Methylglyoxal modifies heat shock protein 27 in glomerular mesangial cells

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Abstract Methylglyoxal (MGO) can modify tissue proteins through the Maillard reaction, resulting in advanced glycation end products (AGEs), which can alter protein structure and functions. Several MGO-derived AGEs have been described, including argpyrimidine, a fluorescent product of the MGO reaction with arginine residues. We detected significant amount of argpyrimidine in rat kidney mesangial cells cultured in media containing high concentrations of glucose. Heat shock protein 27 (Hsp27) was identified by liquid chromatography tandem mass spectrometry as a major anti-argpyrimidine immunoreactive protein. We confirmed this finding by reciprocal co-immunoprecipitation and by Western analysis. Diabetic rats contained more argpyrimidine-modified glomerular Hsp27 than non-diabetic animals. Additional studies showed that MGO-induced modification of Hsp27 decreased its binding to cytochrome *c*. Our results suggest that Hsp27 is a major target for MGO modification in mesangial cells.

Key words: Mesangial cell; Methylglyoxal; Diabetes; Heat shock protein 27; Cytochrome *c*

1. Introduction

The non-enzymatic glycation of proteins in a high glucose environment occurs via the Maillard reaction [1]. Aldehydes and ketones react with amino groups on proteins to produce advanced glycation end products (AGEs), which may include amino acid cross-links, fluorophores, and chromophores [1,2]. AGEs bind to specific receptors on cells, where they can damage the affected tissues by causing intracellular oxidative stress as well as the synthesis of growth factors and cytokines [3,4]. Evidences suggest that AGEs contribute to the development of glomerular lesions of diabetes [5]. In addition, AGE-modified proteins stimulate a variety of cellular responses via specific cell surface receptors on several cell types including glomerular mesangial cells [6–8].

Mesangial cells grown on various AGE-modified matrix proteins exhibit altered functions, such as enhanced production of fibronectin and decreased cell proliferation. Aldehyde-modified matrix macromolecules, such as laminin and fibronectin, can inhibit mesangial cell adhesion, and mesangial cells grown on glyceraldehyde-modified fibronectin have diminished mitogenic activity [9]. Results from these studies clearly implicate AGE-modified proteins in mesangial cell damage in vitro and suggest that similar changes occur in the setting of diabetic renal disease.

Reactive dicarbonyl compounds such as glyoxal and methylglyoxal (MGO) are major intermediates in Maillard reactions [10]. MGO originates from various biochemical pathways, including the dephosphorylation of glycolytic intermediates, metabolites of the polyol pathway, and from aminoacetone metabolism [11]. Several tissues and plasma of diabetic individuals exhibit increased amounts of MGO [12]. MGO can modify amino acids, nucleic acids and proteins, and arginine appears to be a primary target in protein modification [12,13]. Several arginine modifications have been described [14] and one major modification is argpyrimidine which is a blue fluorescent AGE. It has been detected in the human lens and in cultured bovine retinal endothelial cells [15–18].

Because plasma levels of MGO are elevated in diabetes and MGO increases the susceptibility of mesangial cells to apoptosis [19], we believe that MGO-mediated Maillard reactions are involved in diabetic renal disease. In fact some studies have suggested that mesangial cells undergo apoptosis in diabetic kidneys [19,20]. A recent study has suggested a direct role for AGEs in such apoptotic cell death [21]. We investigated the formation of argpyrimidine in rat glomerular mesangial cells that were cultured in high concentrations of glucose and in glomeruli isolated from normal and diabetic animals. We also report argpyrimidine formation in heat shock protein 27 (Hsp27), a stress protein, and the consequences of MGO-mediated Maillard reactions on its binding to cytochrome *c* (cyt *c*).

2. Materials and methods

2.1. Materials

Monoclonal antibody to argpyrimidine was from Dr. Koji Uchida, Nagoya University, Japan; goat antibody to Hsp27 was from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and antibody to cyt *c* was from BD Transduction Laboratories (San Diego, CA, USA). CNBr-activated Sepharose 4B was from Amersham-Pharmacia Bio-

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Abbreviations: AGE, advanced glycation end product; MGO, methylglyoxal; Hsp27, heat shock protein 27; cyt *c*, cytochrome *c*; LC MS/MS, liquid chromatography tandem mass spectrometry

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tech (Piscataway, NJ, USA). All secondary antibodies and protein A/G agarose gel were from Santa Cruz Biotechnology.

2.2. In vivo tissues

Kidneys were isolated from male Sprague–Dawley rats. Diabetes was induced by a single intraperitoneal injection of streptozotocin (60 mg/kg body weight). Diabetic animals received 2 U of NPH humulin once every 4 days to maintain the body weight. Body weight, plasma glucose concentrations and $HbA1_c$ in these animals were measured and shown in Table 1. We compared tissue from diabetic animals (5 months duration) with non-diabetic controls of the same age and weight. The Case Western Reserve University IACAU Committee approved all animal use protocols.

2.3. Mesangial cell culture

Glomeruli were obtained from rat kidneys by a standard sieving technique [22]. Mesangial cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5.6 mM glucose, 110 mg/ml sodium pyruvate, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 15% fetal bovine serum (FBS). Mesangial cells were identified using an antibody against vascular smooth muscle myosin [23]. Mesangial cells (at 6–12 passages) were cultured for a week in DMEM with either 5.6 or 30 mM D-glucose; cultures in 30 mM L-glucose served as osmotic controls. Cultures were replenished with fresh growth media (with or without external glucose) every 48 h. After 1 week of incubation, the medium was replaced with serum-poor medium (0.5% FBS) that contained all other additives, and the cells were incubated for another 48 h. After serum starvation the cells were lysed and protein concentration of lysates was measured using the Micro BCA assay (Pierce, Rockford, IL, USA) [23].

2.4. Argpyrimidine in mesangial cells

Intracellular formation of argpyrimidine was assessed by immunohistochemistry [18]. Mesangial cells were cultured as described above in eight-well chamber slides (Nalge Nunc International, Naperville, IL, USA). The cells were fixed with 4% paraformaldehyde and permeabilized with 80% methanol. The wells were blocked with 5% nonfat dry milk in phosphate-buffered saline (PBS) (pH 7.4), incubated with anti-argpyrimidine antibody followed by rhodamine-conjugated anti-mouse IgG (Chemicon International, Temecula, CA, USA). The slides were mounted in a medium (Biomeda corp., Foster City, CA, USA) and observed under an Olympus fluorescence microscope (Olympus Optical Company, Japan).

2.5. Protein identification by liquid chromatography tandem mass spectrometry (LC MS/MS)

Coomassie blue-stained sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel bands were excised, digested in situ with trypsin and tryptic peptides analyzed by LC MS/MS using a quadrupole time of flight (QTOF2) mass spectrometer equipped with a CapLC system (Micromass) as described [24]. Peptide digests were trapped on a C18 precolumn (e.g. 0.3×1 mm, LC Packing) with 0.1% formic acid in 2% acetonitrile as loading solvent and then eluted onto a capillary C18 column (e.g. PicoFrit 0.050×50 mm, 15 μ tip ID, New Objective, Inc.). Chromatography was performed at 250 nl/min with aqueous acetonitrile/formic acid solvents and 100% of the eluant directed into the mass spectrometer. MS/MS spectra were collected over the range *m/z* 50–2000, ProteinLynx Global Server, MassLynx version 3.5 and the Swiss-Protein and NCBI protein sequence databases (February 2003).

2.6. Immunoprecipitation

Cell lysates were incubated overnight at 4°C with monoclonal argpyrimidine antibody or goat polyclonal antibody to Hsp27. Protein A/G agarose was added, and the mixture was further incubated at 4°C for 2 h. The agarose resin was then washed four times with immunoprecipitation buffer containing 10 mM HEPES (pH 7.4) with 150 mM NaCl, 5 mM ethylenediamine tetraacetic acid (EDTA) and protease inhibitors, and the final pellet was suspended in SDS sample buffer.

2.7. SDS–PAGE and Western blotting

Proteins were separated by electrophoresis on 18% SDS–polyacrylamide denaturing gels and transferred to nitrocellulose membrane. The blots were probed with antibodies to either argpyrimidine or Hsp27. The immunoblots were developed with the appropriate secondary antibody linked to horseradish peroxidase (Sigma and Santa Cruz) and enhanced chemiluminescence (ECL) kit (Perkin-Elmer Life Sciences, Boston, MA, USA).

2.8. Immunostaining of kidney sections

Immunohistochemistry was done according to standard procedures. Methacarn-fixed paraffin-embedded tissue sections were incubated with the argpyrimidine antibody and the reaction was developed with secondary biotinylated antibody (Vector Laboratories, Burlingame, CA, USA). The slides were stained with Vector ABC reagent (Vector Laboratories), then counterstained with hematoxylin and mounted.

2.9. MGO modification of Hsp27 and binding to cyt c

To study the effect of MGO modification on Hsp27, we mixed 25 μg of recombinant human Hsp27 (StressGen Biotechnologies Corp., Victoria, BC, Canada) with 50 and 200 μM of MGO and incubated the mixture at 37°C for 16 h in 50 mM HEPES buffer (pH 7.6). Incubations of Hsp27 with buffer alone served as controls. After incubation, the mixtures were dialyzed extensively against 50 mM HEPES–NaOH (pH 7.6) to remove excess MGO. We assessed binding of control and MGO-modified Hsp27 (10 μg each) to cyt *c* by incubating samples with 20 μg of cyt *c* (Sigma) in the presence of 1 mM deoxyadenosine triphosphate (dATP) [25]. Cyt *c* was immunoprecipitated using a mouse anti-cyt *c* monoclonal antibody [26]. The immunocomplexes were separated by incubation with protein A/G. The pellet was washed five times with 50 mM HEPES–NaOH (pH 7.6) that had protease inhibitors. The immunoblots were developed with antibody to Hsp27 or anti-cyt *c* and ECL kit as described above. In addition to the above method, we studied the binding of cyt *c* to Hsp27 after directly coupling Hsp27 (25 μg/ml) to CNBr-activated Sepharose 4B according to the manufacturer's protocols (Amersham Pharmacia Biotech). The gel was washed extensively with PBS to remove the unbound Hsp27. The Hsp27 bound to Sepharose beads was either used as control (unmodified) or was modified by incubating with 5 mM of MGO for 16 h at 37°C. The binding of cyt *c* to Hsp27 was tested as described above followed by Western blotting.

3. Results

3.1. Argpyrimidine in mesangial cell proteins

Mesangial cells exposed to high concentrations of either glucose (30 mM D-glucose) or MGO (50 μM) accumulate greater quantities of argpyrimidine than cells cultured in unaltered media based on immunofluorescence detection of argpyrimidine with monoclonal antibody (Fig. 1). We detected immunofluorescence throughout the cells but noted highest immunoreactivity in the perinuclear region.

To evaluate whether argpyrimidine formation was associated with specific proteins, we examined the cytosolic proteins by Western blotting. These experiments compared cytosolic proteins from cells grown in 5.6 and 30 mM D-glucose and as shown in Fig. 2, a single argpyrimidine immunoreactive protein band of about 27 kDa was detected under the exper-

Table 1
Body weight, plasma glucose and $HbA1_c$ in control and diabetic rats

Group	Body weight (g)	Plasma glucose (mg/dl)	$HbA1_c$
Control	628 ± 60	76 ± 9	5.2 ± 1.0
Diabetic	299 ± 59	413 ± 39	14.3 ± 1.4

Data are mean ± S.D. Rats were diabetic for 5 months ($n = 10$ in each group).

imental conditions. The intensity of the immunoreactive protein was higher with 30 mM D-glucose-incubated cells when compared to either 5.6 mM D-glucose or 30 mM L-glucose-incubated cells (osmotic control media). It is noteworthy that if the medium was not changed during the 7-day incubation there was no obvious increase in argpyrimidine content in the 27-kDa protein in cells that were cultured in 30 mM D-glucose when compared to those in 5.6 mM D-glucose. A protein band of comparable intensity to that seen in cells incubated with 5.6 mM glucose was detected in preparations from cells incubated with 30 mM L-glucose (data not shown). This could indicate that MGO modifies a 27-kDa protein in cells cultured under basal incubation conditions, and that the protein was further modified in cells exposed to high concentrations of D-glucose.

3.2. Argpyrimidine in rat kidney glomeruli and 27-kDa protein

The next step was to determine if diabetes enhanced formation of argpyrimidine in the kidney. Immunostaining for argpyrimidine in sections from normal and diabetic rat kidneys indicated a greater intensity of staining in glomeruli from diabetic animals than those from non-diabetic controls (Fig. 3A and B). To determine if the 27-kDa protein accounted for the observed immunoreactivity, we isolated glomeruli from normal and diabetic rat kidneys. We then extracted the proteins with a 60 mM Tris buffer (pH 7.0) containing 0.25 M sucrose, 2 mM EDTA and 1 mM β-mercaptoethanol and 10% SDS. Fig. 4 shows a Western blot with antibody to argpyrimidine, indicating that diabetic rats have argpyrimidine associated with the 27-kDa protein while the non-diabetic controls do not.

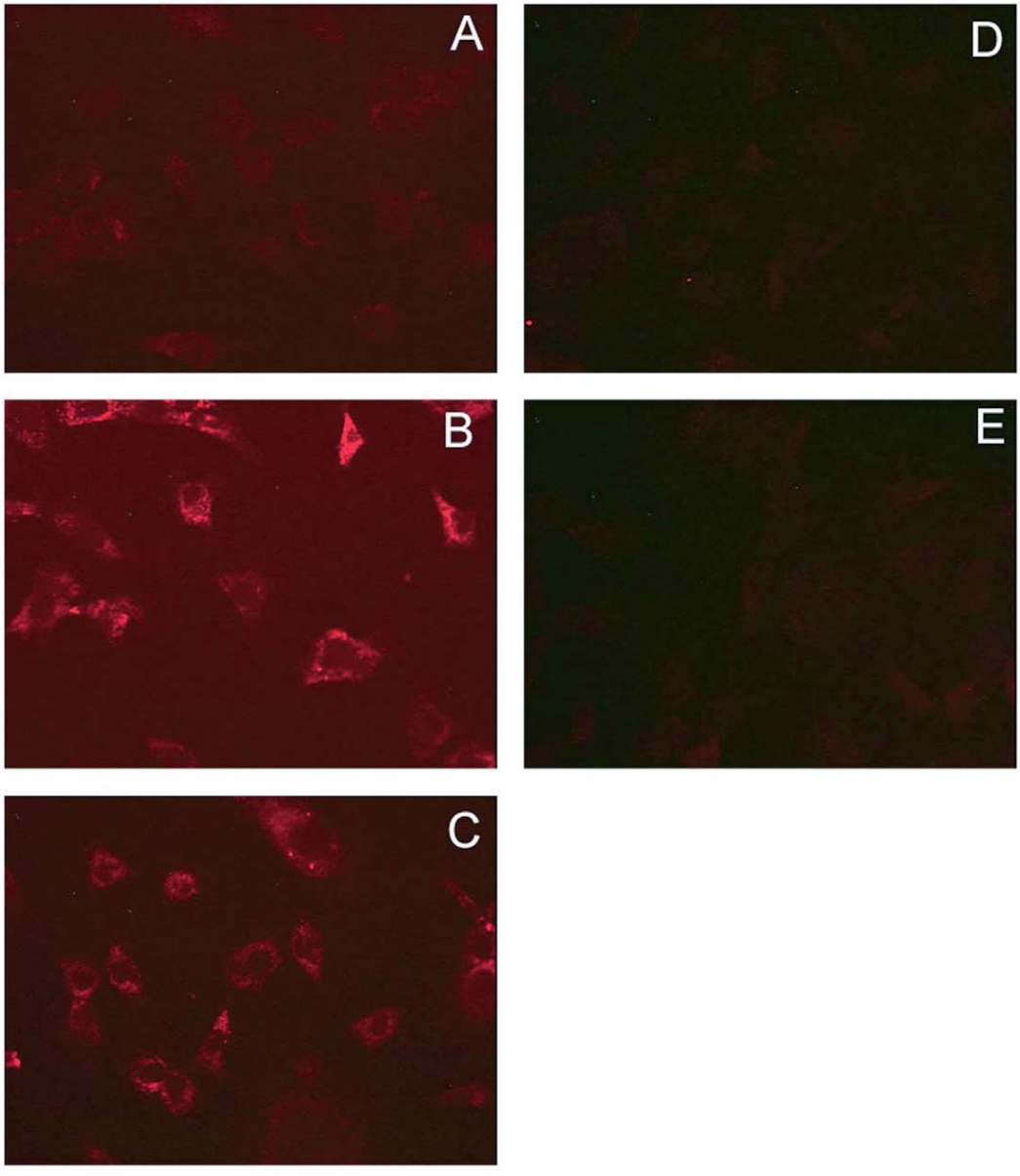

Fig. 1. Detection of argpyrimidine by immunofluorescence. Mesangial cells were cultured in medium with 5.6 (A) or 30 mM D-glucose (B) and stained for argpyrimidine. C shows cells that were incubated with 50 μM MGO for 72 h in standard (5.6 mM glucose) medium. Cells in 30 mM L-glucose were osmotic controls (D). The antibody was blocked with 1 nmol of argpyrimidine before addition to 30 mM D-glucose-treated cells (E). Magnification = 400×.

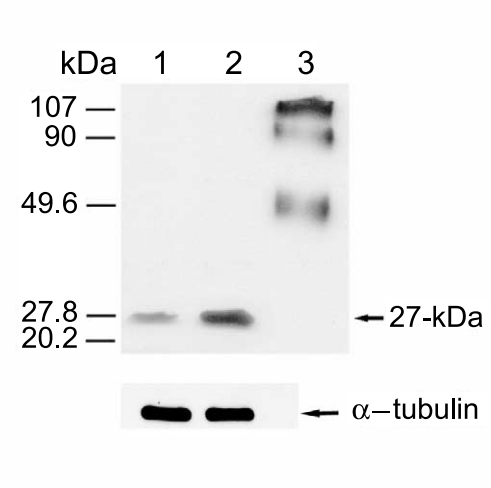

Fig. 2. Argpyrimidine-bearing proteins in mesangial cells. Proteins (100 μg each) from cultured mesangial cells were separated by 15% SDS–PAGE, blotted onto nitrocellulose membranes and probed with antibody to argpyrimidine. Lane 1, mesangial cells cultured in 5.6 mM glucose; lane 2, cells cultured in 30 mM D-glucose; lane 3, MGO-modified bovine serum albumin (BSA) (positive control); α-tubulin (loading control).

3.3. Identification of 27 kDa as Hsp27

Immunoprecipitation and LC MS/MS confirmed that Hsp27 was the argpyrimidine-containing protein. Cell lysate proteins were incubated first with antibodies to Hsp27 or argpyrimidine, then immunoprecipitated by protein A/G agarose, and separated by SDS electrophoresis. Western blot analysis of the immunoprecipitation products with antibodies to either argpyrimidine or Hsp27 (Fig. 5A) showed that Hsp27 co-immunoprecipitated with both antibodies. The argpyrimidine immunoreactive 27-kDa band in the immunoprecipitated proteins was excised from a separate Coomassie blue-stained SDS–PAGE gel, digested in situ with trypsin and the peptides were analyzed by LC MS/MS. The mass spectrometric analysis revealed 10 tryptic peptides that matched with internal sequences of Hsp27. Together these results strongly support the identity of the argpyrimidine-containing 27-kDa protein as Hsp27.

To evaluate whether rat glomerular Hsp27 increases in diabetes, we performed Western blot analysis of glomerular lysates with antibodies to Hsp27 (Fig. 5B) and found that glomeruli from diabetic animals have greater immunoreactivity than those from non-diabetic controls. This may be responsible, in part, for the higher argpyrimidine immunoreactivity in diabetic glomeruli (Figs. 3 and 4).

3.4. MGO modification of Hsp27 reduces its binding to cyt c

Since binding of cyt *c* by Hsp27 inhibits the mitochondrial pathway of apoptosis [26], we speculated that argpyrimidine modification might influence Hsp27 binding to cyt *c*. To examine this possibility, Hsp27 was incubated with 50 μM MGO for 20 h at 37°C, then dialyzed against 50 mM HEPES–NaOH (pH 7.6) and incubated with cyt *c* in the presence of dATP. The Hsp27–cyt *c* complex was immunoprecipitated with antibody to cyt *c* and protein A/G agarose, separated by SDS–PAGE and Western blotted with anti-Hsp27 antibody. Modified Hsp27 bound slightly less cyt *c* than the unmodified protein when exposed to 50 μM MGO (Fig. 6A)

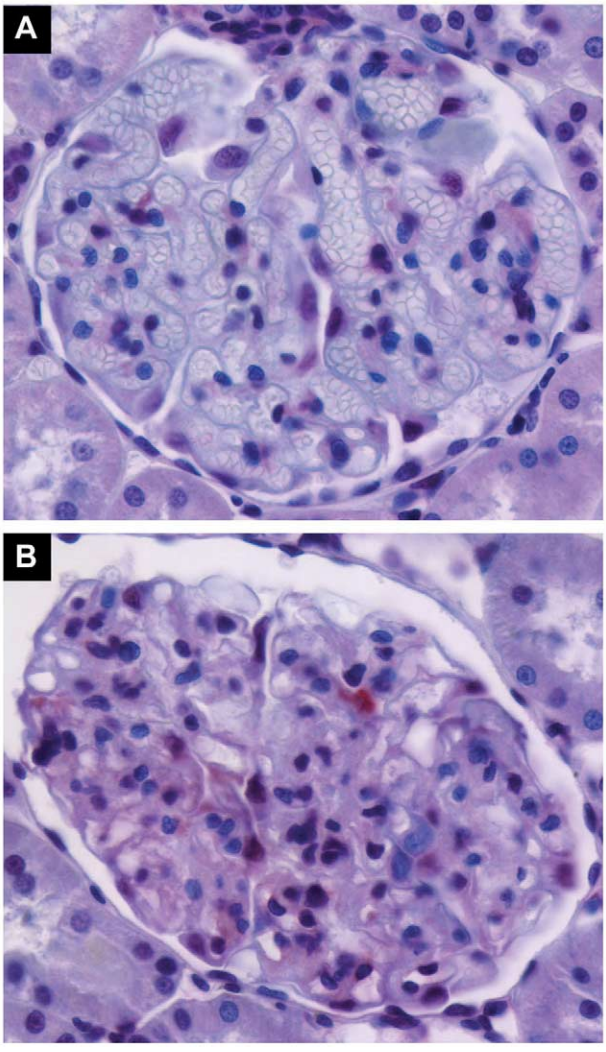

Fig. 3. Argpyrimidine in normal and diabetic rat kidneys. Kidneys from rats that were diabetic for 5 months were compared with non-diabetic controls. Tissues were processed for immunohistochemistry. A shows a representative glomerulus from normal rat kidney section immunostained with antibody to argpyrimidine. B shows a representative glomerulus from diabetic rat kidney section immunostained with antibody to argpyrimidine.

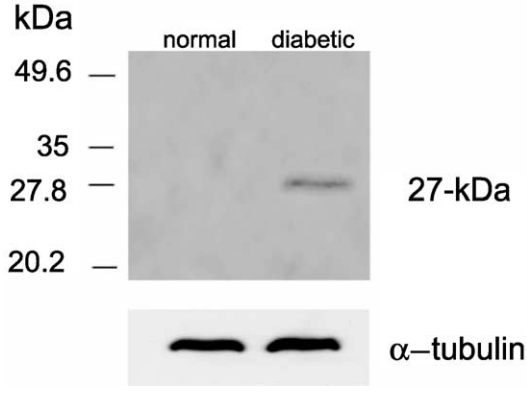

Fig. 4. Western blot of glomerular lysates for argpyrimidine. Equal amounts (50 μg) of glomerular protein from normal or diabetic animals were probed with antibody to argpyrimidine or α-tubulin (loading control).

and significantly less cyt *c* when incubated with 200 μM MGO (Fig. 6B). To further confirm these observations, we conjugated Hsp27 onto CNBr-activated Sepharose 4B. The Hsp27 bound to Sepharose was then modified by incubation with MGO and then reacted with cyt *c*. As shown in Fig. 6C, there was significantly less binding of cyt *c* to MGO-modified Hsp27 when compared to unmodified Hsp27. These results suggest that Hsp27 modification by MGO reduces its ability to bind to cyt *c*.

4. Discussion

We originally planned to identify the major MGO-AGE-bearing proteins in mesangial cells in order to assess how specific changes in these proteins relate to abnormalities of renal function in diabetes. Unexpectedly, we found high levels of argpyrimidine in Hsp27 from cell lysates of cultured rat mesangial cells. It is possible that the arginine residues in Hsp27 are more accessible for reaction with MGO compared to other proteins, and could explain its high immunoreactivity with anti-argpyrimidine antibody. Recently, Sakamoto et al. [27] have reported argpyrimidine formation in Hsp27 in cancer cells that were incubated with high concentrations of glucose. These results support our view that Hsp27 is the primary target for MGO modification in cells.

Our observation that argpyrimidine content in Hsp27 increased under high glucose conditions in rat mesangial cells suggests that this protein is specifically modified in the diabetic kidney glomeruli. In fact, our results from immunostaining of rat kidneys support this view. The presence of argpyrimidine in Hsp27 under normoglycemic conditions suggests the formation of low levels of MGO in these cells. One possible pathway of MGO formation is through oxidative stress. If cells in culture are under a mild oxidative stress, it could lead to formation of MGO. In fact, other investigators showed that oxidative stress promotes MGO production in cultured cells [28]. There are reports that glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the enzyme that converts glyceraldehyde-3-phosphate (G3P) to 1,3-diphosphoglycerate, is inactivated due to oxidative stress and hyperglycemia [29–31]. A loss in GAPDH activity would permit accumulation of G3P, which can be spontaneously converted to MGO by β-elimination of the phosphate group. In addition, oxidative stress may enhance degradation of the Amadori product, an initial condensation product of glucose and lysine residues on proteins, and/or promote autoxidation of sugars [32]. A recent study suggested that activation of thiamine-dependent transketolase, that channels G3P to HMP shunt pathway can lower MGO levels in endothelial cells [33]. Any or all of these processes could contribute to MGO formation. Unfortunately, we were unable to measure MGO directly in mesangial cells, but work from other laboratories supports our idea that MGO formation plays an important role in renal cell damage. For example, Brownlee's group found that normalization of mitochondrial superoxide production attenuates major pathways of diabetic complications, including formation of intracellular AGEs [31].

The ready detection of argpyrimidine in kidneys from diabetic rats, and the association of argpyrimidine with glomerular Hsp27 suggest that in diabetes, MGO production is enhanced within mesangial cells through an increase in oxidative stress and/or enhancement in glucose flux through metabolic pathways. It is not known whether external MGO can enter mesangial cells. Intracellular estimation of MGO would have provided a clearer picture, but we were unable to perform such measurements because of lack of adequate sensitivity of the MGO assays. Moreover, we investigated only one modification of MGO, it is likely that other modifications could occur along with argpyrimidine under hyperglycemic conditions. Whether their synthesis would be enhanced by external

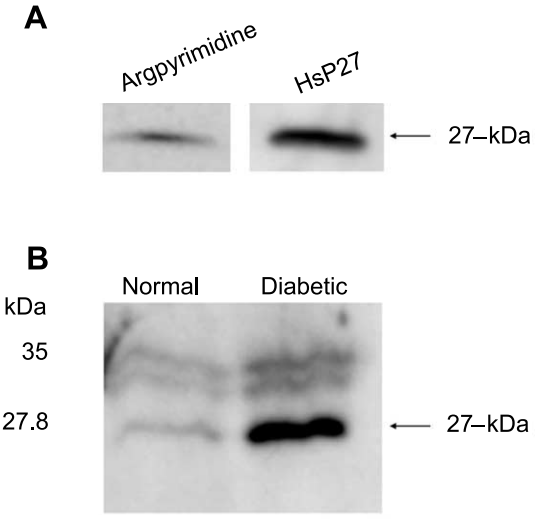

Fig. 5. Hsp27 and argpyrimidine in cell lysates and glomerular extracts. Lysates of mesangial cells cultured in 5.6 mM D-glucose (A) were immunoprecipitated with argpyrimidine antibody and protein A/G agarose, then probed with antibodies to either argpyrimidine or Hsp27. B shows Western blot of kidney glomerular lysates from normal or diabetic animals. Proteins (50 μg each) were separated by SDS–PAGE and probed with antibody to Hsp27.

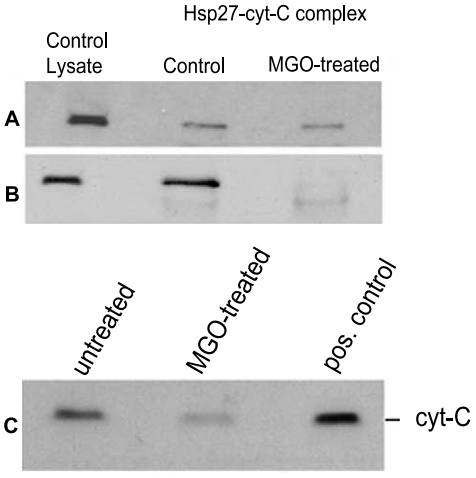

Fig. 6. MGO-induced modification on Hsp27 affects binding to cyt *c*. Hsp27 was reacted with 50 or 200 μM MGO, incubated with cyt *c* and dATP for 1 h at 30°C and the cyt *c*–Hsp27 complex was immunoprecipitated using antibodies to cyt *c*. Western blots were probed with anti-Hsp27 antibody. Control lysates in both panels are lysates from mesangial cells cultured in 5.6 mM D-glucose medium. A shows Hsp27 incubated with 50 μM of MGO and B is for Hsp27 incubated with 200 μM MGO. C: Hsp27 was coupled to CNBr-activated Sepharose 4B and then incubated with or without 5 mM MGO for 16 h. The gel was washed extensively after the incubation and reacted with cyt *c* for 2 h. After extensive washing of the gel, binding of cyt *c* was assessed by Western blotting using an anti-cyt *c* antibody.

MGO is unknown. In the event that MGO is impermeable in mesangial cells, a likely mechanism is the enhanced flux of glucose under hyperglycemic conditions, which could lead to higher concentrations of glycolytic intermediates that can spontaneously produce MGO. Other mechanisms, such as those described above may also play a role. The higher immunoreactivity for argpyrimidine in diabetic glomeruli could also be due to higher content of Hsp27 (see below).

The stress protein Hsp27 is upregulated under certain conditions, e.g. oxidative stress, to protect cells from uncontrolled apoptosis. In fact, our results show that in diabetes, Hsp27 content is increased in the glomerulus. Hsp27 appears to interfere specifically with the mitochondrial pathway of caspase-dependent cell death [26]. Hsp27 inhibits cyt *c*-mediated activation of caspases by binding to cyt *c* and thereby preventing cyt *c*-mediated interaction of Apaf-1 with procaspase-9 [26,34]. Our in vitro experiments showed that cyt *c* binding of Hsp27 is reduced after chemical modification of Hsp27 by MGO, suggesting that argpyrimidine, and possibly other MGO-AGEs, compromises the ability of Hsp27 to prevent apoptotic cell death. A recent report suggested that MGO induces apoptosis of rat mesangial cells [19]. Argpyrimidine formation may compromise other functions of Hsp27 as well. For example, Hsp27 serves as a chaperone to stabilize cytoskeletal proteins [35]. Cytoskeletal breakdown is a key event in the initiation of apoptosis [36].

In summary, results from our experiments show that mesangial cell Hsp27 is chemically modified by MGO; this phenomenon occurs both in cell cultures and in an animal model of diabetes. We find that MGO-mediated modification of Hsp27 reduces its binding to cyt *c*. This structural change of a major stress protein is likely to play a role in altered mesangial cell functions in diabetes.

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