

Human mesangial cells resist glycoxidative stress through an antioxidant response

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Received August 3, 2010; Accepted September 21, 2010

DOI: 10.3892/ijmm.2010.576

Abstract. The generation of advanced glycation end-products (AGE), the interaction with their receptors, the generation of reactive oxygen species, and the modulation of intracellular redox equilibrium are believed to be the main factors causing alterations of mesangial cell physiology leading to diabetic nephropathy. Normal human primary mesangial cells were exposed to glycoxidative stress by culture in high glucose (HG) or treatment with AGE for up to 6 days. In both cases only a moderate generation of reactive oxygen species and production of HNE-protein adducts were induced while protein nitrotyrosination was not affected. Moreover, HG and AGE caused a significant antioxidant response, confirmed by the induction of heme oxygenase 1 and the consumption of vitamin E. Glutathione was decreased only by HG. Mesangial cell proliferation and viability were slightly affected by HG and AGE. Furthermore, both treatments failed to influence TGF- β 1 and MCP-1 secretion and to modulate RAGE and collagen IV expression. We believe that normal human mesangial cells can resist glycoxidative stress by the observed antioxidant response. These results support the concept that mesangial cells are only partly responsible for the onset and progression of diabetic nephropathy and that the role of other cell types, such as podocytes and endothelial cells, should be taken into consideration.

Introduction

Glycoxidation, a process involving both glycative and oxidative mechanisms (1), is widely believed to play a major role in the pathogenesis of several long-term diabetic complications, particularly nephropathy (2). Glycoxidation causes the generation of a wide range of molecular structures,

mainly on proteins, that are able to exert several detrimental effects. These molecular structures are called advanced glycoxidation end-products (AGE). The simple presence of AGE in proteins, not only in long-lived proteins such as crystallins or collagens but also in intracellular proteins (3), can induce structural and functional alterations, leading to increased rigidity, decreased elasticity, reduced turn-over and enzymatic dysfunction. Moreover, the recognition of several cell surface receptors for AGE (4) has aroused increasing interest in investigating the consequences of AGE interaction with their cellular receptors. In particular, the binding of AGE with RAGE has been shown to be connected to signal transduction pathways, leading to increased oxidative stress and various cell responses, which may be involved in the pathogenesis of diabetic complications (5).

Since diabetic nephropathy is characterized by increased deposition of mesangial matrix in the glomerulus, mesangial cells have been attributed a central role in the pathogenesis of this glomerular alteration (6). Exposure of mesangial cells *in vitro* to a high glucose concentration or to glycated proteins has been reported to result in increased production of matrix proteins and decreased production of matrix metalloproteases, indicating that mesangial cells can react to diabetes-like conditions by assuming a profibrogenic attitude (7,8). The mechanisms underlying mesangial cell response probably depend on the increased levels of oxidative stress (9).

We previously demonstrated that certain antioxidants are able to protect streptozotocin-induced diabetic rats from several morphological and molecular aspects of diabetic nephropathy (10,11). Therefore, we decided to investigate the possible protective role of these antioxidants on mesangial cells *in vitro*, when stressed with diabetes-like conditions, such as exposure to a high glucose (HG) culture or to AGE proteins.

However, in the present study, we found that both HG and AGE barely affected cell proliferation and viability and failed to induce a profibrogenic attitude in normal human mesangial cells, which were able to maintain a mild level of oxidative stress, while consuming vitamin E and up-regulating heme oxygenase-1 (HO-1) expression.

Materials and methods

Cell culture and treatments. Normal human mesangial cells (NHMCs) were purchased (2 different batches) from Cambrex

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Key words: diabetes, mesangium, glycoxidation, heme oxygenase

Bio Science and maintained according to the company's instructions in the Mesangial Cell Growth Medium Bullet kit (MsGM™), containing 5 mM glucose, supplemented with 5% FBS and MsGM SingleQuots supplied by the same company. Cells were subcultured every 5 days at 1:4, and the experiments were carried out within the 10th doubling of the cells.

Cells were exposed to the normal growth medium (CTR, control) or to the same medium supplemented with 25 mM glucose (HG, high glucose), 200 µg/ml bovine serum albumin (BSA) or AGE-modified BSA (AGE-BSA), or 100 µM H₂O₂. Exposures were extended for up to 6 days. Every two days, the media were removed, collected and substituted with fresh media with the same characteristics. Some samples were exposed to 0.5–1.0 mM H₂O₂ for 1–3 h.

AGE-BSA was obtained by incubating BSA (10 mg/ml in PBS, 10 mM, pH 7.4) with 50 mM ribose for 6 days. AGE-modification was assessed by fluorescence detection (370 nm ex/440 nm em) and evaluation of carboxymethyl-lysine by Western blot analysis (12).

Cell proliferation and viability. Cell proliferation was evaluated by cell count using trypan blue dye, and cell viability was assessed using the LDH release test (Cytotox 96, non-radioactive cytotoxicity assay; Promega, Madison, WI, USA).

Evaluation of peroxides and superoxide anion generation. Evaluation of peroxides (13) was performed using the dichloro-fluorescein (DCF) test. Briefly, after treatments, cells were washed with PBS and exposed to 5 µM dichloro-dihydro-fluorescein diacetate (DCF-DA) for 30 min at 37°C. Cells were then washed twice and scraped off in 1 ml of PBS; cells were subsequently sonicated. Fluorescence of cell lysates was analyzed using the Perkin-Elmer LS-5 Luminescence spectrometer (498 nm ex/525 nm em). Results are expressed as arbitrary units of fluorescence (AUF)/mg protein.

Superoxide anion production was evaluated using the dihydroethidium test. At the end of the treatments, cells were washed and exposed to 10 µM dihydroethidium for 30 min at 37°C. Cells were then washed and observed by fluorescence microscopy (14).

Analysis of RAGE and HO-1 expression and carboxymethyl-lysine (CML)-, nitro- and HNE-protein adducts. Protein expression levels were evaluated by a Western blot analysis standard technique on Hybond-P PVDF membranes using the following primary antibodies: 1:20000 mouse anti-CML clone no. NF-1G (Wako Chemicals GmbH, Neuss, Germany); 1:1000 mouse anti-nitrotyrosine (Abcam, Cambridge, UK); 1:80000 rabbit anti-HNE (Alpha Diagnostic International, San Antonio, TX, USA); 1:4000 mouse anti-RAGE (Millipore-Chemicon International, Billerica, MA, USA); and 1:250 mouse anti-HO-1 (Abcam). In addition, anti-mouse or anti-rabbit horseradish peroxidase-conjugated antibodies and the ECL Plus detection system (GE Healthcare-Amersham, Milan, Italy) were used according to the manufacturer's instructions.

Analysis of transforming growth factor (TGF)-β1 and monocyte chemoattractant protein (MCP)-1. Evaluation of

TGF-β1 and MCP-1 was performed using ELISA by means of the following kits: DuoSet Human TGF-β1 (R&D Systems, Minneapolis, MN, USA) which also detects latent TGF-β1 and the (h)MCP-1 Biotrak ELISA system (GE Healthcare-Amersham).

Immunocytochemistry for HNE-protein adducts. Immunocytochemistry (ICC) for HNE-protein adducts was performed on chamber slides by standard protocol. Briefly, at the end of the programmed exposure time, cells were fixed in buffered 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Endogenous peroxidase activity was quenched by incubation with 1% H₂O₂, and non-specific binding sites were blocked with 5% goat serum. After overnight incubation with 1:10,000 rabbit anti-HNE antiserum (Alpha Diagnostic International) and a 45-min incubation with 1:1000 anti-rabbit biotinylated antibody, cells were exposed to streptavidin-biotinylated horseradish peroxidase StreptABComplex/HRP (Dako, Glostrup, Denmark), and 3,3'-diaminobenzidine/H₂O₂ (Dako) was applied until the reaction product was visualized (3 min).

Analysis of GSH and vitamin E level. Glutathione (GSH) was evaluated using high-performance liquid chromatography (HPLC) according to Fariss and Reed (15). Briefly, cells were harvested in PBS containing 10% perchloric acid and 1 mM BPDS. After vortexing and precipitation of the proteins by centrifugation, thiol groups were blocked with iodoacetic acid (IAA) at an alkaline pH, and analytes were then converted to 2,4-dinitrophenyl derivatives with 1% 1-fluoro-2,4-dinitrobenzene (FDNB) at 4°C in the dark overnight. Quantitative determination of derivatized analytes was performed by HPLC; the HPLC system was equipped with an NH₂ Spherisorb column and a UV detector set at 365 nm; the flow rate was 1.5 ml/min. The mobile phase was maintained at 80% A (80% methanol) and 20% B (0.5 M sodium acetate in 64% methanol) for 5 min, followed by a 10-min linear gradient to 1% A and 99% B; the mobile phase was maintained at 99% B until GSSG eluted. Total GSH content was evaluated in the chromatograms as GSH+2GSSG and expressed in GSH equivalents (16).

Determination of vitamin E was performed using HPLC according to the method of Lang *et al.* (17). Briefly, cell cultures were harvested in 10 mM PBS and mechanically disrupted; tocopherol acetate (as internal standard, final concentration 1.75 µM), BHT (final concentration 0.25 mg/ml) and SDS (final concentration 10 mM) were added to the mixture. After adding an equal volume of ethanol, the mixture was extracted with heptane (volume/volume). The heptane layer was dried under nitrogen, and the residue was re-dissolved in methanol. An aliquot was analyzed by HPLC (µBondapak C18 column; size, 3.9 x 300 mm; Waters, Milford, MA, USA; pure methanol as mobile phase; UV detection at 292 nm). Quantitation was performed by means of reference chromatograms of a standard solution of vitamin E.

Analysis of heme oxygenase-1 (HO-1) and collagen IV expression. HO-1 and collagen IV mRNA expression was evaluated in a reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted using Trizol



Invitrogen, Milan, Italy) according to the manufacturer's instructions. RNA was reverse transcribed into cDNA by random hexamer priming and SuperScript™ II reverse transcriptase (Invitrogen). Amplification of cDNA by PCR was performed by using 2X PCR Master Mix (Fermentas, Milan, Italy) and specific primers for human HO-1 and collagen IV, synthesized by TIB MolBiol (Genoa, Italy); control reactions were performed with primers specific for human GAPDH. The sequences of the primers were as follows: HO-1 Fw, 5'gct caa cat cca gct ctt tga gg 3'; HO-1 Rv, 5' gac aaa gtt cat ggc cct ggg a 3' (284 bp) (30 cycles: 95°C for 1 min, 62°C for 1 min, 72°C for 2 min) (18); collagen IV Fw, 5' cgg ggt tac aag gtg tca ttg g 3'; collagen IV Rv, 5' gcc aag tat ctc acc tgg 3' (332 bp) (designed on the sequence GenBank NM_001854; 30 cycles: 95°C for 45 sec, 55°C for 45 sec, 72°C for 60 sec); GAPDH Fw, 5' gtc ttc acc acc atg gag aa 3'; GAPDH Rv, 5' atc cac agt ctt ctg ggt gg 3' (266 bp) (30 cycles: 95°C for 1 min, 59°C for 1 min, 72°C for 2 min) (18). The amplification products were separated by electrophoresis on 1.5% agarose ethidium bromide gel and analyzed using the Gel Doc 2000 densitometer by means of 'Molecular Analyst' software (both from BioRad, Milan, Italy).

Statistical analyses. Results were expressed as mean \pm SD from at least three independent experiments. The statistical significance of parametric differences among sets of experimental data was evaluated by ANOVA and Newman Keuls post-test for multiple comparisons. When comparing two groups of data, the t-test was used.

Results

Intracellular oxidative stress. Evaluation of the intracellular oxidative state (Fig. 1) revealed a slight but significant sensitivity of NHMCs to AGE, HG or H₂O₂. Evaluation of peroxides using DCF-DA (Fig. 1a) showed significantly increased production after a 2-day exposure to AGE, HG or 100 μ M H₂O₂; no significant changes in peroxide generation were observed at the other experimental times considered (up to 6 days). However, superoxide production, as assessed by the dihydroethidium test (Fig. 1b), was increased from 6 h up to 6 days of exposure to AGE, HG or 100 μ M H₂O₂. Western blot analysis did not show any significant difference in the level of nitrotyrosine adducts after up to 6 days of AGE, HG or 100 μ M H₂O₂ exposure (Fig. 1c).

Western blot analyses showed moderate increases in the immunoreactivity for HNE-adducts in all treatments from 2 days onwards, particularly for bands between 55 and 72 kDa; after 6 days of treatment with AGE, HG and 100 μ M H₂O₂, the increments were +25, +30 and +35%, respectively, in comparison to the untreated cells (Fig. 1c). Moreover, when evaluated using ICC (Fig. 2), the mesangial cells showed a slight but appreciable increase in HNE-adducts after the same treatments. The accuracy of the ICC assay was confirmed by using mouse anti-actin antibody as positive staining and mouse anti-cytokeratin 18 antibody as negative staining.

Antioxidant defenses. Cell treatment with HG and 100 μ M H₂O₂ for 6 days induced a moderate decrease (-15 and -22%,

respectively) in GSH content in comparison to the untreated cells (Fig. 3a). It should be considered that, under the same culture conditions, fibroblasts contained much less GSH than the mesangial cells, and that the same H₂O₂ exposure induced a dramatic decrease in GSH (-90% vs. control) (data not shown).

Vitamin E was significantly reduced in mesangial cells after a 6-day exposure to AGE (-42%), HG (-44%) or 100 μ M H₂O₂ (-39%) (Fig. 3b).

Regarding HO-1 mRNA expression, AGE, HG and 100 μ M H₂O₂ treatments were able to increase the expression of HO-1 in comparison to the control cells: AGE induced a strong and prolonged induction from 2 days onwards (~5-fold increase in comparison to CTR at the same time); HG caused a slower increase (maximum ~3-fold increase at day 4 in comparison to CTR at the same time), while 100 μ M H₂O₂ was able to induce an 8-fold increase at 24 h (vs. CTR at 24 h), followed by a decrease to lower levels of induction (Fig. 4). Western blot analysis of protein expression provided analogous results (data not shown).

Cell proliferation and viability. Proliferation of NHMCs was evaluated by cell count. Exposure to AGE or HG for 6 days revealed a slight reduction in cell proliferation (-15% in comparison with control cells) while exposure to 100 μ M H₂O₂ induced a more pronounced decrease in cell proliferation (-40% vs. CTR).

Exposure of NHMCs to AGE, HG or 100 μ M H₂O₂ for up to 6 days did not induce any significant alteration of cell viability, as measured by the LDH release test (data not shown).

Mesangial cells proved rather resistant even when exposed to higher H₂O₂ concentrations; 0.5 mM H₂O₂ did not affect cell viability even after 3 h, and after 1 h of treatment with 1 mM H₂O₂, cell viability was still 50% in comparison to the control cells.

TGF- β 1 and MCP-1 production and collagen IV expression. TGF- β 1 production was evaluated both inside the cells and in the medium. NHMCs exposed to the various treatments did not show any variation in intracellular TGF- β 1 content at any time up to 6 days (data not shown). No secreted TGF- β 1 was detected before 24 h of exposure. Afterwards, the quantity of secreted TGF- β 1 increased progressively, but no significant differences were detected at any time among the various exposures (Fig. 5a). Similar results were obtained for MCP-1 secretion (data not shown). Moreover, neither AGE nor HG exposure had an effect on the expression of collagen IV, as revealed by RT-PCR analysis (Fig. 5b).

RAGE expression. No effects of AGE, HG or 100 μ M H₂O₂ exposure on RAGE protein expression level at any experimental time during the 6 days of treatment were noted (Fig. 6).

Discussion

Many researchers have reported that *in vitro* exposure to HG or AGE brings about several pathologic effects on various cell types (e.g. tubular epithelium, vascular smooth muscle

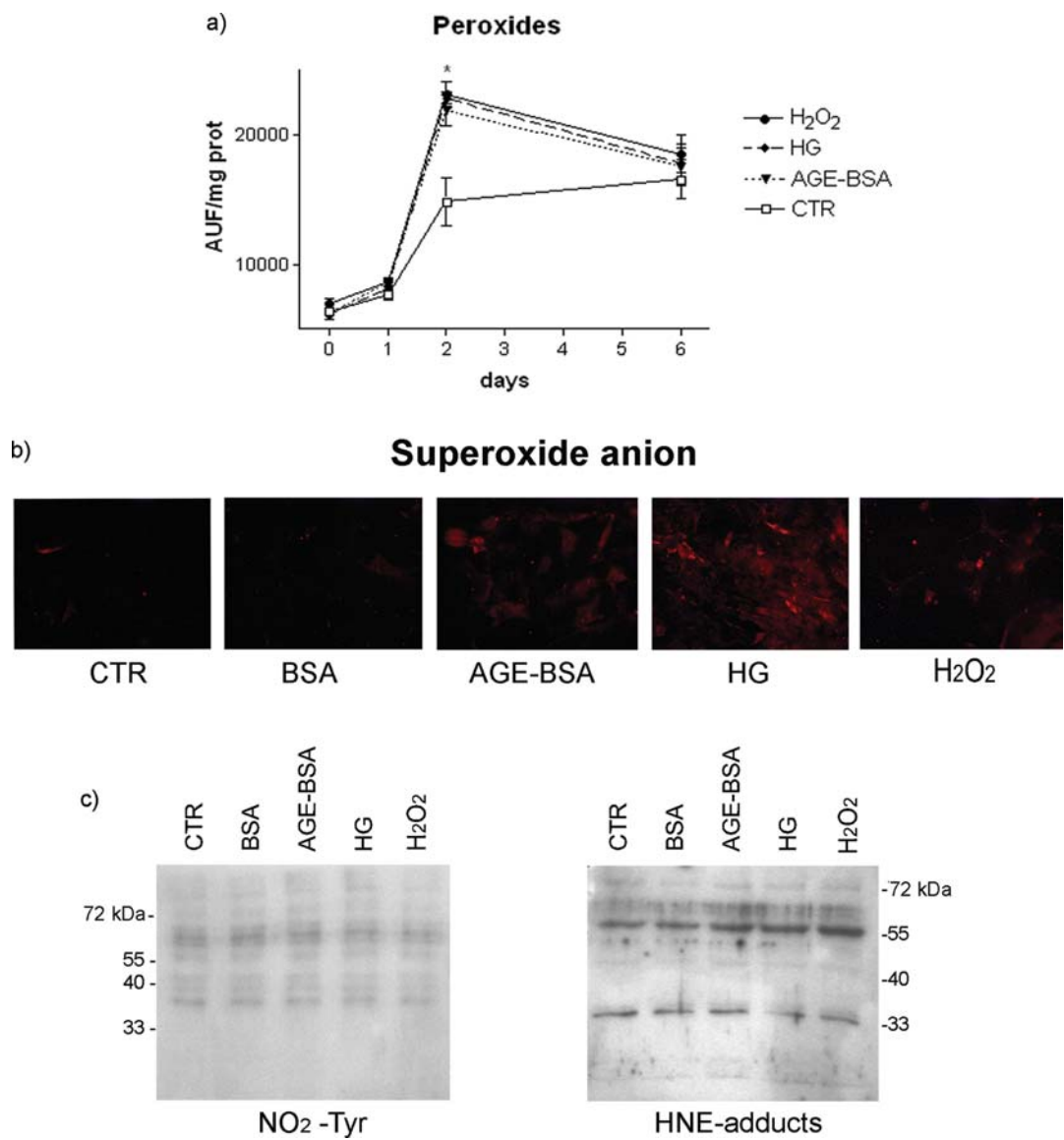


Figure 1. (a) Evaluation of peroxide production by the DCF-DA test during the 6 days of treatment. Statistics included ANOVA and Newman Keuls post-test; * $p<0.05$ vs CTR at the same time. Results are expressed as the mean \pm SEM. (b) Evaluation of superoxide anion production by the dihydroethidium test. The panels are representative of three separate experiments at 48 h of treatment. Analogous results were obtained at each experimental time tested. (c) Evaluation of protein nitrotyrosination and HNE-adducts by Western blotting. The panels are representative of three separate experiments at 6 days of treatment.

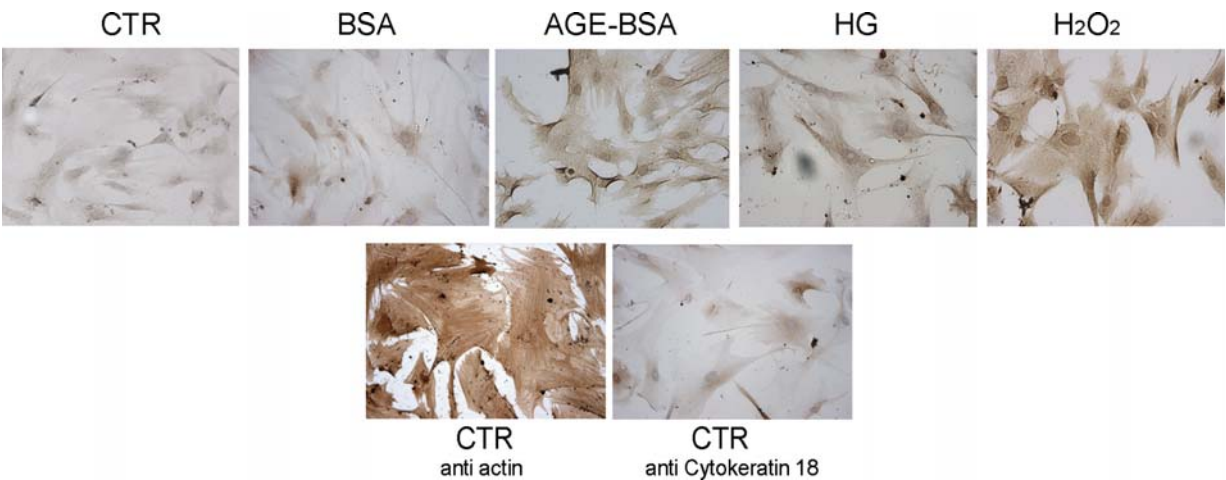


Figure 2. Evaluation of HNE-adducts by immunohistochemistry. The panels are representative of three separate experiments at 48 h of treatment. Analogous results were obtained at each experimental time tested. Anti-actin and anti-cytokeratin reactions are shown as positive and negative staining, respectively.

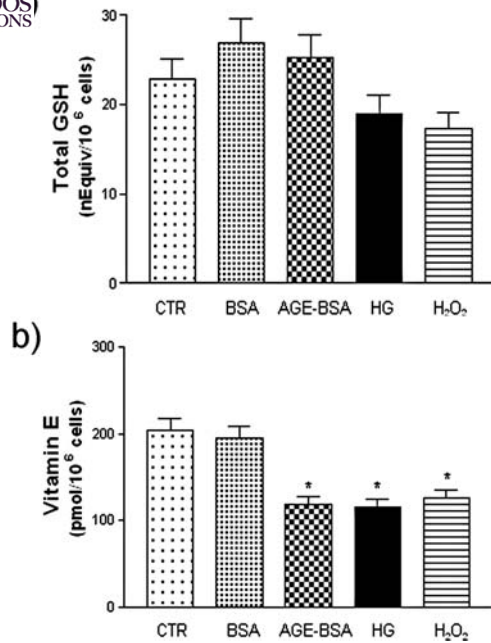


Figure 3. (a) Total glutathione (GSH) content evaluated by HPLC after derivatization with FDNB after 6 days of treatment. Results are expressed as the mean \pm SEM. (b) Vitamin E content evaluated by HPLC after 6 days of treatment. Statistics included ANOVA and Newman Keuls post-test; * $p < 0.05$ vs CTR. Results are expressed as the mean \pm SEM.

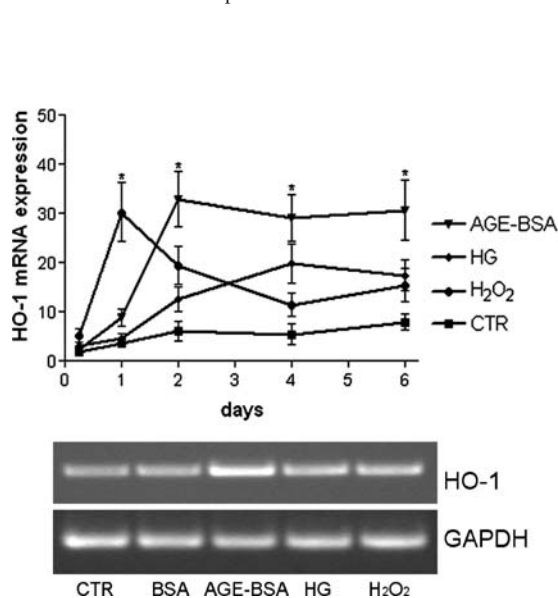


Figure 4. Upper panel: HO-1 mRNA expression evaluated by RT-PCR throughout the 6 days of treatment. Results for BSA were analogous to CTR (data not shown). Statistics included ANOVA and Newman Keuls post-test; * $p < 0.05$ vs CTR at the same time. Results are expressed as the mean \pm SEM. Lower panel: representative gel of HO-1 expression after 6 days of treatments. GAPDH was used as the housekeeping gene.

cells, vascular and glomerular endothelium) (9,19-23). The effects observed by these researchers included alterations in proliferation, viability, cell function and protein expression. Therefore, cell exposure to HG or AGE is believed to be involved in the pathogenesis of diabetic complications.

HG or AGE exposure has been observed to elicit pathologic responses in mesangial cells as well, such as peroxide

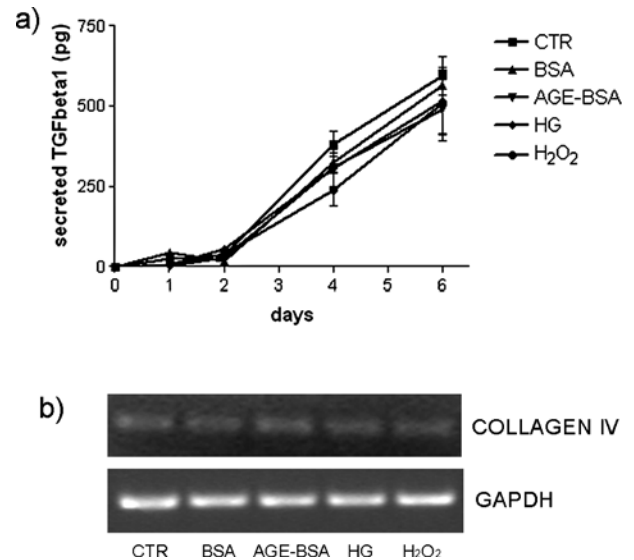


Figure 5. (a) Total TGF- β 1 secreted during the 6 days of treatment. No significant differences were noted at any time. Results are expressed as the mean \pm SEM. (b) Expression of collagen IV, as evaluated by RT-PCR. GAPDH was used as the housekeeping gene. The panels are representative of three separate experiments at 6 days of treatment.

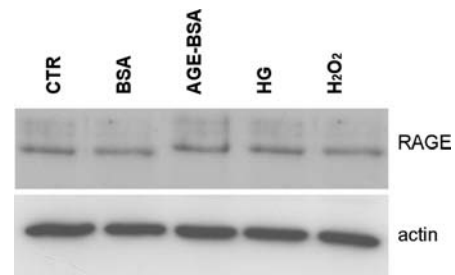


Figure 6. Evaluation of RAGE by Western blotting. β -actin was evaluated as the reference protein. The panel is representative of three separate experiments at day 6 of treatment.

production, activation of transcription factors, modulation of gene expression, matrix deposition and the inhibition of matrix remodeling (6,8,24-28). Since matrix accumulation is a central mechanism of diabetic glomerulopathy, and since glomerular matrix is secreted by mesangial cells, it is widely believed that mesangial cells play a major role in the development of diabetic glomerulopathy (6,9). The reaction of mesangial cells to HG or AGE has, therefore, been regarded as one of the main pathways leading to diabetic glomerulopathy.

In a previous study (10), we observed that some specific antioxidants conferred dramatic protection against morphological and molecular aspects of glomerulopathy in streptozotocin-induced diabetic rats. Therefore, we decided to investigate the effects of the same antioxidants on mesangial cells exposed *in vitro* to diabetes-like conditions (HG or AGE), and we used a commercially available primary cell culture of normal human mesangial cells (NHMCs). Yet, when we tested the effects of HG and AGE exposure on

NHMCs, surprisingly we found that the NHMCs were rather resistant to such treatments.

After AGE, HG or H₂O₂ treatments, peroxide over-production was moderate, with no changes in the level of protein nitrotyrosination and a mild increase in the generation of HNE-protein adducts. This was accompanied by a mild consumption of GSH, a noticeable decrease in vitamin E and a considerable induction of HO-1. Proliferation and viability were virtually unaffected.

Furthermore, HG, AGE and H₂O₂ exposure did not affect the secretion of chemical mediators involved in inflammation and matrix deposition, such as TGF- β 1 and MCP-1; the same treatments were also unable to induce the expression of collagen IV. These results indicated that the NHMCs did not exhibit pro-fibrogenic behavior despite exposure to HG or AGE.

Finally, HG, AGE and H₂O₂ exposure failed to affect the expression of RAGE, which could sensitize the cells to glycoxidative damage. An increase in RAGE expression is generally linked to an intracellular redox imbalance, which acts on the RAGE gene induction through NF- κ B activation (29). Therefore, this result underlines once again the basic resistance of NHMCs to pro-oxidant treatments.

We believe that the resistance of NHMCs to glycoxidative stress is, in all probability, due to an efficacious protective response, shown by the consumption of antioxidants and the induction of HO-1. In this context, it should also be noted that NHMCs demonstrated GSH levels considerably higher than fibroblasts.

We are aware of the amount of scientific data indicating that mesangial cells are susceptible to glycoxidative stress and respond by assuming a fibrogenic attitude (30-33); however, while those data were mainly obtained with 'home-made' primary cultures, foetal, non-human, or virus-modified mesangial cells (8,25,34,35), commercially available normal human mesangial cells were used in the present study, which should be considered to be more reliable.

In conclusion, we are not casting doubts on the role of glycoxidative stress in the pathogenesis of diabetic nephropathy, also confirmed by our previous data obtained *in vivo* (10). Nevertheless, caution should be exercised in attributing an absolutely central role to mesangial cells in the pathogenesis of human diabetic glomerulopathy; mesangial cells may be less sensitive to the diabetes-induced alterations of the microenvironment than is generally believed. Moreover, many important observations on the role of podocytes (36-43) and glomerular endothelial cells (41,44-48) in the pathogenesis of diabetic glomerulopathy have been reported in the literature. Therefore, the role of these other cells should be considered, together with the role of mesangial cells, to fully understand and verify the involvement of glycoxidative stress in the onset and progression of diabetic nephropathy.

Acknowledgements

We thank Mr. Giuseppe Catalano (DIMES, University of Genoa, Italy) for his technical assistance. Grants were provided by MIUR 20077S9A32_002, MIUR 2008N9N9KL_002 and the University of Genoa.

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