Effects of Advanced Glycation End Products on Cytosolic Ca^{2+} Signaling of Cultured Human Mesangial Cells

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Abstract. Advanced glycation end product (AGE) accumulation in a high glucose (HG) environment is thought to mediate some of the vascular complications of diabetes. Transmembrane signaling of contractile cells is generally inhibited by HG, with implications for systemic and target organ hemodynamics. In the kidney, glomerular mesangial cells grown in HG media are hyporesponsive to the effects of vasoconstrictor agents, possibly explaining the hyperfiltration and increased capillary pressure that eventually lead to diabetic glomerulopathy. To verify whether AGE binding to specific mesangial receptors could mediate these effects of HG, cultured human mesangial cells (HMC) were exposed to *in vitro* glycated bovine serum albumin (BSA) for 60 min at 37°C before measurement of cytosolic Ca^{2+} ($[Ca^{2+}]_i$) by microfluorometric techniques in monolayers or single cells. AGE-BSA (2 mg/ml) reduced Ca^{2+} release from intracellular stores by 1 μM angiotensin II from peak $[Ca^{2+}]_i$ levels of 843 ± 117 to 390 ± 50 nM in monolayers and from 689 ± 68 to 291 ± 36 nM in individual cells ($P < 0.05$). Nonglycated BSA and BSA exposed to 250 mM glucose-6-phosphate for 30 d in the presence of 250 mM aminoguanidine (AMGD), an inhibitor of nonenzymatic glycation, had no effect on the angiotensin II-induced $[Ca^{2+}]_i$ spike (peak 766 ± 104 and 647 ± 87 nM, monolayers/single cells, respectively, P = NS). AGE also inhibited store-operated Ca^{2+} influx through plasma membrane channels, assessed by addition of 1 to 10 mM extracellular Ca^{2+} to cells previously held in Ca^{2+}-free media (control 339 ± 46/593 ± 51, +AGE-BSA 236 ± 25/390 ± 56, +AMGD 483 ± 55/374 ± 64 nM $[Ca^{2+}]_i$, monolayers/single cells at 10 mM Ca^{2+}, respectively; +AGE-BSA, $P < 0.05$ *versus* control). Contrary to HG, AGE-BSA did not translocate protein kinase C isoforms α, ζ, and δ to the plasma membrane. Culture of HMC in HG supplemented with 1 mM AMGD prevented downregulation of $[Ca^{2+}]_i$ signaling. These data suggest that glycated macromolecules or matrix components may inhibit transmembrane Ca^{2+} signaling of glomerular cells through binding to a specific AGE receptor, thus mediating some of the known functional effects of HG on the kidney.

Several complications of diabetes mellitus are related to nonenzymatic glycation of proteins in a high glucose (HG) environment via the so-called Maillard reaction (1,2). Compounds in this metabolic pathway include various subfamilies of stable Amadori products, such as pentosidine and pyrraline, collectively termed advanced glycation end products (AGE) (1–4). AGE are known to bind to cell surface receptors coupled to specific signal transduction mechanisms in macrophages and various target organs (5,6). Binding of AGE onto glomerular cells has been demonstrated, possibly accounting for their accumulation in diabetes, along with the known functional and structural changes of diabetic glomerulopathy (7,8).

We and others have shown that substantial postreceptor insensitivity to vasoconstrictors occurs in the microcirculation and in cultured glomerular mesangial cells (MC) exposed to HG media, which might possibly account for renal vasodilation in the early phases of diabetes (9–11). This phenomenon is not linked to altered receptor binding or density (12), but rather to impaired hydrolysis of membrane phosphoinositides by phospholipase C, with decreased inositol phosphate accumulation and Ca^{2+} release from intracellular sources (9–11). Vasoconstrictor-induced Ca^{2+} influx is also decreased (13). The *in vivo* counterpart of these events is elevated blood flow and glomerular capillary pressure, resulting in increased GFR soon after the onset of disease (14). The onset of structural changes is then accompanied by a progressive deterioration of renal function with proteinuria (15,16). Several independent investigators have related the insensitivity to vasoconstrictors to widespread activation of protein kinase C (PKC), a plasma membrane-translocated cytosolic enzyme that controls several branches of transmembrane signaling and the resulting functional responses (17–21). We found that inhibition of PKC restores the cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) responses to vasoconstrictor agents in cultured mesangial cells grown for 3 d in HG media (11,13).

In the present study, we sought to determine whether PKC activation is the sole explanation for $[Ca^{2+}]_i$ insensitivity to vasoconstrictors, or whether other functional events related to nonenzymatic glycation in HG may be involved. To this end, we examined the $[Ca^{2+}]_i$ handling of individual cultured hu-

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man mesangial cells (HMC) or confluent monolayers after direct application of preformed AGE or *in situ* glycation in HG solutions.

Materials and Methods

Cell Culture

Pure lines of HMC were obtained with standard techniques from glomerular explants (22–24). Kidneys not suitable for transplantation or nephrectomy specimens histologically free of lesions were used after obtaining the written informed consent of patients or relatives. Four independent cell lines were used in passages 3 to 16. RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS; Flow Laboratories, Irvine, Scotland, United Kingdom), 5 μg/ml recombinant human insulin (Novo, Copenhagen, Denmark), 10 μg/ml ceftriaxone (Hoffmann-La Roche, Basel, Switzerland), or 100 μg/ml gentamycin (Fournier Pierrel, Milan, Italy) was used for initial plating and propagation of the cultures. The cells were maintained at 37°C in a controlled, humidified atmosphere of 95% O_2/5% CO_2 and subcultured every 4 to 7 d. When appropriate, cells were subcultured in regular complete RPMI medium and 24 h later switched to 5.5 or 30 mM glucose Dulbecco's minimum essential medium (DMEM; Paisley, Scotland, United Kingdom) supplemented with 10% FBS plus antibiotics in the absence of insulin. Media were replaced every 24 h for 5 d.

AGE-Bovine Serum Albumin Preparation

AGE-bovine serum albumin (BSA) was prepared according to standard procedures (25,26) by incubating 20% (wt/vol) fatty acid-free BSA for 30 d with 250 mM glucose-6-phosphate at 37°C in sterile phosphate-buffered saline (PBS), pH 7.2, supplemented with 100 μg/ml gentamycin and 10 μg/ml ceftriaxone. An aliquot of the BSA solution was identically processed in the continued presence of the inhibitor of nonenzymatic glycation, aminoguanidine hemisulfate (AMGD; 250 mM) (27). At the end of the incubation, the solutions were dialyzed overnight under continuous stirring against an identical BSA solution to remove glucose-6-phosphate, AMGD, and the antibiotics. They were then sterile-filtered through 0.2-μm nylon filters (Schleicher and Schuell, Dassel, Germany), aliquoted, and stored frozen until used. The AGE content of the preparations was determined spectrofluorometrically with excitation set at 390 nm and emission set at 450 nm (1–4), and expressed as the percentage of relative fluorescence compared with nonincubated aliquots of the same batch of BSA in glucose-6-phosphate solution, stored frozen immediately after preparation (Table 1).

$[Ca^{2+}]_i$ Measurement in Monolayers

$[Ca^{2+}]_i$ was measured fluorometrically in cells loaded with the intracellular probe, fura-2 (Molecular Probes, Eugene, OR) as described previously (11,13,22–24). Briefly, after withdrawing FBS for the 24 h before an experiment, confluent monolayers grown on plastic Aclar coverslips (Allied Engineered Plastics, Pottsville, PA) were loaded for 40 min at 37°C with 1 μM fura-2 in serum-free DMEM, followed by further incubation for 20 min in the same medium without fura-2. Glucose concentrations were maintained at 5.5 or 30 mM throughout the loading procedure. Fluorescence measurements were performed by inserting the coverslips diagonally in a quartz cuvette filled with 2 ml of modified Krebs-Henseleit solution of the appropriate glucose concentration, buffered with 20 mM Hepes, and supplemented with 0.2% fatty acid-free BSA (KHH). The monolayers were excited at 340 nm with emission collected at 500 nm in a Perkin-Elmer LS5B spectrofluorometer equipped with stirrer and thermostatically controlled cell. Excitation/emission slits were set at 2.5/5 nm, respectively. Calibration of Ca^{2+}-dependent fluorescence was performed by sequential saturation of the dye with 15 to 40 μM ionomycin (Calbiochem-Behring, La Jolla, CA) ± 10 mM $CaCl_2$ to maximum fluorescence (F_{max}), followed by chelation of Ca^{2+} to minimum fluorescence (F_{min}) with 7.5 mM ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid (EGTA) plus 60 mM Tris, pH 10.5. Ratio fluorometry with alternate 340/380 nm excitation at 6-s intervals was used for validation of each set of experiments. Standard formulas were used for the calculation of $[Ca^{2+}]_i$, employing a K_d of fura-2 for Ca^{2+} of 224 nM (22,28). In the Mn^{2+}-quenching experiments, the fluorescence decay of fura-2 was monitored with excitation set at 340 nm and at the 360-nm isosbestic point for fura-2 for at least 3 min after addition of 100 μM $MnCl_2$, and then for an additional 3 min after application of the vasoconstrictors used to promote Ca^{2+} influx.

$[Ca^{2+}]_i$ Measurement in Single Cells

In parallel experiments, $[Ca^{2+}]_i$ was also measured by fluoromicroscopy in single HMC loaded with fura-2. Again, monolayers grown on glass round coverslips were serum-starved for 24 h, loaded for 40 min at 37°C with 1 μM fura-2 in serum-free DMEM, followed by further incubation for 20 min in the same medium without fura-2. Glucose concentrations were maintained at 5.5 or 30 mM throughout the loading procedure. Fluorescence microscopy was performed with a Spex AR-CM apparatus (Spex Industries, Edison, NJ) coupled to a Nikon Diaphot TMD inverted fluorescence microscope equipped with a CF ×40 objective (Nikon Corp., Tokyo, Japan). The coverslip was placed in a thermally controlled chamber bathed with 1 ml of KHH. Individual cells were visualized and excited at alternate 340/380 nm wavelengths with emission collected by a photomultiplier at 510 nm. Calibration of Ca^{2+}-dependent fluorescence was performed by sequential saturation of the dye with 15 to 40 μM ionomycin (±10 mM $CaCl_2$) to maximum fluorescence (F_{max}), followed by chelation of Ca^{2+} to minimum fluorescence (F_{min}) with 7.5 mM EGTA plus 60 mM Tris, pH 10.5. Ratiometrics recordings were stored in an Asem Desk 2010 computer that automatically calculated $[Ca^{2+}]_i$, using standard formulas and a K_d of fura-2 for Ca^{2+} of 224 nM (22,28).

Table 1. Fluorometric AGE assay in glucose-6-phosphate-treated BSA[a]

Characteristic	BSA Control	AGE-BSA	AGE-BSA + AMGD
Fluorescence units	18.2 ± 0.1	68.9 ± 0.9[b]	21.1 ± 0.5
% of control		+277 ± 4	+16 ± 3

[a] Data are expressed as mean ± SEM of quadruplicate measurements on 2 mg/ml BSA solutions (wt/vol) at 390/450 nm excitation/emission. AMGD, 250 mM aminoguanidine present throughout the 30-d incubation with glucose-6-phosphate. AGE, advanced glycation end products; BSA, bovine serum albumin.

[b] $P < 0.001$ *versus* BSA control.

Protein Kinase C Assay

The cellular distribution of PKC isoforms α, δ, and ζ was examined by a conventional immunoblotting technique. Briefly, confluent HMC monolayers treated with AGE-BSA, AMGD-treated BSA, or control BSA were washed twice with ice-cold PBS after removal of the experimental media. The cells were scraped into 1 ml of ice-cold homogenization buffer (20 mM Tris-HCl, pH 7.5, 1 mM ethylenediaminetetra-acetic acid, 1 mM EGTA, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 mM benzamidine, 25 μg/ml leupeptin, and 6 μg/ml aprotinin). All subsequent steps were carried out at 4°C. The cells were lysed by ultrasonication and spun for 1 h at 100,000 $\times$ *g*. Supernatants were used as a source of cytosolic protein. To obtain the solubilized membrane fraction, pellets were sonicated again in 1 ml of the same buffer containing 1% Triton X-100 and centrifuged for 1 h at 100,000 $\times$ *g*. Thirty micrograms of the cell fractions was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8% acrylamide gel), and proteins were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA). Filters were then incubated overnight at 4°C with monoclonal antibodies against PKC-α, -δ, and -ζ (Santa Cruz Biotechnology, Santa Cruz, CA, diluted 1:1000), and then with anti-mouse horseradish peroxidase-conjugated secondary antibody (diluted 1:5000), which was subsequently detected by enhanced chemiluminescence. The filters were then stripped and reprobed with anti-actin antibody (Santa Cruz) to detect this reference gene as an internal control. Molecular weights were calculated on the basis of prestained standards (Bio-Rad, Hercules, CA). Densitometry scanning was carried out on a Bio-Rad GS-700 imaging apparatus, with each value expressed as the ratio of PKC isoenzyme/actin. Rat brain extract was used as a positive control for PKC isoenzymes.

Statistical Analysis

All data were expressed as mean $\pm$ SEM and analyzed by one-way ANOVA.

Results

Consistent with our earlier findings in rat MC (11,13), growth of human MC in HG for 5 d resulted in reduced sensitivity to the effects of angiotensin II (AngII), a typical stimulus of $[Ca^{2+}]_i$ via phospholipase C. As shown in Figure 1 and Table 2, the amplitude of the $[Ca^{2+}]_i$ elevation in response to extracellular Ca^{2+} in cells that were previously equilibrated in Ca^{2+}-free solutions was typically reduced by HG. This relatively slow and persistent rise of $[Ca^{2+}]_i$ results from Ca^{2+} influx through surface channels that are activated by Ca^{2+} depletion. As reported previously (24), these channels belong to the functional group of store-operated Ca^{2+} channels (SOC), responsible for the so-called "capacitative" Ca^{2+} influx, since it enables refilling and subsequent discharge of cytoplasmic stores (13,24,29–33). These storage sites are readily released by inositol (1,4,5)-trisphosphate, which is typically generated in response to AngII in excitable cells, with ensuing "transient" $[Ca^{2+}]_i$ elevation (34). HG also depressed this $[Ca^{2+}]_i$ response to AngII (Figure 1, Table 2), confirming a general inhibition of early $[Ca^{2+}]_i$ signaling in MC. Similar results were obtained with other phospholipase C/Ca^{2+}-transduced agents not related to AngII, such as arginine vasopressin and the endoperoxide/thromboxane A_2 mimetic U-46619 (not shown).

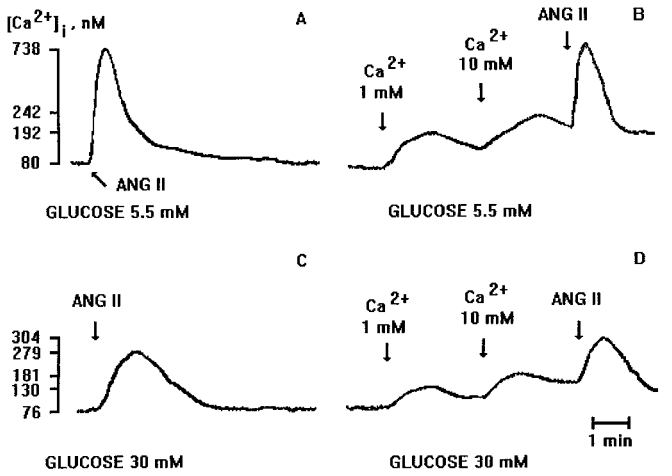

Figure 1. Effects of 5.5/30 mM glucose on cytosolic calcium ($[Ca^{2+}]_i$) of fura-2-loaded monolayers of cultured human mesangial cells equilibrated in Ca^{2+}-free media. (A) $[Ca^{2+}]_i$ elevation upon addition of 1 μM angiotensin II (AngII) to cells grown for 5 d in 5.5 mM glucose, $[Ca^{2+}]_e$ 1.25 mM. (B) Ca^{2+} influx upon addition of 1 to 10 mM Ca^{2+} and 1 μM AngII to cells equilibrated in nominally Ca^{2+}-free solutions after 5 d in 5.5 mM glucose. (C and D) Reduced Ca^{2+} mobilization by AngII and Ca^{2+} influx in 30 mM glucose. Fluorescence tracings are representative of six experiments for each condition.

Next, we tried to duplicate these observations with preformed AGE, based on the hypothesis that nonenzymatic glycation may occur during 5 d of culture in HG, possibly resulting in downregulation of $[Ca^{2+}]_i$ signaling. As shown in Figure 2, 2 mg/ml AGE-BSA added 60 min before microfluorometry of both monolayers (left panels) and single HMC (right) cultured in normal glucose (NG, 5.5 mM) media profoundly inhibited the $[Ca^{2+}]_i$ response to AngII in standard bathing solutions containing 1.25 mM extracellular $[Ca^{2+}]$. An identical amount of native, nonglycated BSA has been added to the control cells (final concentration 0.2%, wt/vol) to account for oncotic and other possible effects of extracellular proteins, including binding to cell surface domains or receptors. AMGD-treated BSA failed to modify the $[Ca^{2+}]_i$ responses to AngII, similar to control BSA. The cumulative results of these experiments, summarized in Figure 3, indicate impaired discharge of Ca^{2+} stores, similar to the cells exposed to HG solutions, pointing to a role of AGE in depressing this immediate signal shared by a number of vasoconstrictors. Basal $[Ca^{2+}]_i$ was not modified by pretreatment with AGE-BSA, which also had no acute effect when added to monolayers not previously exposed to glycated protein (87 $\pm$ 2 *versus* 84 $\pm$ 3 nM, $n = 6$). Because of the intrinsic fluorescence of glycated adducts, interfering with emission of the fura-2 probe, background autofluorescence was recalculated and subtracted in these studies. In Figure 4, Ca^{2+} influx has been examined upon 1-h exposure to AGE-BSA. Similar to the group of experiments of Figure 1, a brief Ca^{2+} deprivation was required to activate Ca^{2+} entry upon graded addition of extracellular Ca^{2+} from nominally free to final concentrations of 1 mM and then 10 mM. Again, both averaged fluorescence signals from

Table 2. Effect of 5 day culture of human mesangial cells in NG/HG media on Ca^{2+} influx and release from intracellular stores[a]

Condition	Media Glucose Concentration			
	5.5 mM	30 mM	5.5 mM	30 mM
			+1 mM Aminoguanidine	
Baseline	63 ± 5	63 ± 6	76 ± 5	67 ± 4
Ca^{2+} 1 mM	263 ± 34	146 ± 23[b]	214 ± 27	225 ± 35
Ca^{2+} 10 mM	485 ± 85	185 ± 22[b]	475 ± 89	399 ± 95
+AngII, 1 μM	657 ± 45	265 ± 27[b]	698 ± 47	777 ± 88

[a] Data are mean ± SEM nM peak $[Ca^{2+}]_i$ from six experiments on independent monolayers equilibrated in Ca^{2+}-free solutions. NG, normal glucose; HG, high glucose; AngII, angiotensin II.
[b] $P < 0.05$ *versus* 5.5 mM glucose.

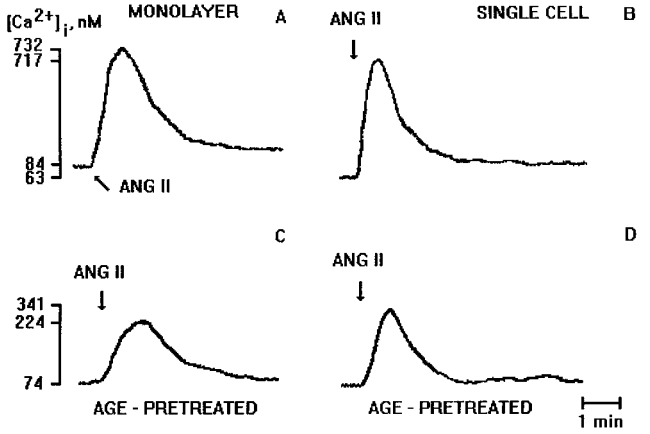

Figure 2. Effects of advanced glycation end products-bovine serum albumin (AGE-BSA) on the release of Ca^{2+} from intracellular stores of fura-2-loaded cultured human mesangial cells by 1 μM AngII. Confluent monolayers (A and C) or individual mesangial cells (B and D) were pretreated for 60 min with 2 mg/ml AGE-BSA (bottom panels) or control nonglycated BSA (top panels). Fluorescence tracings are representative of nine experiments for each condition.

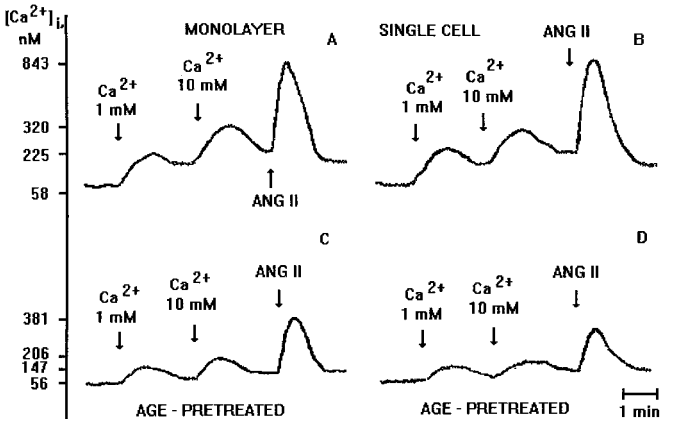

Figure 3. Effects of AGE-BSA on Ca^{2+} influx into fura-2-loaded cultured human mesangial cells previously equilibrated in Ca^{2+}-free media. Confluent monolayers (A and C) or individual mesangial cells (B and D) were pretreated for 60 min with 2 mg/ml AGE-BSA (bottom panels) or control nonglycated BSA (top panels). Fluorescence tracings are representative of nine experiments for each condition.

groups of cells in a monolayer (left) and from individual cells (right) clearly reflect inhibition of Ca^{2+} influx by short-term treatment with AGE-BSA. Interestingly, equivalent amounts of AMGD-treated BSA failed to inhibit both Ca^{2+} influx and subsequent Ca^{2+} release by AngII (Figure 5). The cumulative results of this set of studies are shown in Figure 6. Taken together with the nonglycated BSA controls, these experiments indicate that glycation of BSA is required for inhibition of transmembrane Ca^{2+} fluxes.

We then designed a protocol to validate the Ca^{2+} influx results, by recording fluorescence emission of fura-2 in monolayers exposed to 0.1 mM $MnCl_2$. This divalent cation has been shown to enter most cell types via nonselective plasma membrane channels, including gated Ca^{2+} channels; contrary to Ca^{2+}, however, its binding to fura-2 does not excite the probe's fluorescence emission, but rather irreversibly quenches it (35). Therefore, it has been extensively used to monitor divalent cation activity through decay of the fura-2 emission with excitation set at 340 nm or at the isosbestic point, 360 nm (13,24,30,35). The lower panel of Figure 7 shows reduced Mn^{2+} quenching of fura-2 in monolayers pretreated with 2 mg/ml AGE-BSA, thus confirming the results of direct $[Ca^{2+}]_i$ measurements. Notably, even the rise of fluorescence induced by AngII, resulting from Ca^{2+} release from internal stores, in the absence of extracellular Ca^{2+} is blunted by AGE-BSA (Figure 7).

Finally, we sought to determine whether treatment of the monolayers with AMGD would prevent the inhibitory effects of HG on $[Ca^{2+}]_i$ signaling. As shown in Table 2, culture of HMC for 5 d in HG in the presence of 1 mM AMGD fully restored normal responsiveness of HMC monolayers to both extracellular Ca^{2+} and AngII, pointing to the accumulation of glycated metabolites during prolonged culture as one relevant mechanism of the observed effects of HG. This is confirmed by the basal fluorometric readings obtained in the HG monolayers, which showed significantly greater emission at 390/450 nm wavelengths than cells grown in NG, consistent with gly-

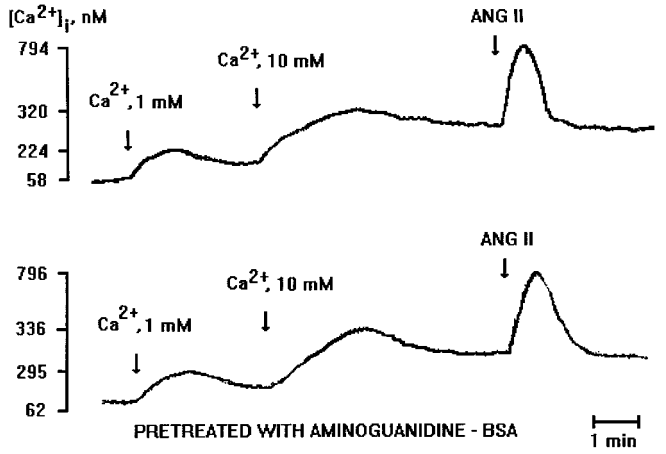

Figure 4. Lack of effect of aminoguanidine-treated BSA (2 mg/ml for 60 min, bottom panel) on $[Ca^{2+}]_i$ regulation in fura-2-loaded monolayers of cultured human mesangial cells. (Top Panel) Sequential addition of 1/10 mM extracellular Ca^{2+} followed by 1 μM AngII to a control monolayer equilibrated in Ca^{2+}-free media. Fluorescence tracings are representative of nine experiments for each condition.

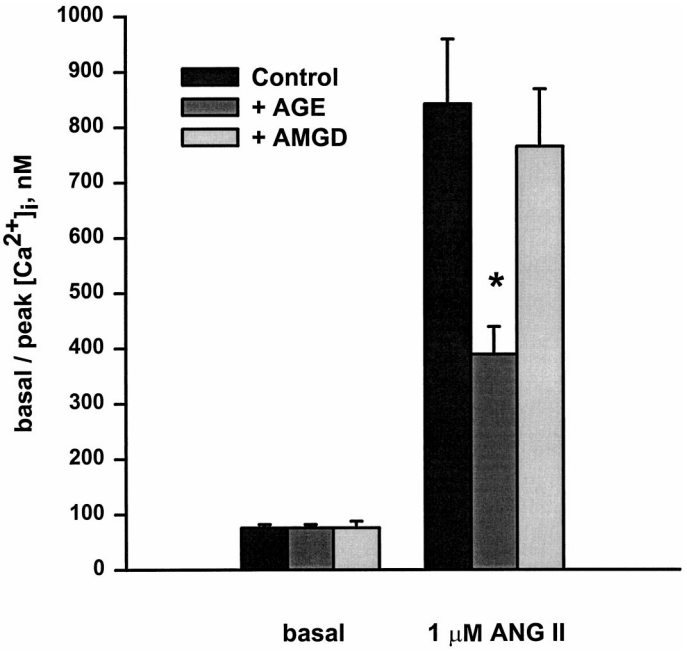

Figure 5. Effects of 2 mg/ml AGE-BSA or aminoguanidine (AMGD)-treated BSA on Ca^{2+} release from intracellular stores by 1 μM AngII in fura-2-loaded monolayers of cultured human mesangial cells. Cumulative results, mean ± SEM from nine experiments for each condition. *$P < 0.05$ *versus* control by one-way ANOVA.

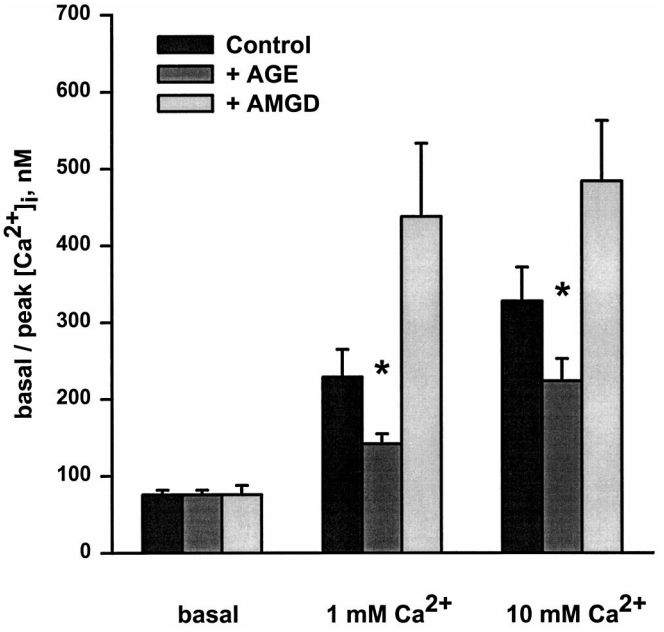

Figure 6. Effects of 2 mg/ml AGE-BSA or AMGD-treated BSA on Ca^{2+} influx into fura-2-loaded monolayers of cultured human mesangial cells. Cumulative results, mean ± SEM from nine experiments for each condition. *$P < 0.05$ *versus* control by one-way ANOVA.

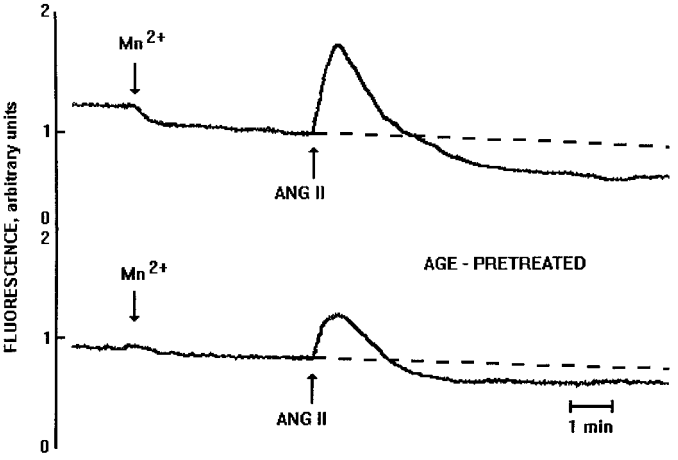

Figure 7. Effects of 2 mg/ml AGE-BSA on Mn^{2+} influx in monolayers of cultured human mesangial cells equilibrated in Ca^{2+}-free media. (Top Panel) Fluorescence decay due to quenching of fura-2 emission by 0.1 mM $MnCl_2$, internalized via divalent cation nonselective channels. Note enhanced fluorescence quenching after a transient $[Ca^{2+}]_i$ elevation induced by 1 μM AngII, due to enhanced Mn^{2+} entry. (Bottom Panel) Inhibition of basal and AngII-induced Mn^{2+} quenching in cells pretreated with AGE-BSA for 60 min. Fluorescence tracings at 340 nm excitation are representative of six experiments, with identical readings obtained at the 360 nm isosbestic point for fura-2.

cation *in vitro* (22.8 ± 4.7 *versus* 16.3 ± 2.1 fluorescence units, HG/NG, respectively, $P < 0.05$ on $n = 24$ monolayers each group).

The effects of AGE could not be attributed to PKC activation as demonstrated previously for HG (11,13), since our immunoblotting experiments indicated that the PKC isoforms α, ζ, and δ are not significantly translocated to the plasma membrane upon 1 h pretreatment with AGE-BSA (Figure 8). As expected, no translocation occurred either in control monolayers exposed to nonglycated BSA, or in cells exposed to AMGD-treated BSA (Figure 8, Table 3).

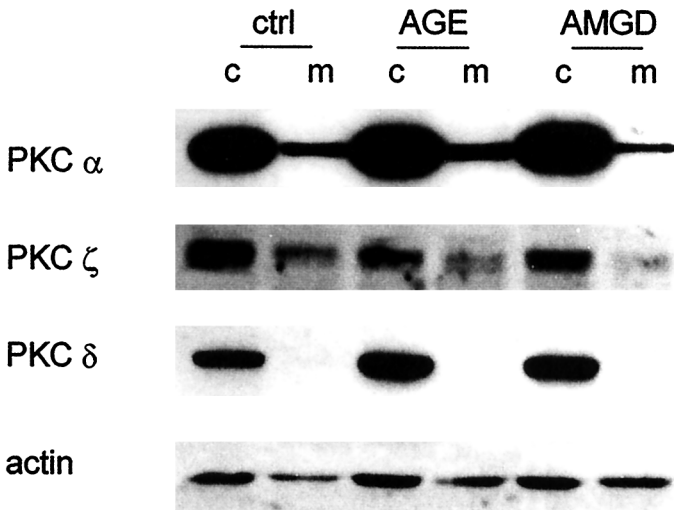

Figure 8. Effects of 60-min incubation of cultured human mesangial cell monolayers with 2 mg/ml AGE-BSA or AMGD-treated BSA on protein kinase C (PKC) isoform α, ζ, and δ translocation to the plasma membrane. c, cytosol; m, plasma membrane. Representative Western blots from three experiments. Molecular weights are 82 kD for PKC α, 65 kD for PKC ζ, and 78 kD for PKC δ.

Table 3. Effect of AGE-BSA on PKC isoform translocation to the plasma membrane in cultured human mesangial cells[a]

Group	BSA Control	AGE-BSA	AGE-BSA + AMGD
PKC α			
cytosol	0.49	0.67	0.67
membrane	0.20	0.19	0.08
PKC ζ			
cytosol	0.83	0.57	0.74
membrane	0.78	0.56	0.39
PKC δ			
cytosol	1.52	2.39	2.41
membrane	0	0	0

[a] Data are actin-normalized densitometry ratios from one experiment representative of $n = 3$, 60-min treatment of independent monolayers with 2 mg/ml BSA protein. PKC, protein kinase C.

Discussion

Nonenzymatic glycation of intrinsic cell components and secretory products, such as extracellular matrix, plays an important role in the vascular and tissue complications of diabetes. The discovery of widespread AGE receptors (RAGE) in both circulating and resident cells suggests a dynamic balance between accumulation and metabolism of glycated proteins, with functional adaptations occurring in target cells recognizing AGE (3–7). MC of the kidney glomerulus are one such cell population, typically responding to the diabetic microenvironment with phenotypic changes, eventually resulting in the deposition of extracellular matrix with mesangial expansion (8,16,25,26,36–40). The hemodynamic overload of the glomerular capillary tuft during the early phase of hyperfiltration may also contribute to the direct effects of HG and altered insulin/receptor coupling, triggering irreversible alterations of the synthetic behavior of MC (41).

Recently, the role of AGE in the control of MC phenotype has been underscored by a number of *in vitro* and *in vivo* studies. Among the observed responses to AGE, receptor binding with uptake, internalization, cell proliferation, and matrix synthesis have been reported (25,26). Enhanced adhesion of monocytic cells to AGE-treated MC has been noted (unpublished results), with possible implications for leukocyte chemoattraction in diabetic vascular and glomerular lesions (42). The present results provide evidence that other functional changes of MC grown in HG are linked to the accumulation of AGE. The incubation of HMC with large amounts of extracellular protein previously glycated *in vitro* rapidly reproduces the inhibitory effects of long-term culture in HG on $[Ca^{2+}]_i$-dependent transmembrane signaling for vasoconstrictors. Under these circumstances, many vascular smooth muscle cell (VSMC) types, including MC, appear less sensitive to the vasomotor action of peptides and autacoids that activate phospholipase C-coupled receptors (43–45). Impaired glomerular contractility in *ex vivo* preparations is believed to result from this phenomenon (46,47), which may explain, at least in part, the glomerular vasodilation of the early phases of diabetes, responsible for hyperfiltration and microalbuminuria. On the basis of the present findings, in addition to smooth muscle-like mesangial cell contraction, a number of other Ca^{2+}-regulated cellular events, ranging from prostanoid biosynthesis to matrix production and degradation, cell proliferation, and apoptosis, may be affected by AGE binding.

Work from several laboratories has established that elevated PKC activity may be a common feature of cells grown in HG media, and might in turn explain several functional aspects of these cells (9,11,13,17–21,36). This may well be the case for inositol phospholipid (InsP)/$[Ca^{2+}]_i$ signaling, which is subject to an inhibitory feedback control from PKC (10,11,13). Earlier studies from our group demonstrated that PKC downregulation rapidly reverses the effects of HG on the immediate $[Ca^{2+}]_i$ responses to vasoactive agents (11,13). Because HG did not alter the size of $InsP_3$-releasable intracellular Ca^{2+} pools, it was concluded that depressed receptor/InsP coupling was responsible for such functional resistance (10,11,13). This was extensively confirmed by other groups in cultured VSMC and MC (9,17,20,43–49).

The effects of HG and preformed AGE on $[Ca^{2+}]_i$ are not restricted to the release of Ca^{2+} from intracellular stores, but involve reduction of Ca^{2+} influx through plasma membrane channels as well. Williams and Schrier have shown inhibition of voltage-gated Ca^{2+} channels in VSMC grown in HG (48). Recent studies from our laboratory demonstrate direct inhibition of a store-dependent Ca^{2+} influx pathway by HG in rat MC (13). Patch-clamp work from Seal *et al.* is consistent with these observations, indicating that a 27-pS nonselective cation channel is inhibited by 30 mM glucose media in the rat (49). This channel, activated by AngII and thapsigargin, may represent one link between store depletion and Ca^{2+} influx in MC

(50). Thus, reduced Ca^{2+} entry may be common to several cell types cultured in HG, but the mechanism of internalization involved could be different, based on the peculiar features of a given cell population. The weaker Ca^{2+} influx in MC may indeed be due to the smaller amounts of Ca^{2+} made available from intracellular stores. Nevertheless, our experiments with PKC activators indicate that Ca^{2+} uptake is also directly downregulated by this enzyme (11,13). Interestingly, the electrophysiology studies by Seal *et al*. indicated that PKC inhibition restores channel responsiveness to AngII in the presence of HG (49). Such inhibition of $[Ca^{2+}]_i$ signaling may represent an endogenous negative feedback, aimed at limiting the state of activation of the cells in the presence of vasoconstrictors, and preventing $[Ca^{2+}]_i$ from rising to uncontrolled levels (51).

In the present investigation, the effects of AGE on $[Ca^{2+}]_i$ transport are specific, as equivalent amounts of native albumin, containing only trace glycated components, and of glucose-treated BSA in the presence of an inhibitor of the Maillard reaction, fail to elicit any significant change in the $[Ca^{2+}]_i$ response to AngII. Actually, the concentration of BSA in these control experiments is identical to that chosen in most $[Ca^{2+}]_i$ studies to maintain the transmembrane oncotic balance and ensure proper membrane surface receptor functionality. Thus, based on the present studies, it is legitimate to envision a model in which depressed $[Ca^{2+}]_i$ signaling occurs as a result of elevated glucose concentrations, partly due to PKC activation via deranged metabolism, and partly because of the progressive accumulation of AGE over several days in culture. The source of these AGE is severalfold. Intrinsic cell components, extracellular matrix and proteins, and even serum components contained in the culture media may accumulate AGE determinants that are ligated at the specific receptor existing in these cells. In our studies, large amounts of preformed AGE may mimic local glycation during culture in HG media. It is of interest that in the experiments with AMGD, blocking glycation during 5 d of culture completely abolished the inhibitory effects of 30 mM glucose on $[Ca^{2+}]_i$ signaling. This has possible clinical implications, as glycation of circulating proteins or intrinsic tissue components may be blocked *in vivo* by administration of this compound.

As far as the mechanisms of action of AGE in downregulating early $[Ca^{2+}]_i$ transmembrane signaling are concerned, our studies do not show any significant effect of AGE on three PKC isoforms within the time frame of the $[Ca^{2+}]_i$ experiments. Other signaling pathways, additional to PKC activation by HG, must therefore be considered. For example, because AGE binding to the dimeric RAGE receptor complex of 60- and 90-kD proteins on various cells, including HMC, triggers a sequence of intracellular signals involving activation of the transcription factor NFκB (5–8,52–54), it is reasonable to attribute to such cascade a negative feedback on phospholipase C-dependent responses to vasoconstrictors. Of course, one could not rule out a possible local effect of AGE cross-linking or clustering at membrane receptors, not involving intracellular signaling, but rather the displacement of receptors for vasoconstrictors and/or ion channels. In either case, the observed effect is likely to be relevant to mesangial function *in vivo*, as deposition of AGE has been shown to occur in this area in diabetic glomerulopathy (3,4,16). RAGE have been suggested to play an active role in this process by uptaking locally generated or circulating glycated macromolecules (3,4,5,55,56).

In conclusion, multiple biologic actions of AGE on target glomerular cells provide a background for the understanding of biochemical events occurring in diabetes, as well as in other pathologic conditions, albeit at a slower rate. Aging, atherosclerosis, chronic renal failure, and renal replacement therapies including hemodialysis and peritoneal dialysis are all settings in which enhanced glycation of tissue and circulating proteins occurs (57). The concept that glomerular cells react to and possibly participate in the metabolism of AGE is of primary importance for clinicians and researchers involved in the prevention and management of human vascular disease.

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