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Regulation of angiotensin II receptors and PKC isoforms by glucose in rat mesangial cells

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Amiri, Farhad, and Raul Garcia. Regulation of angiotensin II receptors and PKC isoforms by glucose in rat mesangial cells. *Am. J. Physiol.* 276 (*Renal Physiol.* 45): F691–F699, 1999.—It has been shown that glomerular angiotensin II (ANG II) receptors are downregulated and protein kinase C (PKC) is activated under diabetic conditions. We, therefore, investigated ANG II receptor and PKC isoform regulation in glomerular mesangial cells (MCs) under normal and elevated glucose concentrations. MCs were isolated from collagenase-treated rat glomeruli and cultured in medium containing normal or high glucose concentrations (5.5 and 25.0 mM, respectively). Competitive binding experiments were performed using the ANG II antagonists losartan and PD-123319, and PKC analysis was conducted by Western blotting. Competitive binding studies showed that the AT₁ receptor was the only ANG II receptor detected on MCs grown to either subconfluence or confluence under either glucose concentration. AT₁ receptor density was significantly downregulated in cells grown to confluence in high-glucose medium. Furthermore, elevated glucose concentration enhanced the presence of all MC PKC isoforms. In addition, PKC β , PKC γ and PKC ϵ were translocated only in cells cultured in elevated glucose concentrations following 1-min stimulation by ANG II, whereas PKC α , PKC θ , and PKC λ were translocated by ANG II only in cells grown in normal glucose. Moreover, no changes in the translocation of PKC δ , PKC ι , PKC ζ , and PKC μ were detected in response to ANG II stimulation under euglycemic conditions. We conclude that MCs grown in high glucose concentration show altered ANG II receptor regulation as well as PKC isoform translocation compared with cells grown in normal glucose concentration.

angiotensin II receptor; protein kinase C; mesangial cell; glucose

SEVERAL LINES OF EVIDENCE indicate that the renin-angiotensin system (RAS) plays an important role in the renal complications seen in patients with insulin-dependent (type I) diabetes mellitus (DM) as well as animals in the early stages of experimental DM, such as with above-normal increases in the glomerular filtration rate (GFR) (6, 40). The RAS mediates these functions through the production of angiotensin II (ANG II) (22), the active element of both the systemic and renal RAS for which all components, namely angiotensinogen, angiotensin I, angiotensin-converting enzyme (ACE), and ANG II, have been localized in the kidney (40). Since ANG II is an important modulator of glomerular filtration and function, it is believed that

glomerular structural injury can be effectively prevented, despite pronounced hyperglycemia, as long as glomerular pressure and flow are maintained within normal limits by the administration of ACE inhibitors (1), suggesting a possible role of ANG II in the development of glomerular injury, a hallmark of DM.

It has long been established that glomerular mesangial cells (MCs) have microfilaments that contract in response to ANG II, mediated by specific ANG II receptor subtypes (49), indicating a plausible role of MCs is the regulation of glomerular size and blood flow via contraction (2). The effects of ANG II are exerted through high-affinity membrane-bound receptors, namely, ANG II type 1 receptor (AT₁) and ANG II type 2 receptor (AT₂), which have been classified recently with the aid of specific nonpeptide antagonists (9, 52). All the known effects of ANG II have been attributed to AT₁, which has a high affinity for the selective nonpeptide antagonist losartan. On the other hand, no functional correlate has been found for AT₂, which has a high affinity for the selective nonpeptide antagonist PD-123319. In addition to these two receptor types, it has been reported that in rodents, AT₁ has two subtypes, namely AT_{1a} and AT_{1b} (9), both of which are present in MCs (7). However, these isoforms cannot be distinguished pharmacologically (8). Both ANG II receptor types have been localized in humans and rats, but their distribution is not uniform in all somatic tissues. Some organs, such as the liver, lung, and kidneys, have a nearly homogenous population of AT₁ receptors, whereas others, such as the pancreas and human uterus, contain almost uniquely the AT₂ subtype (5, 14, 17). A mixture of both receptor subtypes characterizes certain tissues, such as the adrenals and heart (5). The signaling pathways coupled to the AT₁, a G protein-coupled receptor, are diverse. Among these is the phosphoinositide hydrolysis (20) whose products are inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG), which increase intracellular calcium and activate protein kinase C (PKC), respectively. It has been shown that both DAG and PKC are activated in a variety of tissues in experimental diabetes (12, 32) and that hyperglycemia stimulates PKC through de novo synthesis of DAG (11, 40).

Molecular cloning analysis has revealed that the PKC family is comprised of at least 12 isozymes, all having closely related structures but differing in their individual properties. They have been categorized into four classes: conventional or classic PKC isoforms (α , β I, β II, and γ) are Ca²⁺ dependent and phospholipid dependent through their C2 domain; the novel PKC isoforms (δ , ϵ , η , and θ) lack this region and are accordingly Ca²⁺ independent (35). The third class

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consisting of atypical PKC isoforms (ζ , ι , and λ) and the fourth group embodying PKC μ are both Ca²⁺ and DAG independent (35). Different tissue and cellular distributions have been noted for the PKC isoforms, suggesting specific roles for each of them in cell regulation (42). Furthermore, PKC activity in intact cells can be stimulated by tumor-promoting phorbol esters, such as phorbol 12-myristate 13-acetate (PMA), which presumably bind to the same site in the regulatory domain as DAG (33, 42). It is noteworthy that phorbol esters are not metabolized by cells and can therefore produce prolonged PKC stimulation that can be considered non-physiological (33).

ANG II, like many other peptides, has the ability to modulate the density of its receptors in several organs, including those involved in cardiovascular regulation, such as the vascular wall, heart, adrenals, and kidneys (29). In addition, both renal plasma flow and the GFR are blunted in diabetic rats receiving ANG II infusions (46), a finding that can result from either changes in ANG II receptors or alterations in postreceptor actions of the hormone or intracellular signaling pathways. Furthermore, glomerular ANG II receptor density is reduced in diabetic rats (53). These findings could account for the decreased ANG II actions and hyperfiltration observed in DM. Moreover, the impaired contractile responsiveness of diabetic glomeruli to ANG II may be due to MC dysfunction as MCs are responsible for glomerular contraction (30). Likewise, recent advances in renal pathophysiology suggest that MC expansion may play an important role in destruction of the glomerular capillary lumen, resulting in the ultimate cessation of glomerular function, such as that seen in diabetic glomerulosclerosis (26).

The link between the diabetic condition and MCs grown in elevated glucose concentration has been strengthened by several investigations which have demonstrated clearly that MC production of collagen type IV is enhanced under high glucose concentrations (23), a phenomenon possibly leading to mesangium expansion observed in DM (37). In addition, insulin may be required for the contractile response of cultured MCs to ANG II, suggesting that a loss of mesangial contractile activity in a low- or no-insulin environment such as DM could cause a marked increase in glomerular blood flow, ultimately eliciting to glomerulosclerosis (32).

Similar to the diabetic condition, elevated glucose concentration can decrease both MC phosphoinositide metabolism and intracellular calcium signaling (21, 24). This reduction in ANG II-mediated signaling could be due to the negative feedback effect of PKC, which, as aforementioned, becomes activated through de novo DAG formation from glucose (21). However, to date, no unequivocal link has been established between a specific PKC and cellular function, because where isozymes are expressed depends on cell type, the subcellular compartment where it is located, and the ligand used to stimulate it. The purpose of this study was to characterize the regulation of MC ANG II receptors and

PKC isoforms in response to ANG II stimulation under different glucose concentrations.

MATERIALS AND METHODS

Isolation and culture of MCs. MCs were obtained from isolated, collagenase-treated rat glomeruli. In brief, glomeruli were harvested from male Sprague-Dawley rats (225–250 g; Charles River Laboratories, St.-Constant, Quebec, Canada) ($n = 15$ rats per experiment, 3 experiments in total), as described previously (17), by filtration with ice-cold 0.9% NaCl solution through 200-, 150-, 120-, and 50- μ m nylon mesh. Those retained on the sieve were collected, washed by centrifugation (4°C, 2,000 g), and incubated with 250 U/ml collagenase (type I) for 30 min at 37°C under constant, gentle shaking. MCs were plated on plastic tissue culture flasks in DMEM (pH 7.4) with either normal glucose or mannitol (5.5 mM) or elevated glucose or mannitol (25.0 mM) concentrations. The culture medium was supplemented with 20% FBS, 10,000 U/ml penicillin, 10,000 μ g/ml streptomycin, 5 μ g/ml insulin, and 10 μ l/ml of Fungizone, an antimycotic agent. The cells were incubated at 37°C in humidified 5% CO₂-95% air. The cell medium was left untouched for 4 days and then changed every other day until confluence. Only primary cultured cells were used and placed in serum-free medium 24 h prior to the experiments.

Immunohistochemistry. Immunohistochemical analysis was conducted with using specific monoclonal antibodies against cellular markers such as α -actin and vimentin (5 μ g/ml) for smooth muscle cells, von Willebrand factor (10 μ g/ml) for endothelial cells, and cytokeratin (40 μ g/ml) for epithelial cells. Cells were grown on 25-mm round coverslips coated with poly-L-lysine until subconfluence and then fixed in acetone (–20°C). Anti-mouse Ig-fluorescein F(ab')₂ fragment (20 μ g/ml) was used for detection.

Binding experiments. All binding studies were performed out in duplicate, and at least four separate binding experiments were undertaken for each group, in respective serum-free culture medium at 37°C for 90 min. In competition experiments, 25–30 pM ¹²⁵I-labeled [Sar¹,Ile⁸]ANG II was incubated with increasing concentrations of unlabeled displacing compounds, from 10^{–12} to 10^{–6} M for both [Sar¹,Ile⁸]ANG II and PD-123319, and from 10^{–11} to 10^{–5} M for losartan. The volume reaction was 0.5 ml ($\approx 2 \times 10^4$ cells/well). The binding reaction was stopped by washing twice with 0.5 ml of serum-free culture medium. The cells were digested with 0.5 ml of 1 M NaOH, and radioactivity was counted in a gamma counter (LKB, Turku, Finland) with 65% efficiency. Nonspecific binding was determined by the amount of tracer bound in the presence of 1 μ M of unlabeled [Sar¹,Ile⁸]ANG II, and specific binding was defined as total less nonspecific binding.

Cell fraction separation. Confluent MCs were washed with ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.7 mM KH₂PO₄, with a final pH of 7.4) and stimulated with either ANG II (10^{–7} M, for 1 and 3 min) or PMA (10^{–7} M, for 1 min). Unstimulated cells served as controls. After stimulation, the cells were washed and scraped with 50 mM Tris·HCl lysis buffer containing 1 μ M pepstatin A, 1 μ M leupeptin, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM EGTA (pH 8.6), and 2 mM EDTA (pH 6.5) with a final pH of 7.4. Half of the cells were used for the total fraction and thus were incubated under constant shaking for 30 min at 4°C in lysis buffer containing 1% Triton X-100. Subsequently, the total fraction was centrifuged at 145,000 g for 30 min at 4°C, and the supernatant was retained. The remaining portion of the cells was used for cytosolic and particulate fractions, and was thus initially centrifuged at 145,000 g for 30 min at 4°C, thereby isolating the cytosolic fraction in the

supernatant. Subsequently, the particulate fraction was incubated under constant shaking for 30 min at 4°C in lysis buffer containing 1% Triton X-100, and recentrifuged at 145,000 *g* for 30 min at 4°C. Protein concentration for each of the fractions was assessed by a modification of the Bradford method (48), and each fraction was aliquoted and stored at -40°C until Western blot analysis was performed.

PKC isoform analysis. PKC isoforms were detected using PKC isoform-specific monoclonal antibodies. Solubilized proteins, mixed with Laemmli sample buffer, were resolved by 10% SDS-PAGE, transferred to a nitrocellulose membrane and blocked by 90-min incubation at room temperature (22°C) in PBS-T (PBS with 0.1% Tween-20, pH 7.4) plus 2.5% skimmed milk powder and 1.0% polyvinylpyrrolidone. Affinity-purified anti-PKC isoform antibodies were diluted (1:5,000 for α ; 1:2,000 for γ ; 1:1,000 for δ , ι , and μ ; and 1:500 for β , ϵ , θ , λ , and ζ) in PBS-T containing 0.3% BSA. After 90 min of incubation at room temperature, the nitrocellulose membranes were washed five times for 10 min each with PBS-T and incubated with goat anti-mouse IgG horseradish peroxidase conjugate (1:10,000). After extensive washing, bound antibody was visualized on Kodak XRP-1 film, using the Pierce Supersignal substrate chemiluminescence detection kit. Specificity of the bands was assessed by molecular weight markers. The intensity of the bands was quantified by Alpha Ease (Alpha Innotech, San Leandro, CA). The area under the peak of the PKC isoform scanned (both cytosolic and membrane fractions) was determined, and the membrane-to-cytosol ratio was used to calculate fold translocation (or activation).

Chemicals. All materials were of the highest reagent grade available. PMSF, *N*-(α -rhamnopyranosyloxyhydroxyphosphinyl)-Leu-Trp (phosphoramidon), Tween-20, Triton X-100, pepstatin A, EGTA, insulin, BSA, and anti-vimentin antibody were all purchased from the Sigma Chemical (St. Louis, MO). FBS was obtained from Wisent (St. Bruno, Quebec, Canada), and EDTA was from J. T. Baker (Toronto, Ontario, Canada). Penicillin, streptomycin, and Fungizone were from Life Technologies (Rockville, MD) and collagenase type I was from Worthington Biochemical (Freehold, NJ). Anti- α -actin, anti-von Willebrand factor, and anti-mouse Ig-fluorescein F(ab')₂ fragment were procured from Boehringer Mannheim (Laval, Quebec, Canada). Goat anti-mouse IgG horseradish peroxidase conjugate and molecular weight markers were purchased from Bio-Rad (Hercules, CA). [Sar¹,Ile⁸]ANG II was obtained from Bachem California (Torrance, CA), and leupeptin was acquired from Bachem Bioscience (King of Prussia, PA). The potassium salt of 2-*n*-butyl-4-chloro-5-hydroxy-methyl-1-[2-(H-tetrazol-5-yl)biphenyl-4-yl-methyl]imidazole potassium salt (losartan potassium) and 1-(4-amino-3-methylphenyl)methyl-5-diphenylacetyl-4,5,6,7-tetrahydro-1H-imidazole(4,5-c)pyridine-6-carboxylic acid (PD-123319) were synthesized at E. I. Du Pont Nemours (Wilmington, DE). Losartan potassium and PD-123319 were generous gifts from Du Pont Merck Pharmaceutical (Wilmington, DE) and Parke-Davis (Ann Arbor, MI), respectively. PKC isoform-specific antibodies were acquired from Transduction Laboratories (Lexington, KY). The Pierce Supersignal substrate chemiluminescence detection kit was purchased from Pierce (Rockford, IL).

Statistical analysis. Binding data were analyzed by processing the raw data with the EBDA program. The density (B_{max}) and affinity (K_d) of binding sites were then determined with the LIGAND program (41). Statistical analysis was performed with the SigmaStat program (Jandel Scientific, San Rafael, CA), using one-way ANOVA followed by the Student-Newman-Keuls *t*-test to determine significance. The values

presented are means \pm SE. $P < 0.05$ was considered to be significant.

RESULTS

MC characterization studies. Immunohistochemical analysis using specific cellular markers confirmed that MCs were isolated since they stained positive for both vimentin and α -actin and negative for both von Willebrand factor and cytokeratin (Table 1). In addition to immunohistochemical analysis, morphological evaluation of MCs revealed a homogenous population of fusiform cells with prominent fibrillar structures.

ANG II receptor characterization studies. Figure 1 depicts representative competition binding curves of MCs in high-glucose culture medium using the nonspecific ANG II antagonist [Sar¹,Ile⁸]ANG II and the specific ANG II receptor antagonists losartan and PD-123319. On MCs grown in either normal or elevated glucose concentration, only the AT₁ receptor was present, since no displacement was observed with PD-123319.

Figure 2 demonstrates the B_{max} values of AT₁ receptors on MCs grown to subconfluence (14 days) and to confluence (22 days) in normal and high glucose. The density of MC AT₁ receptors in elevated glucose medium was significantly reduced ($P < 0.05$) only after reaching confluence (22 days) compared with MCs in normal glucose medium. Furthermore, to ensure that the observed decrease in AT₁ B_{max} was due to elevated glucose concentration and not to an osmolarity effect, MCs were also grown in normal (5.5 mM) and high (25.0 mM) mannitol concentrations. As expected, no differences in AT₁ B_{max} values were found between normal and elevated mannitol concentrations, even when the cells were grown to confluence (data not shown). Furthermore, no significant difference in K_d was observed in any of the groups, with values ranging from 1.6 ± 0.9 to 2.3 ± 1.1 nM.

PKC isoform analysis. All PKC isoforms were present in MCs grown in normal and high glucose, with the exception of PKC β , which was found only in MCs grown in high-glucose medium (Fig. 3). PKC α was the only classic PKC isoform that was significantly translocated by a 1-min ANG II stimulation under normal glucose condition ($P < 0.05$). However, when under elevated glucose concentration, both PKC α and PKC β quantities were increased two- to fourfold in high-glucose medium, whereas that quantity of PKC γ remained unchanged (Fig. 4). Moreover, after 1 min of ANG II stimulation (10^{-7} M), both PKC β and PKC γ were translocated but returned to basal levels with longer exposure to ANG II (3 min) (Fig. 4). In addition, 1-min

Table 1. Immunohistochemical analysis of mesangial cells grown in normal glucose

Cellular Markers	Staining
α -Actin	+
Vimentin	+
Cytokeratin	-
von Willebrand factor	-

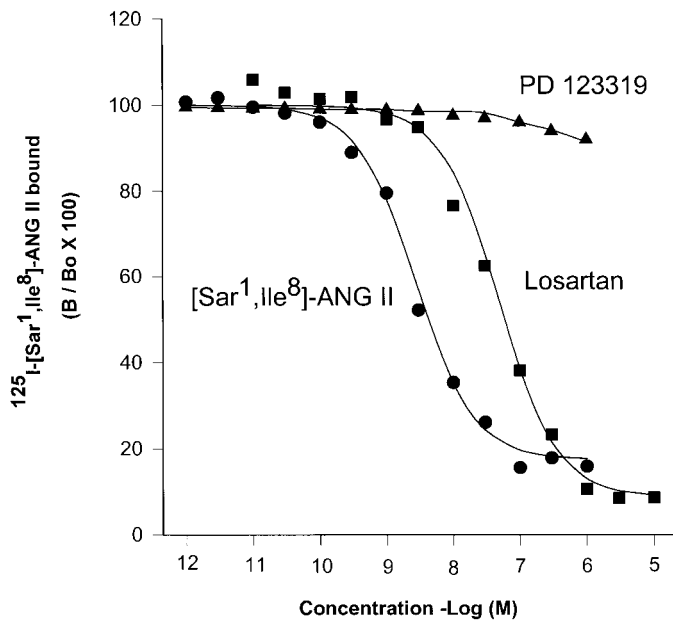


Fig. 1. Representative competition binding curves of mesangial cells (MCs) cultured in high-glucose medium using the nonspecific ANG II receptor antagonist $[Sar^1,Ile^8]ANG II$, the AT_1 receptor antagonist losartan, and the AT_2 receptor antagonist PD-123319. B and B_o represent binding in the respective presence and absence of the competitor.

PMA stimulation translocated $PKC\alpha$ and $PKC\beta$, but not $PKC\gamma$. With respect to novel PKC isoforms in MCs grown in normal glucose media, 1-min ANG II stimulation translocated only $PKC\theta$, whereas PMA stimulation

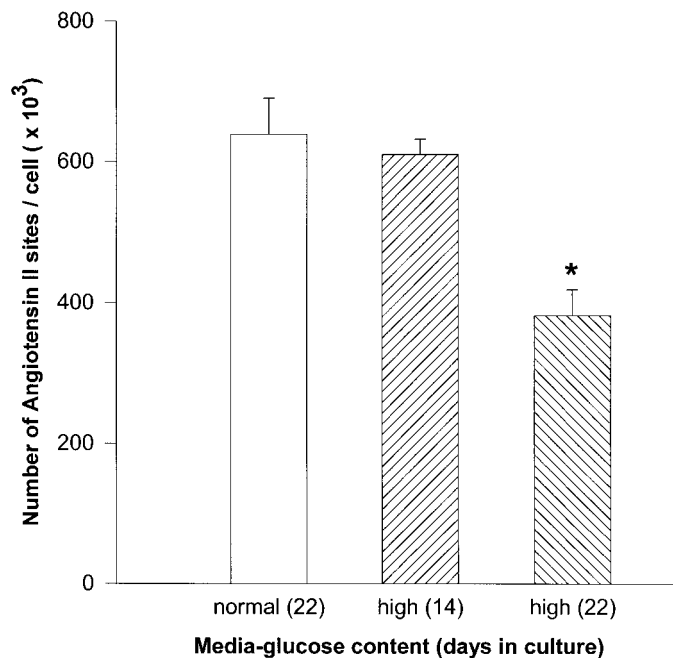


Fig. 2. ANG II receptor density (B_{max}) of MCs cultured for 22 days in normal glucose (5.5 mM) medium or for 14 and 22 days in high-glucose (25.0 mM) medium using the nonspecific ANG II receptor antagonist $[Sar^1,Ile^8]ANG II$. Similar results were obtained with losartan. Values are means \pm SE; $n = 4$ binding experiments performed in duplicate. * $P < 0.001$ vs. normal glucose and high glucose (14 days).

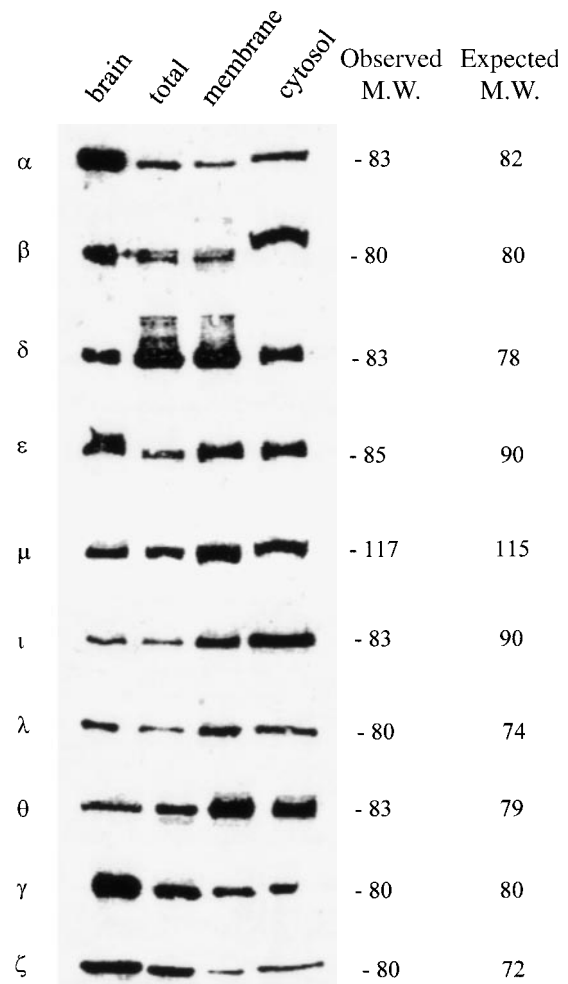


Fig. 3. Representative immunoblots of protein kinase C (PKC) isoforms in MCs cultured under high glucose concentration and in the brain of normal Sprague-Dawley rats. Observed and expected molecular weights are shown on the right.

translocated only $PKC\delta$. Moreover, $PKC\delta$ quantity was the only novel PKC isoform that was increased by an elevation in extracellular glucose concentration (Fig. 5). Similarly to the classic PKC isoforms, ANG II and PMA stimulation caused differential translocation of novel PKC isoforms under hyperglycemic conditions. For instance, $PKC\epsilon$ was translocated by a 1-min ANG II stimulation, whereas $PKC\theta$ was activated only by PMA. On the other hand, $PKC\delta$ translocation was significantly suppressed by either ANG II or PMA stimulation compared with unstimulated cells (Fig. 5).

In a like manner to classic and novel PKC isoforms, atypical PKC isoform quantities were increased in unstimulated MCs grown under hyperglycemic conditions compared with their counterparts cultured under euglycemic conditions. With respect to atypical PKC isoforms, only $PKC\lambda$ was translocated by 1-min ANG II stimulation in MCs grown in normal glucose concentration (Fig. 6). Moreover, all atypical PKC isoforms in MCs cultured in elevated glucose were significantly translocated by 1-min exposure to PMA. As for $PKC\mu$, it was unresponsive to either ANG II or PMA stimulation under normal glucose concentrations, whereas its trans-

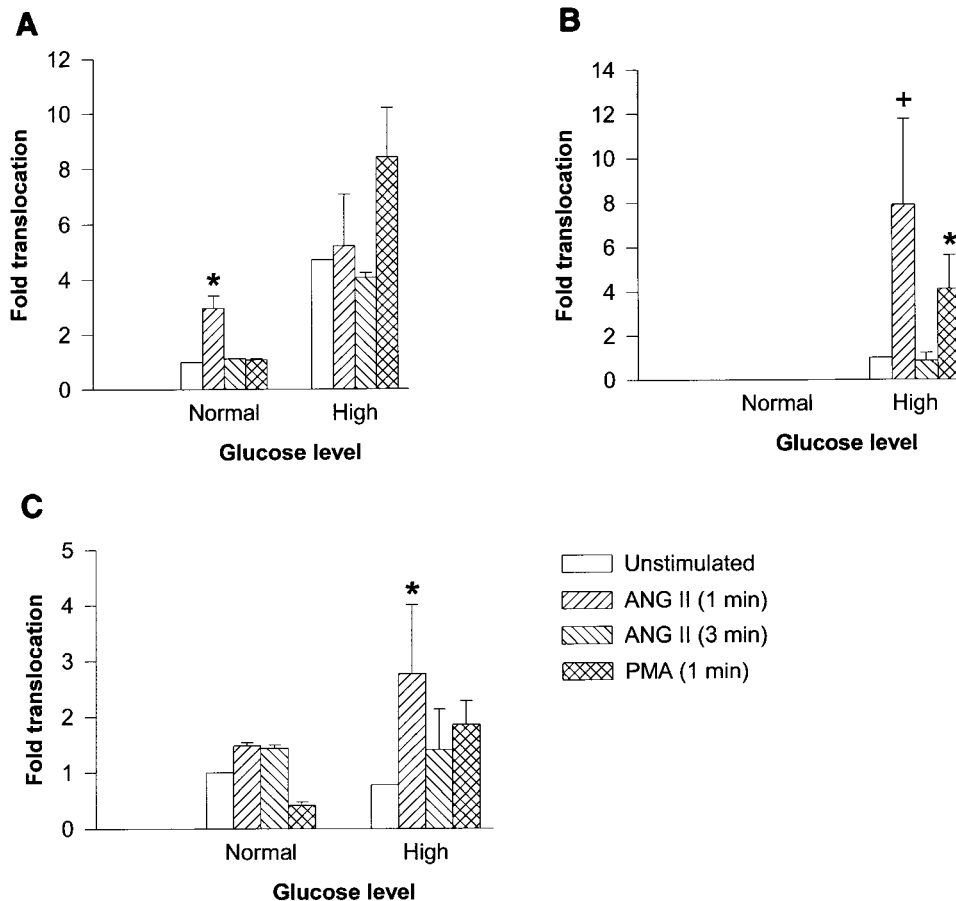


Fig. 4. Fold translocation of classic protein kinase C (PKC) isoforms (α , β , and γ). PKC isoforms from control animals were considered to be 1.0-fold activated. Each graph shows normal and elevated glucose concentration. Values are means \pm SE; $n = 4$ Western blot analyses, each from different cell culture experiment. Area under the peak of the PKC isoform scanned (both cytosolic and membrane fractions) was determined, and the membrane-to-cytosol ratio was used to calculate fold translocation (or activation). PMA, phorbol 12-myristate 13-acetate. * $P < 0.05$ vs. unstimulated cells. + $P < 0.001$ vs. unstimulated cells.

location was significantly suppressed by both agents under hyperglycemic conditions (Fig. 6).

DISCUSSION

In the present study, we have shown that MC ANG II receptors are downregulated in a high-glucose environment, whereas most PKC isoforms in these cells are upregulated. In addition, PKC isoforms are differentially translocated after ANG II stimulation.

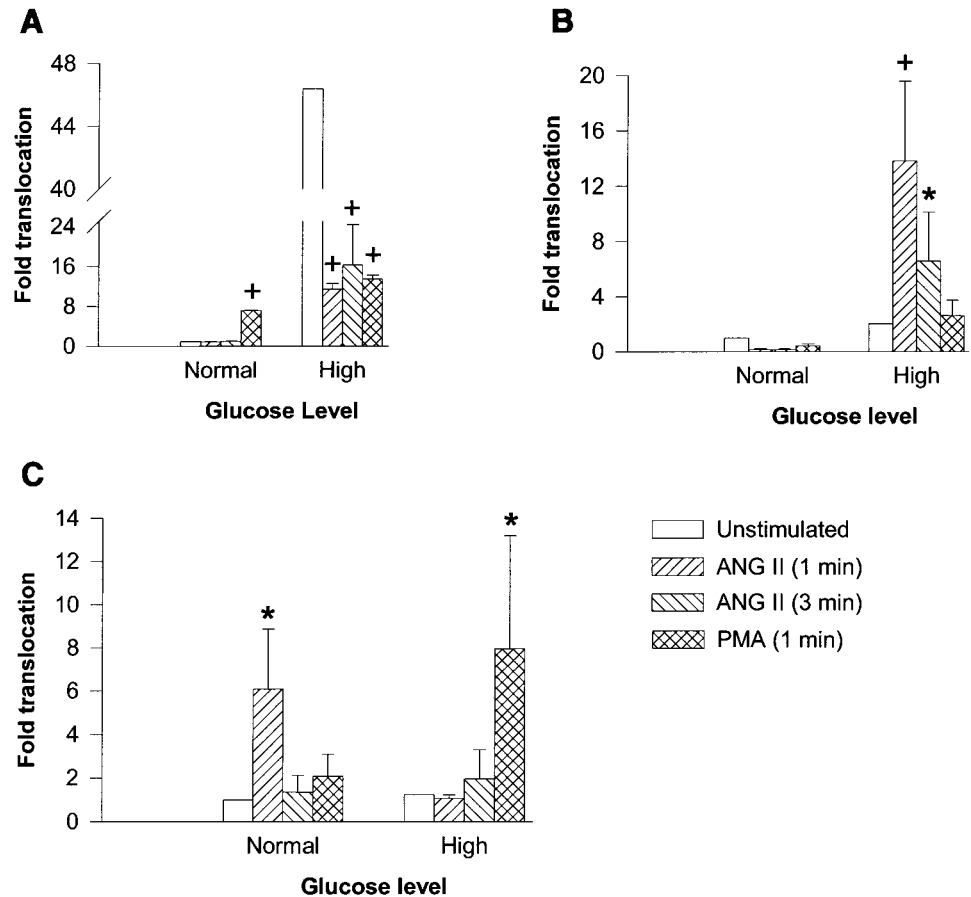
As reported previously (38), MCs stained positively for both α -actin and vimentin, but negatively for cytokeratin and von Willebrand factor, cellular markers for epithelial and endothelial cells, respectively. In addition to immunohistochemical analysis, morphological evaluation of MCs revealed a homogenous population of fusiform cells with prominent fibrillar structures of typical stellate and spindle shape similar to those found by others (36, 38).

Competitive binding studies with the specific AT_1 antagonist losartan and the specific AT_2 antagonist PD-123319 revealed that AT_1 was the only ANG II receptor detected on MCs grown in either normal or elevated glucose concentrations, whereas the AT_2 receptor was not discerned. These results are in total agreement with previous investigations (2, 7), which have clearly demonstrated the absence of AT_2 receptors; but are in contradiction with the findings of Goto et al. (18), who have reported the presence of AT_2 receptors in passaged MCs. The discrepancy between our results

could be due to the fact that Goto et al. (18) studied passaged MCs, whereas we used primary cultured MCs, since it has been shown that passaged MCs undergo dedifferentiation (43). Furthermore, these binding experiments also revealed no significant differences in K_d values among the various experimental conditions.

When AT_1 receptor B_{max} values from MCs grown in normal and elevated glucose concentration (5.5 and 25.0 mM, respectively) were considered, significant downregulation was observed in MCs grown to confluence (22 days) in high-glucose medium when compared either with cells grown to subconfluence in the same medium or cells grown to confluence in normal glucose medium. Since it has been established by several investigators that glomerular (4, 53) and aortic vascular smooth muscle (55) ANG II receptor densities are significantly reduced in diabetic rats, the results that we have obtained clearly demonstrate that MCs grown to confluence in high-glucose medium mimic the in vivo situation of glomerular ANG II receptors in diabetic animals. In support of this, it has been proposed by Sterzel et al. (50) that MCs grown to confluence in a high-glucose medium can serve as an in vitro model of glomerulosclerosis. Moreover, we have also observed reduced glomerular ANG II receptor density in streptozotocin-diabetic rats that were normalized by preventing hyperglycemia with high-dose insulin treatment (Amiri and Garcia, unpublished observations).

Fig. 5. Fold translocation of novel PKC isoforms (δ , ϵ , and θ). PKC isoforms from control animals were considered to be 1.0-fold activated. Each graph shows normal and elevated glucose concentration. Values are means \pm SE; $n = 4$ Western blot analyses, each from different cell culture experiment. Area under the peak of the PKC isoform scanned (both cytosolic and membrane fractions) was determined, and the membrane-to-cytosol ratio was used to calculate fold translocation (or activation). * $P < 0.05$ vs. unstimulated cells. + $P < 0.001$ vs. unstimulated cells.



Furthermore, this downregulation of MC ANG II receptors could explain the decrease in both phosphoinositide generation and intracellular calcium signaling in response to ANG II stimulation (21), suggesting that our experimental conditions sufficiently mimic the in vivo conditions seen in diabetic animals (MCs grown to confluence in high-glucose medium) and nondiabetic animals (MCs grown to confluence in normal glucose medium).

To ascertain whether PKC isoforms were modified by elevated extracellular glucose concentration, we determined the different PKC isoforms present and their respective translocation in unstimulated as well as ANG II- and PMA-stimulated MCs cultured in normal and high-glucose media. We observed a glucose-induced increase of PKC isoforms in addition to differential translocation in response to ANG II stimulation. Since it has been established that in diabetic animals hyperglycemia has the ability to activate/translocate glomerular PKC isoforms through de novo synthesis of DAG (11, 12), our results are in total agreement with previously published data which have clearly shown that PKC isoforms found in MCs are increased under high-glucose conditions (25, 54). This glucose-induced increment in the PKC isoform quantity was most evident with PKC β (Fig. 4), which was not detected in MCs grown in normal glucose medium but was found in unstimulated cells grown in high-glucose medium. These data fully concur with the work of Huwiler et al.

(27), who have also reported the absence of PKC β in MCs grown in normal glucose, but are in disagreement with the results of Saxena et al. (47). This discrepancy could be due to MC confluence at the time of PKC determination. Another plausible explanation for our findings could be the increase in DAG mass caused by de novo synthesis by glucose, as demonstrated by several other investigators (11, 12, 28).

In addition to the PKC-activating effects of hyperglycemia, we have also observed that ANG II through the AT $_1$ receptor has the ability to differentially translocate several PKC isoforms. For instance, we have observed that under elevated glucose concentration, short-duration ANG II stimulation translocates PKC β and PKC γ but not PKC δ and PKC θ . This differential translocation could be explained by the accumulation of intracellular calcium following AT $_1$ stimulation by ANG II. This explanation is supported by Crabos et al. (10), who have demonstrated that calcium is an important requirement for the translocation of calcium-dependent PKC isoforms, since chelation of either intra- or extracellular calcium inhibited agonist-induced PKC activation. In addition, it has been shown that ANG II causes differential PKC activation in smooth muscle cells (13) and that PKC activation is not only tissue dependent but is also affected by hyperglycemia (16). A possible physiological effect of PKC translocation by hyperglycemia and/or ANG II could be AT $_1$ receptor downregulation, as suggested by several other investigators (3, 55).

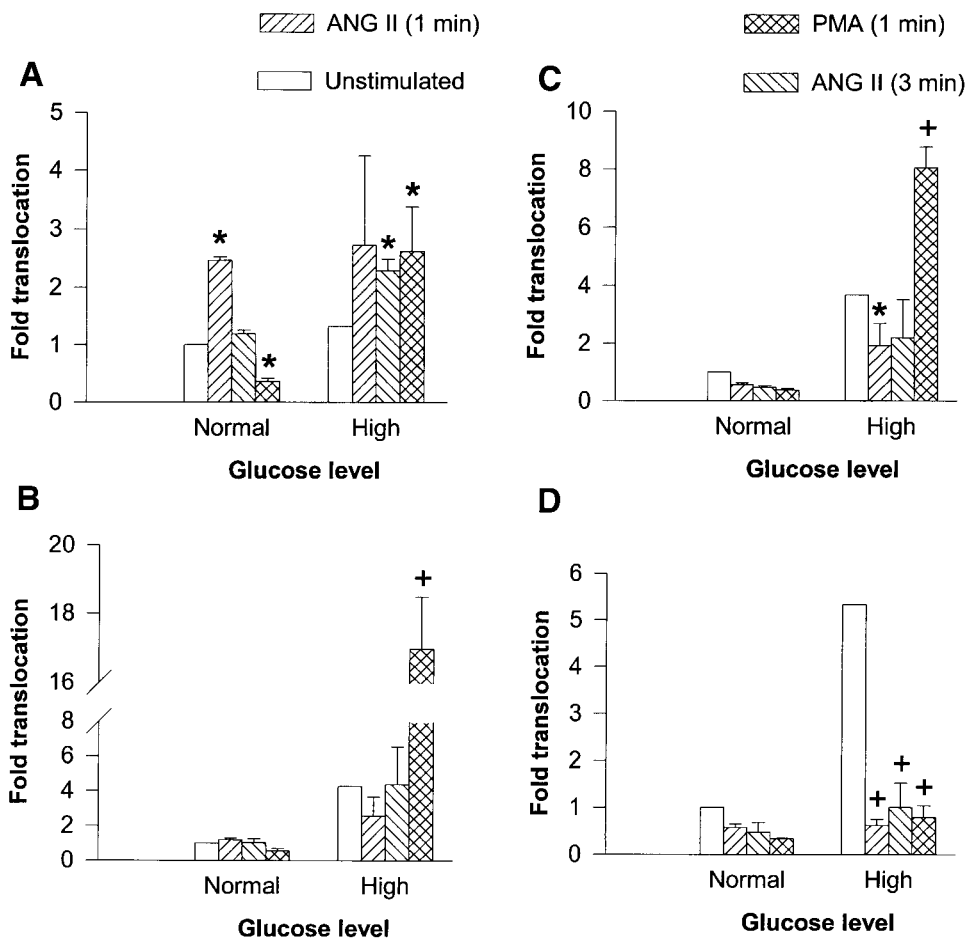


Fig. 6. Fold translocation of atypical PKC isoforms (λ , ι , and ζ) and PKC μ . PKC isoforms from control animals were considered to be 1.0-fold activated. Each graph contains normal and elevated glucose concentration media. Values are means \pm SE; $n = 4$ Western blot analyses, each from different cell culture experiment. Area under the peak of the PKC isoform scanned (both cytosolic and membrane fractions) was determined, and the membrane-to-cytosol ratio was used to calculate fold translocation (or activation). * $P < 0.05$ vs. unstimulated cells. + $P < 0.001$ vs. unstimulated cells.

Furthermore, we found that the phorbol ester, PMA, which was used as a positive control, was more effective in translocating the different MC PKC isoforms in the presence of high glucose concentrations rather than normal glucose concentrations. Since atypical PKC isoforms are generally insensitive to PMA stimulation due to the lack of the DAG binding domain (33, 42), a possible explanation for such translocation could be that under hyperglycemic conditions, PMA has the ability to activate atypical PKC isoforms, whereas under euglycemic conditions, exposure to PMA downregulates the majority of PKC isoforms (51). Another plausible explanation could be that PMA-sensitive PKC isoforms could activate atypical PKC isoforms under hyperglycemic conditions, which is supported by Kim et al. (31), who have suggested possible cross-talk between PKC isoforms. Moreover, it has been proposed that augmented PKC activation/translocation could play an important role in diabetic vascular dysfunction and complications (34) and that this activation is a key regulator of many important vascular functions found to be abnormal in diabetes, including cell growth, permeability, contractility, and synthesis of extracellular matrix proteins (15).

In addition, we saw that prolonged ANG II stimulation (3 min) does not translocate most PKC isoforms and that this pattern was not affected by extracellular glucose concentration. In other words, we generally

found that a 1-min exposure to ANG II stimulates PKC translocation, whereas a 3-min exposure does not. This lack of activation for the longer stimulation period could be explained by PKC-mediated receptor desensitization, as documented by other investigators (44, 51). Interestingly, Griendling et al. (19) have demonstrated that ANG II receptor internalization in vascular smooth muscle cells is directly related to ANG II-induced DAG accumulation secondary to PKC activation accompanied by an increase in IP₃. When DAG accumulation is inhibited, ANG II receptor internalization is blocked, suggesting that DAG stimulation is a necessary step to ANG II receptor internalization and regulation. It is also noteworthy that PKC-dependent receptor regulation is not only dependent on the ANG II receptor, since it has also been reportedly involved in desensitization and downregulation of non-G protein-coupled receptors such as those of natriuretic peptides (45).

In conclusion, we report that in MCs cultured in elevated glucose concentration, ANG II receptor density is greatly reduced, and this effect is specific to glucose and not to increased osmolarity. In addition, we also found that glucose not only affected the quantity of PKC isoforms present but also their translocation with respect to PMA. Furthermore, ANG II stimulated most PKC isoforms present in MCs when used for a short period of stimulation (1 min) but caused desensitization when used for longer stimulation (3 min).

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