis and fibrosis in diabetic nephropathy (52). TGFβ1 exerts important tumor suppressive effects, and as such csGRP78 inhibits TGFβ1 signaling in cancer cells, primarily through its association with Cripto, a small GPI-anchored protein and negative regulator of TGFβ1 (53). Tsai et al. have also demonstrated that csGRP78, through its association with another GPI-anchored protein CD109, prevents TGFβ1 signaling in HeLa cells by routing its receptor to caveolae for degradation (40). The role of csGRP78 in the regulation of TGFβ1 in primary cells, however, is likely to differ. The establishment of TGFβ1 as an important mediator of HG-induced matrix upregulation in MC (54), coupled with our findings that csGRP78 is required for HG-induced matrix synthesis, strongly suggest that csGRP78 promotes TGFβ1 effects, at least in MC. Indeed, we previously showed that Akt mediates TGFβ1 synthesis in response to HG (20-22). It is thus likely that csGRP78, through Akt activation, mediates TGFβ1 synthesis in this setting, although this requires further study.

Collectively, our data reveal csGRP78 as a novel regulator of fibrosis in response to HG through its regulation of FAK/Akt signaling. In vivo relevance is supported by the observed increase in csGRP78 in diabetic kidneys. Cell surface GRP78 thus represents a potential novel target for the treatment and/or prevention of DN. Future studies will seek identification of a ligand for csGRP78 which enables its HG-induced signaling thus expanding opportunities for the development of novel therapeutics.

**Experimental Procedures**

**Cell Culture**

Primary MCs were isolated from Sprague-Dawley rats, as described previously (55-59). MCs were cultured in Dulbecco’s modified Eagle’s medium supplemented with 20% fetal bovine serum, streptomycin (100 μg/ml) and penicillin (100 μg/ml) (Invitrogen), at 37°C in 95% air, 5% CO2. Cells between passages 8 to 15 were used. Cells were serum deprived at 80-90% confluence overnight prior to treatment with 24.4 mM glucose (Sigma) (to a final concentration of 30 mM glucose) for HG treatment. Mannitol (24.4 mM, Sigma) was used as an osmotic control. Prior to treatment with HG, cells were treated with the inhibitors: 4 sodium phenylbutyrate (4-PBA, Scandinavian Formulas Inc), salubrinal (Selleckchem, 1614), integrin β1 neutralizing antibody CD29 (Biolegend, 102209), integrin β1 non-neutralizing antibody (Abcam, EPR16895), GRP78 antibody C20 (Santa Cruz, SC1051), GRP78 antibody N20 (Santa Cruz, SC1050), Subtilase cytotoxin A (SubA), the SubA inactive mutant SubAA272, and PF573228 (Tocris, 3239). SubA and SubAA272 were purified as described previously (27). Time and dose of treatments are provided in the figure legends.

**Protein Extraction**

Cells were lysed in buffer containing 50 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10% glycerol and protease/phosphatase inhibitors. Cellular debris was cleared from cell
lysate by centrifugation at 13,000 rpm for 10 minutes at 4°C. Proteins were separated by SDS-PAGE and probed for: PDGFR (1:10000, Santa Cruz, SC432), GRP78 (1:1000, BD Transduction, 610979), phosphorylated Akt(pAkt) Ser473 (1:1000, Cell Signaling, 9271), Akt (1:1000, Cell Signaling, 9272), phosphorylated focal adhesion kinase (pFAK) Y397 (1:1000, Millipore, 07-012), FAK (1:1000, Santa Cruz, SC558), MTJ-1 (1:1000, Santa Cruz, SC104898), tubulin (1:10000, Sigma, T6074), integrin β1 (1:1000, Abcam, 102209), collagen I (1:1000, Novus Biologicals, NB600-408), and fibronectin (1:5000, BD Transduction, 610078).

Cell Surface Biotinylation

Cells were washed with ice-cold PBS and incubated with EZ-link Sulfo-NHS-LC-Biotin (Pierce, 21331) (0.5 mg/ml in PBS) for 20 minutes. Biotinylation was terminated with 0.1 M glycine in PBS. Cells were lysed and biotinylated proteins precipitated with 50% neutravidin slurry (Fisher, PI29200) overnight. Beads were then washed and cell surface proteins analyzed.

Flow Cytometry

Following treatment, cells were washed three times with cold PBS and harvested with Accutase (Innovative cell technologies, AT104), followed by three further washes (1% FBS/PBS). Cells were incubated with C20 GRP78 antibody (5 μg/10⁶ cells) for 1 hour at 37°C, washed with FACS buffer (0.2% BSA/PBS), and then incubated with 1 μg/10⁶ cells of AF488 anti-goat secondary antibody (Molecular Probes) for 1 hour at 37°C in the dark. Cells were then washed with FACS buffer and resuspended in 1% paraformaldehyde in PBS. Staining was assessed using the LSRII software from BD Biosciences and analyzed using FlowJo software from Tree Star.

RNA Interference

Rat MTJ-1 on-target plus Smart Pool siRNA and non-specific control siRNA were obtained from Dharmaco ). MCs were plated to 70% confluence and transfected with 50nM siRNA utilizing GeneEraser siRNA reagent (Stratagene, 204152). After 48 hours, they were serum-deprived for 24 hours, treated and harvested for protein.

Immunoprecipitation

After cells were washed with ice cold PBS, plasma membrane protein was isolated using the Minute™ Plasma Membrane protein isolation kit (Invent Biotechnologies, SM005) per manufacturer’s protocol. Two μg of the C20 antibody was used to immunoprecipitate GRP78, and antibody-protein complexes were purified using protein G sepharose beads. These were washed prior to analysis by immunoblotting. Alternatively, after HG treatment, live cells were exposed to GRP78 antibody or preadsorbed nonspecific IgG for 2h on ice, followed by washing, cell lysis and pull-down using protein G beads.

Animals

Animal studies were carried out in accordance with McMaster University and the Canadian Council on Animal Care guidelines. Male CD1 mice (Charles River Laboratories) underwent a left nephrectomy at 9 weeks of age. One week following this, diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ) (Sigma, S0130) at 200 mg/kg. Control mice were injected with an equal volume of citrate buffer. Blood glucose was tested the following week and mice with values of > 17 mM were enrolled in the study. Diabetic mice that developed ketonuria (assessed by dipstick (Bayer Multistix)) were implanted with an insulin pellet (LinShin Canada) to maintain body weight and hyperglycemia. CD1 mice were sacrificed following 12 weeks of diabetes. Male diabetic C57BL/6-Ins2Akita/J (Jackson Laboratory mice and their wild type (WT) littersmates were assessed at 30 weeks of age.

Imaging

Immunohistochemistry was performed on 4 μm deparaffinised paraffin sections using the GRP78 rabbit antibody (1:1000, Abcam, ab21685) at 4°C overnight after steaming. Pictures were taken at 1000x magnification. Immunofluorescence (IF) was performed on 6 μm OCT sections. Sections were stained with the GRP78 rabbit antibody (1:20,000, Abcam) together with the α8-integrin antibody (1:500, R&D, BAF4076) overnight, followed by secondary antibody (rabbit AF647, goat AF488, Molecular Probes; 1:500 for 1 hour) Pictures were taken at 1000x magnification using an Olympus 1X81 microscope. Colocalization
between GRP78 and α8 integrin was determined by using the colocalization plugin available for ImageJ. Areas of colocalization are presented as white in the mask+overlay images.

**Live tissue staining**

Kidney samples were diced and digested with collagenase P (1 mg/ml, Roche, 11213857001) and DNAse (0.1 mg/ml, Roche, 10104159001) in HBSS for 20 minutes at 37°C. Tissue was then passed through a 100 μm sieve, followed by a 70 μm sieve (BD Falcon) and glomeruli captured on a 53 μm sieve (VWR). These were suspended in wash buffer (1% FBS in HBSS), then incubated with both the GRP78 rabbit antibody (1:40,000, Abcam) and α8-integrin antibody (1:500, R&D) overnight, followed by secondary antibody (rabbit AF647, goat AF488, Molecular Probes; 1:500) for 2 hours in the dark. After washing with FACS buffer, cells were fixed in 4% paraformaldehyde for 30 minutes and counterstained with DAPI for 5 minutes before mounting in Fluoro-Gel (Electron Microscopy Sciences). Colocalization between GRP78 and α8-integrin was determined using the colocalization plugin available for ImageJ.

**Statistical analysis**

Statistical analysis was performed using the two-tailed t-test for experiments with only two experimental groups. Experiments with more than two groups were analyzed by one-way ANOVA with Tukey’s multiple comparison test for post hoc analysis. A $P<0.05$ was considered significant. Data are presented as mean ± SEM.

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**Conflict of interest** There is no conflict of interest for any of the authors.
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Figure 1. High glucose promotes the expression of GRP78 at the cell surface. (A) MC were treated with HG for the indicated times, after which cell surface (cs) GRP78 was assessed by a biotinylation assay as described in Methods. PDGFR was used as a loading control. (B) csGRP78 was assessed by biotinylation after HG or mannitol (24h)(n=7). (C,D) Expression of csGRP78 after HG for 24h was confirmed by flow cytometry (n=4). (E) Markers of ER stress were assessed by immunoblotting after HG treatment for the indicated times (n=3). (F) Effects of ER stress inhibitors 4-PBA (2.5 μM) or salubrinal (Sal, 30 μM) on HG-induced csGRP78 expression were assessed by biotinylation (n=5). (G) The effect of MTJ-1 downregulation using siRNA on HG-induced csGRP78 expression was assessed by biotinylation (n=3). MTJ-1 downregulation was assessed by immunoblotting of whole cell lysate. *p < 0.05 HG vs control, †p < 0.05 vs HG.
Figure 2. Cell surface GRP78 mediates HG-induced Akt and FAK activation in response to HG. MCs were treated with high glucose (HG) for 3 hours, a time at which we previously showed these to be activated upstream of profibrotic effects. FAK and Akt activation were assessed by their phosphorylation on Y397 and S473 respectively. (A) MCs were pretreated with antibodies targeting the C- (C20, 2 μg/ml) or N-terminus (N20, 2 μg/ml) of GRP78 for 1 hour. (n=5) (B) MCs were pretreated with subtilase cytotoxin A (SubA, 25 ng/ml) or an inactive mutant (Mut, 25 ng/ml) for 1 hour. (n=10) (C) MTJ-1 was downregulated using siRNA prior to treatment with HG. (n=3) *p < 0.05 HG vs control, †p < 0.05 vs HG.
**Figure 3. HG induces integrin β1 interaction with GRP78 at the cell surface.** (A) After treatment with HG (24h), GRP78 was immunoprecipitated from plasma membrane isolates and association with integrin β1 assessed by immunoblotting. (n=5) (B-E) After HG, either csGRP78 or membrane integrin β1 were immunoprecipitated from live cells, and association assessed by immunoblotting. Nonspecific IgG was used as a control. In D, MCs were cotreated with antibodies targeting the C- (C20, 2 μg/ml) or N-terminus (N20, 2 μg/ml) of GRP78 and in E, they were pretreated with subtilase cytotoxin A (SubA, 25 ng/ml) or an inactive mutant (Mut, 25 ng/ml) for 1 hour. *p < 0.05 vs control, †p<0.01 vs control, †† p<0.01 vs HG, ‡p<0.001 vs HG.
Figure 4. GRP78 interaction with integrin β1 mediates FAK and Akt activation by HG. MCs were treated with HG for 3 hours and FAK and Akt activation assessed by their phosphorylation, with MCs pretreated either with an integrin β1 neutralizing antibody (CD29) or nonspecific IgG (both 10 μg/ml) for 1 hour (A, n= 3), a non-neutralizing integrin β1 antibody (EPR16895, EPR, 10 μg/ml, 1 hour) (B, n=3) or with the FAK inhibitor PF573228 (PF, 1 μM) for 1 hour (C, n=5) *p < 0.05 vs control, ‡p < 0.05 vs HG, #p<0.01 vs control, †p<0.001 vs control
csGRP78 is a mediator of diabetic nephropathy

Figure 5. Cell surface GRP78 mediates the expression of ECM protein in response to HG. MCs were treated with high glucose (HG) for 72 hours, with this longer HG incubation required for synthesis of matrix proteins. Collagen I and fibronectin (FN) protein were determined in whole cell lysate. (A) MCs were pretreated with antibodies targeting the C- (C20, 2 μg/ml) or N-terminus (N20, 2 μg/ml) of GRP78 or an IgG control antibody for 1 hour. (n=4) (B) MCs were pretreated with subtilase cytotoxin A (SubA, 25 ng/ml) or an inactive mutant (Mut, 25 ng/ml) for 1 hour. (n=3) (C) MTJ-1 was downregulated using siRNA. (n=4) *p < 0.05 vs control, ‡p < 0.05 vs all HG.
Figure 6. Plasma membrane GRP78 is seen in glomeruli of type I diabetic CD-1 mice. Kidneys were harvested from vehicle or STZ-treated CD-1 mice after 12 weeks of diabetes (n=3 per group). (A) Paraffin-embedded sections were stained for GRP78. Black arrows indicate areas in which GRP78 appears to be localized to the plasma membrane. (B) OCT sections were stained for GRP78 (red) or the plasma membrane marker α8-integrin (green). Areas of colocalization between GRP78 and the plasma membrane are indicated by white in the overlay+mask images.
Figure 7. GRP78 is expressed at the plasma membrane in type I diabetic Akita mice. Kidneys were harvested from wild-type or Akita mice at 30 weeks of age (n=3 per group). (A) Paraffin-embedded sections were stained for GRP78. Black arrows indicate areas in which GRP78 appears to be localized to the plasma membrane. (B) OCT sections were stained for GRP78 (red) or the plasma membrane marker α8-integrin (green). Areas of colocalization between GRP78 and the plasma membrane are indicated by white in the overlay+mask images.
Figure 8. GRP78 is expressed at the plasma membrane in live type I diabetic Akita mouse glomeruli. (A) Kidneys from one wild-type and one diabetic Akita mouse aged 40 weeks were digested and stained for α8-integrin (green) to identify MCs and GRP78 (red). Fixation was performed after primary antibody incubation to enable more specific cell-surface staining. Colocalization between α8-integrin and GRP78 is indicated by white in the mask images. (B) 30 glomeruli from each mouse were assessed for quantification of colocalization. *p < 0.05
Figure 9. Schematic of cell surface GRP78 signaling in response to high glucose in kidney mesangial cells. In response to high glucose, GRP78 is translocated from the ER to the cell surface through an ER stress-dependent mechanism. This is mediated by the chaperone MTJ-1. At the cell surface, GRP78 interacts with integrin β1 which mediates the phosphorylation and activation of downstream FAK and Akt. This contributes to fibrosis through promoting the expression of ECM proteins including fibronectin and collagen I.
Cell surface expression of 78 kDa glucose regulated protein (GRP78) mediates diabetic nephropathy

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