Bimodal Effect of Advanced Glycation End Products on Mesangial Cell Proliferation Is Mediated by Neutral Ceramidase Regulation and Endogenous Sphingolipids

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Advanced glycation end-products (AGE) are generated by chronic hyperglycaemia and may cause diabetic microvascular complications such as diabetic nephropathy. Many factors influence the development of diabetic nephropathy; however, dysregulation of mesangial cell (MC) proliferation appears to play an early and crucial role. In this study, we investigated the effects of AGE on rat MC proliferation and the involvement of sphingolipids in the AGE response. Results show a bimodal effect of AGE on MC proliferation. Thus, low AGE concentrations (<1 μM) induced a significant increase (+26%) of MC proliferation, whereas higher concentrations (10 μM) markedly reduced it (−24%). In parallel, AGE exerted biphasic effects on neutral ceramidase expression and activity. Low AGE concentrations increased neutral ceramidase activity and expression, whereas high AGE concentrations showed opposite effects. Surprisingly, neutral ceramidase modulation did not result in changes of ceramide levels. However, the AGE (10 μM)-inhibitory effect on MC proliferation was associated with accumulation of sphingosine and was specifically prevented by blocking glucosylceramide synthesis, suggesting that the high AGE concentration effects are mediated by sphingosine and/or glycolipids. On the other hand, treatment of cells with low AGE concentrations led to an increase of sphingosine kinase activity and sphingosine-1-phosphate production that drove the increase of MC proliferation. Interestingly, in glomeruli isolated from streptozotocin-diabetic rats, a time-dependent modulation of ceramidase activity was observed as compared with controls. These results suggest that AGE regulate MC growth by modulating neutral ceramidase and endogenous sphingolipids.

Chronic hyperglycaemia promotes advanced glycation end-products (AGE) formation and accumulation in vitro. It has been largely documented that glycation, as a nonenzymatic reaction between glucose and amino acid residues of long living proteins, leads to structural and functional alterations of structural as well as circulating proteins, such as hemoglobin, IgG, or albumin (1–3). AGE have been shown to contribute to peripheral microvascular alterations, leading to one of the major complications of diabetes mellitus, diabetic nephropathy and end stage renal failure. Indeed, a number of AGE such as carboxymethyllysine or pentosidine have been identified in kidneys of diabetic patients, and their renal accumulation was positively correlated with the disease’s severity (4). AGE glomerular accumulation was also reported in animal models such as the streptozotocin-induced type I diabetes mellitus in rat (5, 6).

Compelling evidence suggests that high ambient glucose-induced AGE accumulation contributes importantly to mesangium pathogenesis (7). For example, one of the dominant histological features of the diseased or diabetic kidney is the expansion of the extracellular matrix. In this regard, an imbalance in the control of mesangial cell (MC) proliferation appears to play an early and crucial role in the initiation and progression of glomerulosclerosis. After entering an early and limited step of hyperplasia, MC arrest in the G1 phase and undergo de novo synthesis and accumulation of constitutive proteins (such as collagen IV, fibronectin, and laminin B1), leading to cellular hypertrophy (8). At this point, AGE have been recently shown to inhibit human MC proliferation (9) and to promote apoptosis. Additionally, excessive matrix protein secretion and deposition into the conjunctive space by MC has been associated with autocrine overexpression of transforming growth factor β (10). Interestingly, transforming growth factor β overproduction was suggested to be triggered by AGE (11).

Sphingolipid metabolism, in particular the ceramide (Cer) pathway, has been proposed to regulate many biological responses, including cell survival and cell death. Cer has also been implicated in the pathogenesis of diabetes (12, 13). A balance between Cer and other bioactive metabolites such as sphingosine (Sph) and sphingosine-1-phosphate (S1P) has been pointed out as a sensitive rheostat controlling growth and death of various cell types (14, 15). Thus, in contrast to the growth-inhibitory and proapoptotic effects of Cer and Sph (16–20), S1P has been shown to promote cell growth in various cell types including MC (21, 22). Many studies have reported the involvement of sphingomylinas, the ceramide-generating enzymes, in the regulation of Cer levels in response to proinflammatory cytokines, growth factors, or stress stimuli (23, 24). Ceramidas (CDases), the ceramide-degrading enzymes, could also play an important role in the regulation of Cer levels. CDases hydrolyze the Cer N-acyl linkage between the fatty acyl and the sphingosine base. Current knowledge suggests that...
this catabolic pathway represents the prevailing source of cellular Sph (25–27), which can in turn be phosphorylated by sphingosine kinase (SphK) to form S1P. In this way, CDase activity may be a key step in determining the intracellular levels of Cer and Sph/SIP, thus playing a crucial role in the regulation of cell survival or death in response to external stimuli (reviewed in Ref. 28).

Indeed, recent studies have implicated CDase activity in MC proliferation in response to platelet-derived growth factor stimulation (29). Franzen and et al. (30) showed that chronic interleukin-β (IL-β) treatment of rat mesangial cells resulted in neutral CDase activation, thereby counteracting sphingomyelinase activation, Cer accumulation, and apoptosis. Nikolaova-Karakashian et al. (31) reported in primary cultures of rat hepatocytes a dose-dependent effect of interleukin-1β on CDase activity. Further, this CDase regulation provided a “switch” determining the net levels of Cer and Cer-downstream active metabolites, Sph or S1P, and the subsequent effects on expression of specific genes.

In the present study, the involvement of sphingolipid metabolism in the growth response cultured MC exposed to AGE was investigated. Results show that AGE, most likely through the receptor for AGE (RAGE), exerted a bimodal effect on MC proliferation. These effects were associated with modulation of neutral CDase (N-CDase) and SphK activities and resulted in the receptor for AGE (RAGE), exerted a bimodal effect on MC proliferation in response to platelet-derived growth factor stimulation (29). Franzen and et al. (30) showed that chronic interleukin-β (IL-β) treatment of rat mesangial cells results in neutral CDase activation, thereby counteracting sphingomyelinase activation, Cer accumulation, and apoptosis. Nikolaova-Karakashian et al. (31) reported in primary cultures of rat hepatocytes a dose-dependent effect of interleukin-1β on CDase activity. Further, this CDase regulation provided a “switch” determining the net levels of Cer and Cer-downstream active metabolites, Sph or S1P, and the subsequent effects on expression of specific genes.

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Bimodal effects of AGE on mesangial cells proliferation. $[^{3}H]$Thymidine incorporation was evaluated on confluent quiescent MC after 72 h of incubation in the presence of the indicated age or control BSA concentrations. $[^{3}H]$Thymidine uptake was evaluated during the last 18 h of treatment. Results are expressed as percentage of relative control BSA and represent means ± S.D. of seven or eight experiments, each performed in duplicate (*, p < 0.05 versus BSA control).

(Amersham Biosciences), and the radioactive spots corresponding to S1P were visualized by autoradiography. Bands corresponding to $[^{33}P]$S1P were quantified, and SphK specific activity was expressed in arbitrary units/min/mg of protein.

Lipid Analysis and Quantification—Quiescent confluent MC cultured in 10-cm Petri dishes were treated for 72 h with AGE or control BSA. Culture medium was removed, cells were washed twice with ice-cold PBS and then scraped in 1 ml of methanol, and total lipids were extracted by the method of Bligh and Dyer (38). Total lipids in the organic phase were then dried, and an aliquot was taken from each sample for total lipid phosphate determination as described (39).

Ceramide Measurement Using the Dsacylglycerol Kinase Assay—Measurement of Cer levels was performed using the DAG kinase assay as described (39, 40). Briefly, lipid samples and standards were sonicated in 20 μl of mixed micelles (7.5% β-n-octyl-glucopyranoside, 25 mM dioleyl phosphatidylglycerol) and incubated for 30 min at 37°C. Then 40 μl of enzyme reaction buffer (75 mM imidazole, pH 6.8, 71 mM LiCl, 17.8 mM MgCl2, 1.5 mM EDTA, 0.25 mM diethylthiocarbamoyltaurate acid, 2.8 mM dithiothreitol, and 3 μg of Escherichia coli DAG kinase) and 10 μl of ATP mixture (10 mM ATP and 0.2 μCi/μl ($\gamma$-33P)ATP in 5 mM imidazole) were added to samples, and the mixture was incubated for 1 h at room temperature. Phosphorylated Cer and DAG were extracted with chloroform/methanol. Lipids and DAG and Cer standards (0–600 pmol) were then spotted onto TLC plates and developed in chloroform/acetone/methanol/acetic acid/water (50:20:15:10:5, v/v/v/v/v). Radioactive DAG 1-phosphate and Cer 1-phosphate were visualized and quantified by autoradiography.

HPLC Assay of S1P and Sph—HPLC assay was used to quantify the released S1P and Sph, as described by Min et al. (41). Cells from 3 × 109 cm diameter dishes were scraped, pooled, and centrifuged, and pellets were resuspended into 100 μl of PBS, and an aliquot was kept for protein determination. After adding 250 μl of methanol, 0.6 μl of concentrated HCl, and 20 pmol of C18-sphingosine 1-phosphate as an internal standard, samples were sonicated for 5 min at 4°C. 500 μl of chloroform plus 1 μl NaCl (1.1%, v/v) and 25 μl of 3 N NaOH were added, and the mixture was vortexed and centrifuged to allow phase separation. S1P is water-soluble at alkaline pH and partitions into the aqueous phase. After centrifugation, the lower phase was re-extracted twice with 250 μl of methanol plus 1 μl NaCl (1:1, v/v), 15 μl of 3 N NaOH. Aqueous phases were combined, mixed thoroughly with 130 μl of reaction buffer (200 mM Tris-HCl, pH 7.4, 75 mM MgCl2, 2 mM glycine, pH 9), 50 units of alkaline phosphatase and incubated for 1 h at 37°C on a 200-μl chloroform layer placed at the bottom of the reaction mixture. Phosphorylated sphingoid bases were extracted twice with 300 μl of chloroform. Pooled organic layers were further washed three times with 300 μl of alkaline water, placed in amber glass microvials, dried under N2, and dissolved in 120 μl of ethanol. Released Sph was derivatized with 15 μl of freshly prepared o-phthalaldehyde reagent (OPA) (5 ml of 3% (w/v) boric acid, pH 10.5, 10 μl of β-mercaptoethanol, and 100 μl of ethanol containing 5 mg of OPA). The mixture was allowed to stand for 20 min at room temperature before analysis. HPLC was conducted using a Hewlett Packard 1100-HPLC model fitted with a 5-μm RP-C18 column (25 × 4) combined with a guard column cartridge. The solvent was acetonitrile/water (90:10, v/v), and elution was achieved during 50 min with a flow rate of 1 ml/min. A spectrofluorometer detector was used with an excitation at 340 nm and emission at 455 nm. The retention times of C17- and C18-S1P were around 9 and 12 min, respectively. Chromatographic profiles were then analyzed using the HP Chemstation software. S1P release in the culture medium of MC was measured as described above, except for the volume of solvent used for extraction.

For Sph measurement, standard C17-Sph was added in the residual organic phase after primary S1P extraction. Sph derivatization and analysis was then performed as described for S1P.

Western Blot Analysis—Quiescent confluent MC in 10-cm diameter dishes were treated with the indicated concentrations of AGE or control BSA for 72 h. The culture medium was removed, and the cells were washed with ice-cold PBS. Cells were then scraped directly into lysis buffer (25 mM Heps, pH 7.5, 5 mM EDTA, 0.2% Triton X-100, 1.5 mM sodium fluoride, 1 mM sodium vanadate, 10 μM protease inhibitor mixture) and homogenized by brief sonication. The homogenate was centrifuged for 10 min at 10,000 × g, and the supernatant was taken for protein determination. Equal amounts of cell lysates (50–100 μg of protein) were boiled in Laemmli’s sample buffer, separated on 7.5% SDS-PAGE, and blotted onto polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat dry milk in 0.1% Tween 2000-33P]ATP in 5 mM imidazole) were added to samples, and the mixture was incubated for 1 h at room temperature. Phosphorylated Cer and DAG were extracted with chloroform/methanol. Lipids and DAG and Cer standards (0–600 pmol) were then spotted onto TLC plates and developed in chloroform/acetone/methanol/acetic acid/water (50:20:15:10:5, v/v/v/v/v). Radioactive DAG 1-phosphate and Cer 1-phosphate were visualized and quantified by autoradiography.

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For Sph measurement, standard C17-Sph was added in the residual organic phase after primary S1P extraction. Sph derivatization and analysis was then performed as described for S1P.
20/PBS (PBST) for 1 h, and incubated overnight with an affinity-purified polyclonal rabbit anti-CDase antibody (1:1000). The antibody was raised against the 311–327 peptide KNRGYLPGQGPFVANFA and was a generous gift from Dr. Yusuf A. Hannun (Medical University of South Carolina, Charleston, SC). The next day, membranes were washed for 30 min in 0.1% PBST and incubated in the same buffer containing 5% nonfat dry milk with goat anti-rabbit horseradish peroxidase-conjugated IgG (1:1000) (DAKO, Trappes, France). Immune complexes were detected by enhanced chemiluminescence (ECL™ Western blotting detection reagents kit; Amersham Biosciences). CDase bands were quantified using ImageQuant software, and data are expressed as intensity arbitrary units. Results were corrected for total proteins loaded using /H9251-actin detected separately for each sample lane with a mouse monoclonal antibody (Oncogene Research Products, San Diego, CA) at a dilution of 1:1000.

Western blot was also performed to analyze RAGE expression using a primary goat RAGE polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a dilution of 1:1000.

**Induction of Diabetes in Rats**—Wistar male rats, weighing 176–200 g, were injected intravenously with 65 mg/kg STZ in citrate buffer, pH 4.5. Control rats were injected with citrate buffer alone. Animals were housed in cages with standard chow and water ad libitum. STZ and control rats were sacrificed either 4 days or 4 weeks after injection. Blood glucose levels were measured by a glucose-oxidase colorimetric assay, body weight was checked at this point, and the kidneys were harvested. Glomeruli were isolated by mechanical sieving and frozen at −70 °C until further analysis. Glomeruli were resuspended in lysis buffer (25 mM Hepes, pH 7.5, 5 mM EDTA, 0.2% Triton X-100, 1.5 mM sodium fluoride, 1 mM sodium vanadate, protease inhibitor mixture) and homogenized by serial passes through a 23-gauge needle fitted on a 1-ml syringe. After centrifugation at 10,000 g for 5 min, the supernatant was used for protein determination, CDase assay, and Western blot analysis.

**Statistical Analysis**—Results are expressed as means ± S.D. Comparisons were done using the paired Student’s t test and analysis of variance performed with SPSS for Windows version 11.0 (SPSS, Paris, France) and Statview 4.1 (StatView software, Cary, NC) software, respectively. Statistical significance was accepted at a level of p < 0.05.

**FIG. 3.** Concentration-dependent and time course effects of AGE on neutral ceramidase in mesangial cells. Confluent quiescent MC were treated for the indicated time with the indicated concentrations of AGE or control BSA. In A, C, and D, cells were treated for 72 h, and N-CDase activity and Western blot analysis were performed as described under “Experimental Procedures.” Under control conditions (BSA treatment), N-CDase specific activity was 1.18 ± 0.18 pmol/min/mg. Data in A and B are expressed as a percentage of relative control BSA and represent means ± S.D. of seven or eight experiments (*, p < 0.05 versus BSA control). Data in D represent Western blot quantifications and are expressed as means ± S.D. (normalized to actin) of four or five experiments (*, p < 0.05 versus BSA control).
RESULTS

Effects of AGE on MC Proliferation—In vivo under physiological conditions, MC are quiescent, and only during glomerular disease such as the early phase of diabetic nephropathy do these cells start to proliferate. Therefore, nearly confluent MC were made quiescent by incubation in media containing low serum concentrations (0.5%) for 24 h before treatment. The time of treatment was chosen according to previous results obtained in our laboratory showing that AGE affect growth responses in culture only after several days of treatment (42).

When MC were treated with AGE for 72 h, an unexpected biphasic growth response of these cells was observed (Fig. 1). At low AGE concentrations (0.25–1 Μ), MC proliferation was significantly increased (+26% with 0.75 Μ AGE), whereas higher AGE concentrations (>3 Μ) induced a marked inhibition of proliferation (−23% with 10 Μ AGE). Longer duration (>72 h) of treatment with AGE showed similar results (data not shown). Thymidine incorporation results were confirmed by DNA quantification using the CyQuant assay (results not shown). These results indicate that AGE modulate MC proliferation and suggest that the high and low concentration effects are probably mediated through different mechanisms.

AGE Mediate Their Effects on MC Proliferation through RAGE—It has been shown that AGE mediate their effects through specific receptors such as RAGE. Since MC are known to express RAGE (43), we further explored, using specific siRNA designed against rat RAGE sequence, whether the AGE effects on MC proliferation were mediated through RAGE. In preliminary experiments, cells were transfected with increasing concentrations of siRNA and then collected, and RAGE protein expression was evaluated. The expression of RAGE protein was inhibited by −50% after 24–96 h with 400 nM siRNA (Fig. 2A and data not shown). siRNA concentrations

![Fig. 4. Effects of AGE on ceramide formation in mesangial cells.](image)

Confluent quiescent MC were treated for 72 h with 0.25–10 Μ AGE. Cer levels were measured on total lipid extracts using the DAG kinase assay as described under “Experimental Procedures.” Phosphorylated Cer were separated on TLC, quantified, and expressed as a percentage of relative control BSA. Data represent means ± S.D. of five experiments (*, p < 0.05 versus BSA control). Cer levels in BSA treated control cells were 4.88 ± 0.82 pmol/nmol of phosphate.

![Fig. 5. Sphingosine 1-phosphate levels in AGE-treated mesangial cells.](image)

Confluent quiescent MC were treated for 72 h with 0.25–10 Μ AGE. S1P was measured in cells (A) or culture media (B) by HPLC analysis after extraction and OPA-derivatization as described under “Experimental Procedures.” S1P levels in BSA-treated control cells were 12.76 ± 1.3 pmol/mg of protein. Data are expressed as percentage of relative control BSA and represent means ± S.D. of six experiments (A). In B, data are expressed as concentration in the culture media and represent means ± S.D. of three experiments (*, p < 0.05 versus BSA control).
higher than 400 nM showed modest toxicity; thus, in the following experiments MC were transfected with 400 nM siRNA targeting RAGE expression. Following experiments, MC were transfected with 400 nM siRNA targeting RAGE expression and allowed to recover for 24 h before treatments. When cells were treated with AGE, RAGE expression was increased significantly at high AGE concentrations (Fig. 2B). This increase was no longer observed in siRNA-transfected cells (Fig. 2B). Next, under these conditions, cell proliferation in response to AGE was measured. As shown in Fig. 2C, the effects of AGE (low and high concentrations) on MC proliferation were inhibited (Fig. 2C), suggesting that AGE exert their effects by acting specifically on RAGE. The significant reversal effects might be explained by the fact that siRNA would also inhibit the AGE-induced increase of RAGE expression.

AGE Exert Bimodal Effects on Neutral CDase Activity and Protein Level—Sphingolipids, and in particular Cer and S1P, have been shown to play important roles in the regulation of cell growth and cell death. Further, current knowledge suggests that CDases are key enzymes in the regulation of the net balance between Cer and Sph/S1P levels. Therefore, we investigated the following experiments the involvement of CDases and sphingolipid metabolites (Cer and S1P) in the observed AGE effects.

Results indicated that AGE also exert a biphasic effect on N-CDase activity (Fig. 3A). Low AGE concentrations (<1 μM) increased N-CDase activity with a peak at 0.75 μM, and high AGE concentrations (>3 μM) showed opposite effects. Maximal inhibition was obtained with 10 μM AGE (-23%). Time course studies were also performed at low and high concentrations. Results in Fig. 3B indicated that AGE effects were significant after 72 h of treatment. On the other hand, AGE treatment (high and low) did not affect alkaline (measured at pH 9.5) CDase activity (data not shown), suggesting a specific effect of AGE on the neutral isomerase.

To test whether the effects of AGE on CDase activity were caused by variation in the amount of N-CDase protein, CDase expression was quantified by Western blot analysis using an affinity-purified polyclonal antibody raised against a peptide fragment of the homologous rat brain CDase (36). The antibody revealed a band of 110–120 kDa, in agreement with the recently described size in rat mesangial cells (30) and in rat kidney (44). The upper band of 140 kDa was not identified. As shown in Fig. 3, C and D, N-CDase expression detected in MC homogenates closely followed the modulation of the enzyme activity (+60% with 0.75 μM and -41% with 10 μM AGE).

Ceramide Levels in Response to AGE—Considering the bimodal effects of AGE on CDase activity, the intracellular Cer levels were measured using the diacylglycerol kinase assay.

Figure 6: Effects of AGE on sphingosine kinase activity in mesangial cells. Confluent quiescent MC were stimulated with the indicated AGE concentrations (0.25–10 μM). Therefore, SphK activity was assayed on cell lysates containing 100 μg of protein as described under “Experimental Procedures.” Data are expressed as a percentage of relative control BSA and represent means ± S.D. of six experiments (*, p < 0.05 versus BSA control).

Figure 7A: mitogenic effects of low S1P concentrations. [3H]thymidine incorporation was evaluated in confluent quiescent MC after a 72-h incubation in the presence of the indicated S1P-BSA or control BSA concentrations. [3H]thymidine uptake was performed during the last 18 h of treatment. Results are expressed as a percentage of relative control BSA and represent means ± S.D. of four experiments (*, p < 0.05 versus BSA control). B, prevention of the AGE effects on MC proliferation. Proliferation of MC was evaluated in confluent quiescent MC after a 72-h incubation in the presence of the indicated AGE or control BSA concentrations, in the presence or absence of DMS (1 μM), a SphK inhibitor; PTX (100 ng/ml), an inhibitor of S1P G-protein-coupled receptor; PPMP (1 μM), a glucosylceramide synthase inhibitor. Results are expressed as a percentage of relative control BSA and represent means ± S.D. of four or five experiments, each being performed in duplicate (*, p < 0.05 versus BSA control).

Surprisingly, Cer levels were unchanged after AGE treatment, whatever the AGE concentration (Fig. 4). Similar results were obtained when cells were labeled with [14C]serine, and Cer was quantified after lipid separation by TLC (data not shown). Thus, Cer levels were not affected despite CDase activation or inhibition, suggesting that compensatory pathways regulate Cer content in MC exposed to AGE.

Low AGE Concentrations Induce S1P Production and SphK Activation in MC—Cer hydrolysis by CDase leads to Sph generation. Sph can, in turn, be converted by SphK into S1P, which has been described to directly trigger various cellular proliferative responses. To this end, we examined the hypothesis that CDase activation induced by low AGE concentrations promotes S1P production and increases MC proliferation. As shown in Fig. 5A, MC treated with AGE exhibited higher amounts of intracellular S1P, with a 1.7-fold increase at 0.75 μM AGE. An increase of S1P levels was also observed in the culture media of cells treated with low concentrations of AGE (Fig. 5B). Next, SphK activity was measured, and Fig. 6 shows that MC treated with low AGE concentrations exhibited increased SphK activity, suggesting that the observed increase of S1P levels was
caused by the concomitant activation of CDase and SphK. Interestingly, in cells treated with high AGE concentrations (10 μM), S1P levels were reduced (Fig. 5A), and SphK was inhibited (Fig. 6) as observed for CDase. This inhibition of SphK might suggest an increase of Sph levels, which could mediate the inhibition of MC proliferation (see Fig. 8).

S1P Mediates the Low AGE-induced Proliferation of MC—To confirm whether S1P is responsible for mediating the low AGE-induced MC proliferation, cells were treated for 72 h with exogenous S1P, and proliferation was measured. Maximal [3H]thymidine uptake (2-fold) was observed after incubation of MC with 0.25 μM S1P, and attested for the mitogenic effects of S1P on its own (Fig. 7A). Interestingly, these concentrations are within the range of the S1P levels measured in culture media of MC treated with low AGE concentrations (Fig. 5B).

High S1P concentrations inhibited proliferation, possibly through the conversion of S1P to other sphingolipid metabolites such as Sph. The involvement of S1P was also supported by results using the SphK inhibitor, N,N-dimethylsphingosine. DMS selectively blocked the low AGE concentration-induced MC proliferation (Fig. 7B) and did not affect the high AGE concentration effects.

Because S1P effects have been suggested to be mediated through specific S1P receptors, we checked this hypothesis in MC using PTX, a commonly used inhibitor of S1P receptor-mediated effects. Indeed, as shown in Fig. 7B, PTX inhibited the increase of proliferation induced by low AGE concentrations. However, PTX did not affect high AGE concentration effects, indicating that the effects of PTX are specific for S1P-mediated effects.

High AGE-induced Growth Inhibition Implicates Sph Accumulation and Cer Conversion into Other Bioactive Sphingolipids—Treatment of MC with high AGE concentrations (3–10 μM) resulted in down-regulation of N-CDase expression and activity but did not lead to increased Cer levels. This suggests a compensatory pathway responsible for limiting Cer accumulation under these conditions. Cer can alternatively be used as substrate for sphingomyelin synthase and be turned into sphingomyelin, with a simultaneous generation of diacylglycerol. In [3H]serine labeling experiments, no increase of SM levels was observed in response to high AGE concentrations (data not shown), and also DAG was not augmented when quantified by the DAG kinase assay (data not shown). These results suggest that the SM pathway is not involved in the high AGE response.

Another possibility for Cer metabolism is its conversion by the action of glucosylceramide synthase into glycospingolipids, particularly gangliosides. When MC were treated with high AGE concentrations in the presence of 1 μM PPMP, a glucosylceramide synthase inhibitor, the AGE effect was clearly reversed (Fig. 7B). These results suggest that Cer is most likely converted into other bioactive glycolipids that would mediate at least partially the inhibition of MC proliferation in response to high AGE. This hypothesis is also supported by unpublished data from our laboratory showing an inhibition of MC proliferation in ganglioside-treated cells.2

On the other hand, when Sph levels were measured in AGE-treated MC, a 2.5-fold increase was observed in response to high AGE concentrations (Fig. 8A). This accumulation of Sph could result from the observed SphK inhibition or, alternatively, from other degradative/salvage pathways of glycolipids (45). The latter is supported by the complete reverse inhibition of AGE effects with PPMP. Interestingly, low AGE concentrations did not affect Sph levels (Fig. 8A). Sph has been shown to induce cell death and apoptosis in various cell types; therefore, the effect of exogenous Sph on MC proliferation was tested next. As expected, Sph inhibited MC proliferation (Fig. 8B).

These results suggest that Sph may also be involved in the inhibition of MC proliferation in response to high AGE concentrations.

CDase Activity and Expression Are Modulated in Vivo under Diabetic Conditions—To attest the physiological relevance of our results, CDase activity and expression were monitored ex vivo in the streptozotocin diabetic rat model. In addition, as AGE accumulates progressively during the course of diabetes, experiments were performed very early (4 days) and late (28 days) after the induction of diabetes. Body weight and blood glucose levels were measured in both the 4- and 28-day groups. As expected, the fasting plasma levels of glucose in the STZ-treated groups was higher than that in the control group (5.82 ± 0.73 versus 3.79 ± 0.54 mm after 4 days; 9.35 ± 1.37 versus 4.93 mm after 28 days), and the body weight of the STZ-induced diabetic groups was slightly reduced compared with controls at 4 days (183.25 ± 5.38 versus 193.5 ± 5.21 g) and markedly reduced after 28 days (205 ± 22.54 versus 333 ± 17.3 g).

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2 E. Masson, D. Ruggiero, N. Wiernsperger, M. Lagarde, and S. El Bawab, unpublished data.
Next, glomeruli from control or diabetic animals were isolated by sieving and homogenized, and N-CDase activity and expression were measured on 4- and 28-day treated animals. Results show that 4 days after induction of diabetes, N-CDase activity and expression were increased in the diabetic glomeruli as compared with controls (Fig. 9, A–C). On the contrary, N-CDase activity and expression were inhibited after 28 days of diabetes (Fig. 9, A–C). These data indicate that CDase activity is also regulated ex vivo in glomeruli of STZ rats, as observed in vitro in MC.

**DISCUSSION**

MC proliferation is one of the most common renal histological lesions in various glomerular diseases and is closely related to glomerulosclerosis. In diabetic animal models, an increase of MC proliferation has been shown to occur very early in the kidney (46, 47). This initial and transient proliferation is followed by MC cell cycle arrest, hypertrophy, and overdeposition of extracellular matrix proteins into the glomerular basement membrane. Ultimately, at late stages of DN, MC die by apoptosis and/or necrosis. The present study has established that AGE regulate cultured rat mesangial cell proliferation by modulating sphingolipid metabolism, and it accounts for the first time (as far as we know) for concentration-dependent effects of AGE in vitro. The results showed mitogenic effects of AGE at low concentrations (<1 μM), whereas high AGE concentrations resulted in inhibition of proliferation. These effects were most likely mediated through interaction between AGE and RAGE, since inhibition of RAGE expression resulted in attenuation of the AGE effects.

Over the last decade, cell growth, survival, and death processes have been associated with regulation of sphingolipid metabolism. Among these complex ubiquitous lipids, Cer and two of its main metabolites, Sph and S1P, are now recognized as essential messengers (17, 49). Therefore, the involvement of
sphingolipids in the regulation of MC proliferation in response to AGE was investigated. Results showed that CDase and SphK, key enzymes regulating net levels of cellular Cer, Sph, and S1P, are implicated in the AGE response. Indeed, AGE concomitantly exerted dose-dependent effects on N-CDase and SphK activities.

CDases are known to exist as isoenzymes that differ with respect to their genes, optimum pH, subcellular localization, and biological role. AGE treatment did not affect alkaline CDase activity but increased N-CDase at low concentrations and inhibited it at high concentrations, suggesting a specific effect on the neutral isoform. In addition, AGE modulated N-CDase activity through modulation of its expression. How AGE regulates N-CDase expression is not known; however, recent studies in MC by Franzen and co-workers (50) have suggested the involvement of protein kinase C. Thus, protein kinase C-dependent phosphorylation of N-CDase was associated with increased activity, probably through protection against ubiquitination and subsequent degradation via the ubiquitin–proteasome complex (51).

Experiments using DMS, a SphK inhibitor, strongly suggested the involvement of SphK in MC proliferation in response to low AGE concentrations. Many studies showed that SphK activation is implicated in counteracting apoptosis engagement (52), and it is now well established that the formation of Sph, which is in turn a substrate for SphK to produce SIP, is generally not issued from the de novo synthesis but via the degradation of Cer (27) by CDase. Thus, the increased S1P production observed in response to low AGE concentrations resulted from N-CDase and SphK activation and induced MC proliferation. The increase of S1P levels in response to low AGE concentrations was further observed both intracellularly and extracellularly. Interestingly, the low S1P concentrations (≤250 nM) necessary to induce MC proliferation were within the range of S1P concentrations measured in the media after AGE treatment. This may suggest that MC proliferation can be activated from outside the cells via their proper S1P production, implicating an autocrine/paracrine stimulation. Indeed, MC have been shown to express receptors for S1P, the G-protein-coupled receptor endothelial differentiation gene EDG-1, EDG-2, and EDG-5 (53). In agreement with this hypothesis, low AGE-induced MC proliferation was efficiently reversed by co-treatment with pertussis toxin, a common G-protein-coupled receptor inhibitor. S1P secretion by MC was not observed in response to 10 μM AGE (data not shown), probably as a result of the inhibition of N-CDase and SphK.

Concomitant activation of CDase and SphK has been shown in response to several stimuli (platelet-derived growth factor, interleukin-1β, and oxidized low density lipoproteins) and in various cell types. This raises the intriguing possibility of a concerted regulation of these two enzymes. In support to this hypothesis, protein kinase C-dependent activation of SphK1 has recently been shown (54).

High concentrations of AGE (10 μM) decreased MC proliferation and N-CDase and SphK activities and resulted in Sph accumulation. The inhibition of SphK may explain Sph accumulation. However, the inhibition of N-CDase upstream suggests that the decrease of Sph levels in response to AGE probably proceeds from Cer degradation through other metabolic pathways. Alternatively, CDase inhibition might shift Cer into glycolipid biosynthesis, particularly gangliosides, which have been shown to inhibit cell proliferation. Another possibility explaining Sph accumulation is through the salvage or degradative pathways of glycolipids, as it has been recently reviewed (45). This latter hypothesis is supported by the total reversal of high AGE concentration effect on MC proliferation using PPMP, a glucosylceramide synthase inhibitor. Whatever the pathway of Sph accumulation, as shown in the present study, Sph could inhibit MC proliferation and thereby mediate the effect of high AGE concentrations. Further, preliminary experiments in MC treated with high AGE concentrations did not demonstrate apoptotic cell death as measured by annexin V staining (data not shown), at least in our conditions after 72 h of treatment. This suggests that AGE act rather through cell cycle-mediated events (8). Interestingly, micromolar concentrations of Sph have been shown to induce hypophosphorylation of retinoblastoma protein, which under this form blocks cell cycle progression at the G1/S transition point, thus mediating cell growth inhibition (55). The hypophosphorylated state of retinoblastoma protein has also been implicated in promoting cell cycle-dependent growth arrest and hypertrophy of MC under diabetic conditions (56). Altogether, our results are
in agreement with those from Gnenno et al. (21), documenting a role of S1P and Sph in the regulation of subconfluent MC proliferation.

Experiments performed on STZ-induced diabetic animals have highlighted the potential physiological relevance of these findings. In a previous study, a hyperproliferative state of MC was shown 4–5 days after STZ administration in rats (46), whereas blockade of MC proliferation was observed starting at 2 weeks. Results from the present ex vivo experiments showed that the time course modulation of CDase expression and activity (increase at 4 days and inhibition at 2 weeks) in STZ-gromeleruli parallels MC proliferation in vivo in this model. AGE accumulate slowly in vivo with normal aging and at an accelerated state in diabetes mellitus. In addition, there is good evidence suggesting that AGE through their receptors (RAGE) play an important role in the early events of diabetic nephropathy (48, 57, 58). Unpublished data from our laboratory has revealed an increase of RAGE expression in glomeruli isolated from 4-day STZ animals. These observations raise the hypothesis that AGE accumulation in vivo in the STZ model might be regulated by AGE levels/accumulation and sphingolipids as observed in response to low and high AGE concentrations in vitro (Fig. 10).

In conclusion, the present study suggests that AGE per se are implicated in the regulation of MC proliferation through N-CDase and SphK regulation and the downstream endogenous sphingolipids.

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