AGEs induce oxidative stress and activate protein kinase C-β II in neonatal mesangial cells

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Sciavittaro, Vincenzo, Michael B. Ganz, and Miriam F. Weiss. AGEs induce oxidative stress and activate protein kinase C-β II in neonatal mesangial cells. Am J Physiol Renal Physiol 278: F676–F683, 2000.—Increased activation of specific protein kinase C (PKC) isoforms and increased nonenzymatic glycation of intracellular and extracellular proteins (the accumulation of advanced glycation end products (AGEs)) are major mechanistic pathways implicated in the pathogenesis of diabetic complications. Blocking PKC-β II has been shown to decrease albuminuria in animal models of diabetes. To demonstrate a direct relationship between AGEs and the induction and translocation of PKC-β II, studies were carried out in rat neonatal mesangial cells, known to express PKC-β II, in association with rapid proliferation in post-natal development. Oxidative stress was studied by using the fluorescent probe dichlorfluorescein diacetate. Translocation of PKC-β II was demonstrated by using immunofluorescence and Western blotting of fractionated mesangial cells. Induction of intracellular oxidative stress, increase in intracellular calcium, and cytosol to membrane PKC-β II translocation (with no change in PKC-α) were demonstrated after exposure to AGE-rich proteins. These data support the hypothesis that AGEs cause mesangial oxidative stress and alterations in PKC-β II, changes that may ultimately contribute to phenotypic abnormalities associated with diabetic nephropathy.

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proxyl radicals created during sugar autoxidation degrade proteins by breaking covalent bonds, or lead to cell membrane lipid peroxidation (26). In contrast AGEs are believed to induce cellular oxidative stress through interaction with specific cellular receptors (23, 30).

PKC-βII expression is temporally associated with rapid mesangial cell proliferation during development (1, 33). With further renal maturation this isoform becomes downregulated in mesangial cells (33). Conversely, inhibition of PKC-βII expression in vitro blocks the rapid proliferation of rat glomerular mesangial cells (1). Because neonatal rat mesangial cells consistently express PKC-βII, our experiments demonstrate a direct relationship between AGEs, the induction of oxidative stress, and the translocation of a PKC isoform with demonstrated pathophysiological importance in diabetic nephropathy.

METHODS

Isolation and culture of mesangial cells. Mesangial cells were obtained from kidneys of neonatal Sprague-Dawley rats (days 1–5 after birth), as previously described for adult mesangial cells (33). Pregnant females were purchased from Harlan (Indianapolis, IN). Kidneys were dissected out from anesthetized rats under aseptic conditions. After the capsule was removed, the entire kidney was homogenized in ice-cold Hanks’ balanced salt solution (HBSS; Gibco, Grand Island, NY) and passed through sieves (Tyler, Mentor, OH) to isolate glomeruli. Glomeruli were then washed twice with HBSS and were plated onto 75-cm² tissue culture flasks (Falcon, Lincoln Park, NJ) in 15 ml of DMEM (Gibco) containing 5 µg/ml gentamicin. After 2 days, cells were washed and incubated with a collagen solution (Collaborative Research, Bedford, MA), 25 mM glucose, 400 ng/ml penicillin (Sigma Chemical), and 25 mM NaHCO₃ with 20% FBS (Gibco). For all experiments only primary (unpassaged) neonatal cells were used. The method of obtaining unpassaged neonatal mesangial cells was as follows. Glomerular explants from neonatal rats were continued in flasks (for Western blot experiments) or plated onto 3-well chamber slides (Naperville, IL; for determination of intracellular calcium, or immunofluorescence), or into 96-well plates (for determination of oxidative stress). Once the glomerular explants were attached to the surface (within 24 h), the flasks or wells were washed thoroughly every other day with HBSS, followed by the addition of fresh growth medium containing 20% FBS. Neonatal mesangial cells were ready for immunostaining when they became 60–70% confluent (usually 2–3 wk). As previously described for adult mesangial cells, neonatal rat mesangial cells in culture were also immunostained for the Thy 1.1 epitope, a defining characteristic of rat mesangial cells (14), and vascular smooth muscle myosin antigen (13). Seventy-two hours before experiments, the growth medium was changed from 20% to 0.5% FBS to halt cell growth.

Production and characterization of AGE proteins. AGE proteins were produced using pyrogen-free human serum albumin (HSA) approved for human clinical use (Albumark, Baxter, McGaw Park, IL). Glycated human serum albumin (AGE-HSA) was prepared by incubation of HSA (50 mg/ml) in 0.5M glucose at 37°C for 30 days (25). Control HSA consisted of HSA (50 mg/ml) incubated without additional glucose at 37°C. HSA with a high specific content of Nε-carboxymethyl)lysine (CML) was prepared according to the method of Dunn (CML-HSA) (10). Before incubation, the glucose-HSA solution was filtered sequentially over 0.44- and 0.2-µm Gelman Acrodisc filters. After incubation the solutions were dialyzed against deaerated Chelex-treated phosphate buffered saline, aliquoted and stored at −80°C. At the time of storage, all batches were checked for potential contamination by lipopolysaccharide (LPS), and batches containing >0.25 µU endotoxin/ml in the limulus lysate assay (Sigma Chemical) were discarded. The CML (16) and pentosidine (11) content of the preparations and controls were determined by HPLC as previously described. The CML content of the CML-HSA was 122,450 pmol/mg protein; the pentosidine content, 2.8 pmol/mg protein. The CML content of the AGE-rich HSA was 22,512 pmol/mg; the pentosidine content, 24.9 pmol/mg. The control-HSA contained CML at 495 pmol/mg protein, and pentosidine at 2.9 pmol/mg.

Determination of intracellular calcium concentration ([Ca²⁺]) by using the fura 2 loading procedure. [Ca²⁺]i was ascertained by using the fluorescent calcium indicator fura 2 (Molecular Probes, Eugene, OR) as described by Grynkiewicz et al. (18). Fura 2 was initially dissolved in DMSO at 5 mM and diluted to a final concentration of 5 µM in a saline solution containing 0.1% BSA. Neonatal mesangial cells grown on glass coverslips were loaded with fura 2 for 30 min at 37°C and inserted into a thermostatically controlled cuvette holder in the Perkin-Elmer LS-58 spectrofluorometer (Norwalk, CT). The contribution of dye leakage (extracellular fura 2) to the fluorescence signal was eliminated by continuous superfusion of the coverslips with the bathing solution at a rate of 2.5 ml/min. The dye was then excited at two different excitation wavelengths, 340 and 380 nm, with a constant emission of 510 nm. Once a stable baseline had been established, the AGE protein or control protein was added. The Perkin-Elmer LS-58 is a single-wavelength instrument that necessitates shuttering between the two different excitation wavelengths. However, the interval delay between changing wavelengths is short enough to permit an accurate determination of the time course of change in cytosolic calcium concentration. Using the ratio of fluorescence at 340 and 380 nm, the fractional increase in [Ca²⁺]i can be determined, as previously described (13).

Measurement of oxidative stress in neonatal mesangial cells. AGE-induced cellular oxidative stress was detected in neonatal cells by using the fluorescent probe dichlorofluorescein diacetate (DCFH-DA; Molecular Probes) (17, 20, 21) according to methods previously described for the detection of reactive oxygen metabolites in mesangial cells (6). DCFH-DA is a membrane-permeant diacete derivative of dichlorofluorescein (DCFH). On entering the cells, the diacete group is cleaved off enzymatically and DCFH is trapped inside the cell. Both DCFH-DA and DCFH are nonfluorescent fluorescein analogs that are oxidized to highly fluorescent 2,7’-dichlorofluorescein (DCF) by reactive oxygen species. DCFH oxidation may be caused by several reactive intermediates (H₂O₂, O₂, NO, and so on). Consequently the fluorescent signal produced by DCFH is an index of overall oxidative stress in biological systems.

For each experiment, mesangial cells were washed once with Dulbecco’s phosphate-buffered saline (DPBS), and reloaded with 20 µM DCFH-DA in DPBS. After 15-min incubation at 37°C, cells were washed with DPBS, and HSA-control, AGE-HSA, or CML-HSA was added. The development of fluorescence at different time points was measured in a microplate fluorometer (Cytofluor II, Perspective Biosystems, Framingham, MA) interfaced with a computer for data processing. For each experiment, proteins were also added to empty wells to detect protein autofluorescence. The autofluorescence signal was subtracted from the signal generated by
the mesangial cells in response to the test proteins. Results were expressed as relative fluorescence units (RFU) monitored at 485-nm excitation and at 530-nm emission wavelength. Because the cells in the plates were not disturbed by the measuring procedure, the plates could be incubated for additional time periods and reread. Fluorescence photobleaching under this condition was negligible.

Western blot analysis of PKC isoforms. PKC-α and -βII isoform expression in serum-starved mesangial cells were detected by using polyclonal antibodies against PKC-α and -βII purchased from GIBCO as previously described (15). Antibody specificity was established by competing with excess peptide. Neonatal mesangial cells were lysed, and the cytosolic and membrane fractions were isolated. In brief, mesangial cells were washed twice and incubated with 200 µl of a balanced saline solution containing 0.8 meq/dl Ca2+. The cells were scraped, transferred to prechilled Eppendorf tubes, and centrifuged at 5,000 rpm for 5 min. at 4°C. Cell pellets were resuspended in ice-cold homogenization buffer (60 mM Tris, 0.25 M sucrose, 10 mM EGTA, 2 mM EDTA, 10 mM β-mercaptoethanol) containing protease inhibitors, 200 µM phenylmethylsulfonyl fluoride, and 2 µg/ml aprotinin. The cell suspensions were sonicated, and the degree of cell lysis was assessed by light microscopy. Lysate and membrane fractions were separated by high-speed centrifugation. The supernatants were used as cytosol fractions while the pellets were resuspended in ice-cold homogenization buffer containing 1% Triton X-100 and protease inhibitors and centrifuged at 100,000 g for 1 h at 4°C. The pellets were discarded, and supernatants contained crude (i.e., nuclear, plasma, and mitochondrial membranes) membrane fractions. Rat brain cell PKC or recombinant PKC-α or -βII was used as positive controls in appropriate experiments. Membrane and cytosol fractions from each time period were separated by electrophoresis on 12% SDS-polyacrylamide slab gels and the separated peptides were transferred by electrophoresis to nitrocellulose sheets by using Semi Dry Trans Blot Cell (Bio-Rad, Hercules, CA). Nonspecific binding sites were blocked by incubating nitrocellulose sheets with 5% nonfat dry milk in PBS for 1 h at room temperature. Thereafter, the nitrocellulose strips were incubated with polyclonal isoform-specific antibodies in appropriate dilutions in PBS (1:1,000) overnight at 4°C. The sheets were washed three times for 10 min with PBS and incubated with secondary antibodies (anti-rabbit IgG-horseradish peroxidase; diluted 1:1,000 in PBS) for 1 h at room temperature. The sheets were washed, and the activity of enzyme peroxidase was detected by the chemiluminescent reaction of the substrate luminol by using the enhanced chemiluminesence technique (Amersham, Arlington Heights, IL). Rat brain and C6 glioma cell PKC were used as a positive confirmation of the presence of PKC-α or -βII in the neonatal mesangial cells (12, 15).

Immunofluorescent detection of PKC-βII translocation. Rat neonatal mesangial cells were stimulated for 30–180 min in the presence of two concentrations of control and AGE-proteins at 37°C, washed with PBS, and then fixed with 37% formaldehyde for 30 min. After washing and blocking with PBS-1% BSA, rabbit polyclonal anti-PKC-α or rabbit polyclonal anti-PKC-βII (both from Santa Cruz Biotech, Santa Cruz, CA) were added for 2 h, followed by a specific secondary antibody conjugated to FITC. Cells were observed by using the microscope and the fluorescent images captured with an Optronic Video-digital Image System (Optronics, Goleta, CA) connected to an IBM computer.

RESULTS

Intracellular calcium [Ca2+]i is increased in neonatal mesangial cells exposed to AGEs. Stable baseline calcium concentration was achieved in the neonatal mesangial cells. Then, AGE-HSA, CML-HSA, or control proteins were added to the cells at protein concentrations ranging from 1 nM to 1 µM. A representative experiment demonstrates the effects of adding the AGE-HSA, CML-HSA, and control proteins (arrow) at a concentration of 1 µM (Fig. 1A). A highly significant increase in intracellular calcium content developed over the next 1–2 min in response to the glycated proteins, but not the HSA-control protein. To define whether the increase in intracellular calcium was due to release from intracellular stores or a channel-mediated effect, we preincubated cells with 1.2-bis (2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid (BAPTA; Sigma Chemical). BAPTA acts inside the cell to chelate calcium on release from smooth endoplasmic reticulum. As shown in a representative experiment (Fig. 1B), preincubation with BAPTA blocked the increase in intracellular calcium in response to CML-HSA. The effect of AGEs was not altered by adding...
nifedipine, a calcium channel blocker. The peak calcium response to CML-HSA reached 160 ± 8.3 nM in the presence of 1 mM nifedipine, compared with 163 ± 12.1 nM in its absence.

The dose-response relationship between peak intracellular calcium and the addition of AGE-proteins or control is demonstrated in Table 1. Addition of both CML-HSA and AGE-HSA resulted in significant increases in intracellular calcium content compared with the control HSA. The effect of CML-HSA was slightly, but not significantly, greater than the effect of AGE-HSA, reflecting the greater content of the chemically identified AGE structure, CML. A threshold effect for the increase in intracellular calcium is noted between 1 nM and 1 µM of protein for both AGE-HSA and CML-HSA.

Intracellular oxidative stress is increased in neonatal mesangial cells exposed to AGES. The threshold for the dose of AGE-proteins stimulating increased intracellular oxidative stress was comparable to the dose that increased intracellular calcium (Table 1). The effect reached a plateau at 10 µM and demonstrated a decrease in peak intracellular oxidative stress above 20 µM, reflecting quenching of the fluorescence signal by increasing concentrations of protein. The time course of stimulation of increased intracellular oxidative stress by AGE-proteins was considerably slower than the increase in intracellular calcium. A maximum increase was seen at 60 min (Fig. 2). At 60 min, the fluorescence signal produced by neonatal rat mesangial cells with no added protein was 237 ± 30 RFU. In the presence of 100 µM phorbol myristate acetate (PMA), a maximal signal of 4,032 ± 226 RFU was produced. In contrast to the rapid increase in intracellular calcium, the time course of intracellular oxidative stress was comparable to the translocation response of PKC-βII (see below).

Translocation of PKC-βII in response to AGES. Translocation of PKC-βII was examined across a range of protein concentrations. Each value represents the mean ± SD of 14 experiments (12 control experiments), expressed as densitometric readings (Table 2). Highly significant differences in cytosol to membrane translocation of PKC-βII were found in response to both CML-HSA and AGE-HSA at protein concentrations above 1 µM. Similar to the results for increased intracellular calcium, at least 1 µM of CML-HSA or AGE-HSA was required to stimulate translocation of PKC-βII. Translocation of PKC-βII between cellular and membrane fractions was examined at 0, 10, 30, 60, and 120 min after the serum-starved neonatal mesangial cells were incubated with either 1 µM CML-HSA, AGE-HSA, or control protein. Each value represents the mean ± SD of 12 experiments (4 control experiments), expressed as densitometric readings (Table 3). A greater translocation of PKC-βII was seen in response to CML-HSA than to AGE-HSA. In contrast to PKC-βII, no change in translocation of PKC-α could be detected, at maximal translocation time for any concentration of the CML-HSA (8 experiments). These results did not differ from HSA-Control protein (13 experiments; Table 4).

A representative Western blot of the 80-kDa PKC-βII signal in cytosolic and membrane fractions of neonatal rat mesangial cells is shown in Fig. 3. In this figure, the density of the membrane fraction signal for PKC-βII can be seen to increase at 60 min and remain significantly elevated at 120 min in response to 1 µM CML-HSA.

A representative experiment using immunofluorescence to demonstrate the translocation of the signal for PKC-βII from cytosol to membrane is shown in Fig. 4. These photomicrographs were obtained after 60 min of exposure to AGE-proteins at 10 µM. In four experiments, luminosity measurements comparing the cytosol with membrane fractions were performed. The

![Fig. 2. Time course of intracellular oxidative stress in neonatal mesangial cells exposed to 5 µM AGE-HSA, CML-HSA, or control-HSA. Values are means ± SD of 4 experiments.](https://example.com/fig2.png)

**Table 1. Dose-dependent peak intracellular calcium (nM) or oxidative stress signal (RFU) in neonatal rat mesangial cells**

<table>
<thead>
<tr>
<th>Protein Concentration</th>
<th>CML-HSA</th>
<th></th>
<th></th>
<th>Control-HSA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Calcium, nM</td>
<td>RFU</td>
<td>Calcium, nM</td>
<td>RFU</td>
</tr>
<tr>
<td>1 nM</td>
<td>103 ± 5</td>
<td>92 ± 37</td>
<td>97 ± 7</td>
<td>903 ± 84</td>
</tr>
<tr>
<td>1 µM</td>
<td>143 ± 7†</td>
<td>1,750 ± 132</td>
<td>129 ± 8†</td>
<td>1,572 ± 55</td>
</tr>
<tr>
<td>5 µM</td>
<td>2,173 ± 155*</td>
<td>2,093 ± 91†</td>
<td>1,770 ± 216*</td>
<td>1,700 ± 84*</td>
</tr>
<tr>
<td>10 µM</td>
<td>2,183 ± 81</td>
<td>1,183 ± 81</td>
<td>1,101 ± 161</td>
<td>1,024 ± 32</td>
</tr>
<tr>
<td>20 µM</td>
<td>146 ± 8†</td>
<td>134 ± 7†</td>
<td>86 ± 7</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD of 8 experiments for intracellular calcium at 2 min, and 4 experiments for relative fluorescence units (RFU) at 60 min. CML-HSA, N-carboxymethyl)lysine-rich human serum albumin; AGE-HSA, advanced glycation end product-rich HSA. *P < 0.05, †P < 0.001, compared with control human serum albumin (control-HSA).
Table 2. Dose-dependent translocation of protein kinase C isoform-β1 in neonatal rat mesangial cells

<table>
<thead>
<tr>
<th>Protein Concentration</th>
<th>CML-HSA</th>
<th>AGE-HSA</th>
<th>Control-HSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 nM</td>
<td>Cytosol</td>
<td>Membrane</td>
<td>Cytosol</td>
</tr>
<tr>
<td></td>
<td>11.7 ± 4.2</td>
<td>21.9 ± 4.0</td>
<td>28.1 ± 5.6</td>
</tr>
<tr>
<td>1 µM</td>
<td>17.9 ± 3.1</td>
<td>18.3 ± 2.8</td>
<td>19.9 ± 2.7</td>
</tr>
<tr>
<td>1 mM</td>
<td>23.5 ± 2.1</td>
<td>13.7 ± 2.2</td>
<td>19.9 ± 2.7</td>
</tr>
</tbody>
</table>

Data represent densitometric readings, expressed as means ± SD of 14 experiments for each experimental condition and 12 experiments for control HSA condition at 1 nM, studied at maximal translocation time (30 min for protein kinase C-β1 (PKC-β1)). *P < 0.05, †P < 0.001 compared with control HSA.

DISCUSSION

These data demonstrate a dose-response relationship between proteins with a high content of AGES, the induction of intracellular oxidative stress, and translocation of the specific isoform PKC-β1 in neonatal mesangial cells. AGES also cause an immediate increase in mesangial cell intracellular calcium, independent of induction of a calcium channel, as demonstrated by blockade with BAPTA but not with nifedipine. These changes and intracellular oxidative stress are unrelated to alterations in the glucose concentration of the growth media, which was kept constant in all control and experimental conditions. Paradoxically, long-term exposure to AGES causes a decrease in [Ca²⁺] flux, when flux is stimulated by angiotensin II (27). Further studies are needed to investigate the relationship between initial and late effects on [Ca²⁺], flux induced by AGES. AGEs upregulate an array of genes expressed in diabetic glomerular disease (40). In addition, one of the known receptors for AGE-modified proteins has significant sequence homology with an 87-kDa protein substrate for PKC (24). However, the data presented in this paper are the first to illuminate a direct relationship between AGEs and this important subcellular signaling pathway, without implicating the effect of hyperglycemia on DAG.

Most workers are unable to demonstrate translocation of PKC isoforms in adult rat mesangial cells exposed to AGE-proteins (27). Defining subcellular processes responsible for changes in mesangial cell phenotypic behavior in disease is difficult because mesangial cells from diseased animals are not readily subcultured. As a result a number of laboratories have compared immature cells to diseased cells. Expression of specific phenotypic behavior is suppressed after completion of embryological and early postnatal development, but reappears in disease states. For example an increase in the PKC isoforms β1, βII, and ζ are found in immature hematopoietic cells. Expression of these isotypes may, in part, be responsible for the uncontrolled growth seen in leukemia cells (8). These phenotypes are also responsible for the rapid growth of fetal epithelial cells and have been reported in association with changes in differentiation and cellular growth in other tissues as well (31). Cultured adult rat mesangial cells, whether primary adult mesangial cells or passing cells, can be demonstrated to possess the α-, β1-, and γ-isosofms of PKC, but not PKC-βII. A distinctive temporal pattern of PKC isoform translocation in mesangial cells is suggestive of the idea that each of the PKC isoforms subserves a specific biological role. The α-isofm may be responsible for early membrane events such as Na-H exchange and channel activity (32). The β1-, βI-, and γ-isosofms of PKC are associated with cellular events such as mitogenesis, matrix production and differentiation (15). The data in this report demonstrate that exposure to AGE-HSA or CML-HSA induces translocation in a PKC isoform, which is more likely to effect alterations in cell phenotype than those that occur in response to changes in Na-H exchange or channel activity.

In the pre- and postnatal period of kidney development, proliferation with subsequent functional maturation of intrinsic glomerular mesangial cells continues within the existing framework. The differential expres-

Table 3. Time-dependent translocation of PKC-β1 in neonatal rat mesangial cells

<table>
<thead>
<tr>
<th>Incubation Time, min</th>
<th>CML-HSA, 1 µM</th>
<th>AGE-HSA, 1 µM</th>
<th>Control HSA, 1 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cytosol</td>
<td>Membrane</td>
<td>Cytosol</td>
</tr>
<tr>
<td>0</td>
<td>21.7 ± 3.5</td>
<td>6.9 ± 4.2</td>
<td>24.9 ± 3.9</td>
</tr>
<tr>
<td>10</td>
<td>19.2 ± 2.9</td>
<td>11.2 ± 5.7</td>
<td>22.1 ± 4.2</td>
</tr>
<tr>
<td>30</td>
<td>14.5 ± 4.1</td>
<td>19.1 ± 3.1</td>
<td>18.2 ± 2.1</td>
</tr>
<tr>
<td>60</td>
<td>11.2 ± 5.2</td>
<td>19.0 ± 5.1</td>
<td>15.1 ± 6.1</td>
</tr>
<tr>
<td>120</td>
<td>16.0 ± 2.9</td>
<td>14.3 ± 4.9</td>
<td>19.1 ± 6.0</td>
</tr>
</tbody>
</table>

Data represent densitometric readings, expressed as means ± SD of 12 experiments for each experimental condition and 4 experiments for each control condition. *P < 0.02, †P < 0.05, compared with time 0.
Table 4. Lack of translocation of PKC isoform \( \alpha \) in neonatal rat mesangial cells

<table>
<thead>
<tr>
<th>Protein Concentration</th>
<th>CML-HSA</th>
<th>Control-HSA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cytosol</td>
<td>Membrane</td>
</tr>
<tr>
<td></td>
<td>Cytosol</td>
<td>Membrane</td>
</tr>
<tr>
<td>1 nM</td>
<td>20.3 ± 3.5</td>
<td>5.6 ± 2.5</td>
</tr>
<tr>
<td>1 µM</td>
<td>20.0 ± 4.0</td>
<td>5.2 ± 3.3</td>
</tr>
<tr>
<td>1 mM</td>
<td>21.8 ± 4.0</td>
<td>4.6 ± 2.0</td>
</tr>
</tbody>
</table>

Data represent densitometric readings, expressed as means ± SD of at least 8 experiments for each condition. No significant differences were found.

The mechanism(s) by which AGE-proteins induce abnormal cellular responses remains poorly understood because AGEs are a heterogeneous group of structures, and cells have many AGE "receptors." The group of receptors identified to date is diverse and includes the receptor for AGE (RAGE), lactoferrin, galectin-3/p60/p90 (24), and the macrophage scavenger receptor (2). AGE-induced activation has been demonstrated in a variety of cell types. RAGE expression on vascular endothelium is increased in diabetes, where it leads to increased expression of VCAM-1 (34) and is regarded as a hallmark of atherogenesis. Both antibodies against RAGE and soluble RAGE, a truncated form of the receptor, inhibit AGE-albumin induced VCAM-1 expression. Incubation of mesangial cells with AGE-proteins induces mRNA and protein for transforming growth factor-\( \beta \) (TGF-\( \beta \)), insulin like growth factor-I (IGF-I), and extracellular matrix components (fibronecin, laminin, collagen IV). An antibody against the p60/OST AGE receptor can block these effects (30).

AGEs binding to RAGE induce cellular oxidative stress, as demonstrated in endothelial cells (39) and monocytes (28). Intracellular oxidative stress leads to the activation of the transcription of NF-\( \kappa \)B and upregulation of various NF-\( \kappa \)B-controlled genes, an effect that can be blocked by transfecting the antisense to RAGE (5). In smooth muscle cells from rat pulmonary artery, AGE-albumin activated p21ras and enhanced MAP kinase activity, effects that were enhanced by depleting intracelular glutathione, and blocked by anti-RAGE antibody (23). The specific receptor mediating the translocation of PKC-\( \beta_{II} \) by AGEs remains undefined. The temporal relationship between DCFH fluorescence and PKC-\( \beta_{II} \) translocation is suggestive of a cell-signaling process involving intracellular oxidative stress (23). Independent of AGEs, the link between oxidant stress and activation of PKC can be broken by antioxidants such as \( \alpha \)-tocopherol (22). More research is needed to define the relative importance of hyperglycemia with its activation of DAG-PKC pathways, and AGE-mediated activation of PKC-\( \beta_{II} \) in the development of diabetic nephropathy.

In summary, translocation of PKC-\( \beta_{II} \) can be demonstrated in neonatal rat mesangial cells after exposure to AGE-rich proteins. The time course parallels the increase in intracellular oxidative stress induced by these proteins. These effects are not mediated by hyperglycemia but represent a direct response to AGE proteins. In the present study, the demonstration of a specific AGE effect may not have been possible without the use of actively proliferating cells that express PKC-\( \beta_{II} \). These data lead to the hypothesis that the dedifferentiation of mature mesangial cells may be necessary before AGEs can trigger intracellular calcium flux, intracellular oxidative stress, and the translocation of PKC-\( \beta_{II} \) in diabetic nephropathy.

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