

Regulation of Glucose Uptake in Mesangial Cells Stimulated by High Glucose: Role of Angiotensin II and Insulin

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Mesangial cells (MCs) play a central role in the pathogenesis of diabetic nephropathy (DN). MC dysfunction arises from excessive glucose uptake through insulin-independent glucose transporter (GLUT1). The role of the insulin-dependent transporter (GLUT4) remains unknown. This study evaluated the effect of high glucose on GLUT1, GLUT4, and fibronectin expression levels. Glucose uptake was determined in the absence and presence of insulin. Angiotensin II has been implicated as a mediator of MC abnormalities in DN, and its effects on the GLUTs expression were evaluated in the presence of losartan. MCs were exposed to normal (NG, 10 mM) or high (HG, 30 mM) glucose for 1, 4, 12, 24, and 72 hrs. Glucose uptake was elevated from 1 hr up to 24 hrs of HG, but returned to NG levels after 72 hrs. HG induced an early (1-, 4-, and 12-hrs) rise in GLUT1 expression, returning to NG levels after 72 hrs, whereas GLUT4 was overexpressed at later timepoints (24 and 72 hrs). HG during 4 hrs induced a 40% rise in glucose uptake, which was unaffected by insulin. In contrast, after 72 hrs, glucose uptake was increased by 50%, only under insulin stimulus. Losartan blunted the effects of HG on GLUT1, GLUT4, and fibronectin expression and on glucose uptake. Results suggest that MCs can be highly susceptible to the HG environment since they uptake glucose in both an insulin-independent and insulin-dependent manner. The beneficial effects of angiotensin II inhibition in DN may also involve a decrease in the rate of glucose uptake by MCs. *Exp Biol Med* 234:1095–1101, 2009

Key words: glucose transport; mesangial cell; GLUT1; GLUT4; insulin; diabetic nephropathy

Introduction

Mesangial cell (MC) proliferation and excessive production of mesangial matrix components constitute an initial pathophysiologic mechanism involved in the glomerulosclerosis that is typical of diabetic nephropathy (DN) (1). MC dysfunction arises in part due to abnormally high cellular glucose uptake in response to a high extracellular glucose concentration. The transport of glucose into these cells is mediated by facilitative glucose transporters, and previous studies have shown that MCs express both an insulin-sensitive glucose transporter (GLUT4) and an insulin-insensitive glucose transporter (GLUT1), with GLUT1 being the predominant isoform expressed in MCs under standard glucose conditions (2–5). Thus, it is expected that excess extracellular glucose will spontaneously enter the cell *via* GLUT1 and result in a shift in the metabolic pathway with activation of the polyol cascade (6), leading to cellular defects such as excessive production of the extracellular matrix. In a previous study, Heilig and coworkers demonstrated that MCs overexpressing the GLUT1 transporter presented increased production of mesangial matrix proteins even under normal glucose conditions (3), indicating a key role of GLUT1 in mediating MC dysfunction in diabetes. However, it was also observed that the biochemical pathway used by GLUT1-transfected cells was distinct from that used by MCs stimulated with high glucose (HG), including the induction of the profibrotic molecule transforming growth factor- β (TGF- β) (7), suggesting that the increased glucose influx induced by GLUT1 does not completely explain the diabetic behavior of MCs. It is clear that GLUT1 is the main pathway for glucose uptake in MCs, but the role of GLUT4 in the MC abnormalities that occur in response to the HG environment is less well understood.

It is well established that angiotensin II (Ang II) has an important role in the development of DN, and the activation of the intrarenal renin-angiotensin system (RAS) appears to be critical (8). MCs are a site of intrarenal generation of Ang II as they contain all of the elements necessary to generate Ang II independently (9, 10). In addition, our laboratory and

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others have shown that MCs exposed to HG concentrations produce higher levels of Ang II (11, 12). Ang II induces an increase in TGF- β expression in experimental and human diabetes as well as in MCs in culture (13–15). This, in turn, promotes cell hypertrophy and stimulates mesangial matrix production (16), leading to glomerular sclerosis. However, the role of Ang II in the metabolic alterations in MCs induced by diabetes is less well explored. It has been shown previously that Ang II increases glucose uptake in MCs by inducing the expression of GLUT1 (2), but the effect of endogenous Ang II generation in response to HG environment on glucose uptake remains unclear.

Thus, the present study evaluated the short-term effect of HG concentration on the expression levels of GLUT1, GLUT4, and fibronectin in MCs. The role of both glucose transporters was further analyzed by the determination of glucose uptake rates in the presence and absence of insulin. The role of locally generated Ang II in response to an HG milieu on the expression of glucose transporters and glucose uptake was estimated using the angiotensin receptor blocker losartan.

Methods

Primary Culture of Mesangial Cells. Mesangial cells were cultured using standard techniques already established in this laboratory involving glomerular isolation through differential sieving (17). Briefly, the glomeruli were isolated from freshly removed kidneys from adult male Wistar rats and then plated at a density of approximately 300 glomeruli/cm in RPMI 1640 culture medium containing a standard glucose concentration of 10 mM and supplemented with 20% fetal bovine serum, 50 U/ml penicillin, 11 mM HEPES, and 2 mM glutamine. Cells were used between the third and fifth subcultures. The purity of cultures was periodically checked by immunostaining: cultures should be negative for human factor VIII antigens and cytokeratin, and positive for α -actin and Thy 1. Also, the presence of contaminating juxtaglomerular cells was assessed based on their characteristic spherical shape, which contrasts with the stellate appearance of MCs. At semiconfluence, cells were incubated in a culture medium determined by their experimental grouping as follows: control group, cells were maintained under standard D-glucose concentration (NG, 10 mM); and HG (30 mM) groups, cells were exposed to RMPI culture medium (containing 10 mM D-glucose) supplemented with 20 mM of D-glucose for 1, 4, 12, 24, and 72 hrs. Osmolarity was controlled by adding 20 mM mannitol to the culture medium instead of D-glucose for 24 hrs. Additional NG and HG groups were treated with losartan (100 nM) for 24 hrs. All reagents, except when otherwise specified, were obtained from Sigma Aldrich Chemical Company (St. Louis, MO).

Glucose Uptake ($[^3\text{H}]$ -2-Deoxyglucose Transport Measurement). Glucose transport was assessed by measuring the uptake of radiolabeled $[^3\text{H}]$ -2-deoxyglucose

(Amersham Biosciences UK Limited, UK), as previously described (18). The effects of glucose concentrations (10, 20, and 30 mM) and of the exposure time to HG from 1 to 72 hrs were analyzed. MCs were seeded in 12-well culture plates and confluent cells were treated according to their respective group. Cells were rinsed with Krebs's Ringer phosphate buffer (KRH) containing 0.1% bovine serum albumin (BSA) (136 mM NaCl; 4.7 mM KCl; 1.25 mM CaCl_2 ; 1.25 mM MgSO_4 ; and 10 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$; pH 7.4), and were then incubated at 37°C for 15 mins in KRH buffer. Nonspecific uptake of $[^3\text{H}]$ -2-deoxyglucose was determined by the addition of 10 μM cytochalasin B into 2 wells. To estimate the insulin-stimulated glucose transport, 0.8 units (60 $\mu\text{g}/\text{ml}$ of culture medium) of insulin (Iolin NPH 100 IU/ml, Novo Nordisk, Brazil) was added in some wells. Fifty microliters of a mixed solution containing 1 mM unlabeled 2-deoxyglucose and 5 μCi $[^3\text{H}]$ -2-deoxyglucose were added into all wells. After 5 mins of incubation, the radioactive mixture was rapidly aspirated and the cells were rinsed with ice-cold phosphate-buffered saline (PBS) and then solubilized in 500 μl of 0.05% sodium dodecyl sulphate (SDS). To determine the amount of $[^3\text{H}]$ -2-deoxyglucose that was incorporated, 400 μl of each sample was added to 2 ml of scintillation liquid (Optiphas Hisafe 3, Perkin Elmer Life and Analytical Sciences, Boston, MA) and the radioactivity was counted in a β -counter (Tri-Carb 2100 TR, Packard, Meriden, CT). The remainder of each sample was used for protein determination by the Folin method. The results were expressed as nanomoles of glucose transported per milligram of protein per minute. Samples were processed in triplicate.

Quantitative Real-Time PCR. Total RNA was purified from MCs by the phenol and guanidine isothiocyanate-cesium chloride method using a kit (Trizol, Gibco BRL, Rockland, MD). Two micrograms of total RNA were treated with DNase (Promega, Madison, WI) to avoid genomic DNA contamination. The RNA pellet was resuspended with RNase-free water and reverse transcribed into cDNA by the addition of a mixture containing 0.5 mg/ml oligo d(T), 10 mM DTT, 0.5 mM deoxynucleoside triphosphates (Amersham Pharmacia Biotech, Uppsala, Sweden), and 200 units of reverse transcriptase enzyme (SuperScript RT, Gibco BRL). The mRNA expression levels for GLUT1, GLUT4, and fibronectin were estimated *via* real-time reverse transcriptase polymerase chain reaction (RT-PCR) using the GeneAmp 5700 System (Applied Biosystems, Foster City, CA). Briefly, 1 μl of cDNA was added to the PCR mixture containing the dye SYBER Green I (Applied Biosystems, Warrington, UK). Primers were designed and chosen based on their efficiency and were the following (forward and reverse, respectively): GLUT1: 5'-agaccagcgcctgtccagac-3' and 5'-tgaccacctgtgtctcag-3'; GLUT4: 5'-tgccacatagactctgggtg-3' and 5'-agggtggagcctacagcagc-3'; Fibronectin: 5'-tggtgacagtgtgtgcctg-3' and 5'-tgtttggacacagccacagg-3'; β -actin: 5'-ccctctatgccacacagtgc-3'; and 5'-acatctgctggaaggtggac-3'. Real-time PCR product

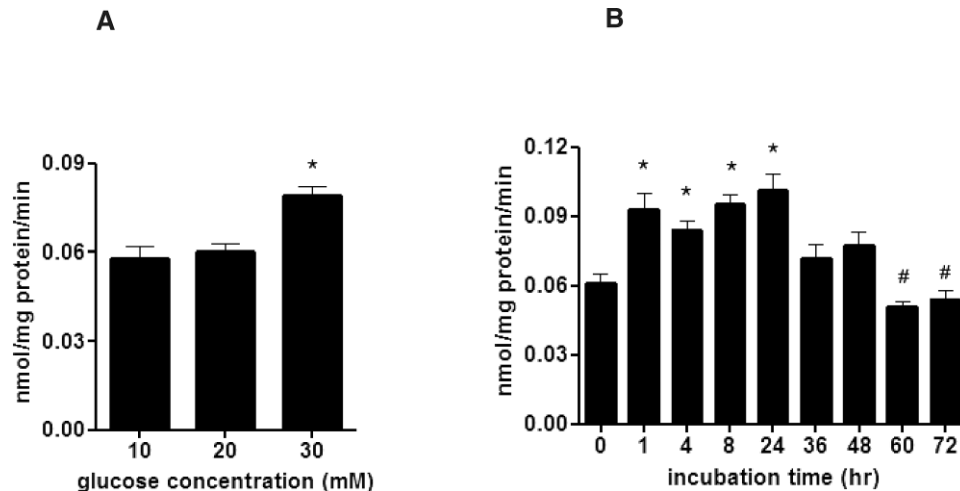


Figure 1. Effects of glucose concentration and incubation time under high glucose milieu (HG, 30 mM) on glucose uptake. MCs were stimulated with different glucose concentration during 24 hrs (A). The time-dependent effect of the HG milieu is shown in panel B. After the experiments, cells were rinsed and incubated with 3H-DOG for glucose uptake rate determination. Results were obtained in triplicate from 5 to 8 cultures for each measurement. $P < 0.05$: * vs. NG (A) or time 0 (B); # vs. 24 hrs.

accumulation was monitored using the intercalating dye, SYBR Green I, which exhibits a higher fluorescence upon the binding of double-strand DNA. Fluorescence for each cycle was quantitatively analyzed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems). The melting curve analysis was performed at the end of all PCR runs to verify the presence of a single amplification product. Relative gene expression was calculated using early PCR stage conditions under which the amplification curve is logarithmic. The mRNA expression levels were normalized to β -actin and the results were expressed as arbitrary units compared to the NG group as the standard.

Western Blot. Analysis of the protein expression levels of GLUT1, GLUT4, and fibronectin was performed by Western blot. Cells were lysed (buffer: 50 mM Tris, pH 8.0; 150 mM NaCl; 1% Nonidet P-40; 0.5% sodium deoxycholate; 0.1% SDS; 2.5 mM EDTA; 1 mM phenylmethylsulfonyl fluoride (PMSF); and 44 mM o-phenanthroline) and centrifuged, and the supernatant was stored at -80°C until use. Protein concentration was determined using the Folin method, with reagents from Bio-Rad (Bio-Rad DC Protein Assay, Richmond, CA). Then, 50 μg of protein was separated on 10% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) and transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ), at 4°C , using transfer buffer containing 25 mM Tris-HCl, 192 mM glycine, and 20% methanol. Nonspecific binding was blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) containing 10 mM Tris-HCl, pH 7.5, and 200 mM NaCl, followed by washing in TBS-T (Tween 0.05%) at room temperature. Immunoblots were then incubated overnight at 4°C with primary polyclonal antibodies anti-GLUT1, anti-GLUT4, and anti-fibronectin (Santa Cruz Biotechnology Inc., Santa Cruz, CA), all of them diluted 1:200 in TBS-T containing 3% BSA. The

monoclonal anti- β -actin antibody (Abcam Inc., Cambridge, UK) was diluted 1:400 in TBS containing 3% BSA. After washing with TBS-T, membranes were incubated for 1 hr at 4°C with the appropriate HRP-conjugated secondary antibody (Sigma Aldrich). Detection of specific protein bands was accomplished by chemiluminescence using a detection system (Immobilon Western, Chemiluminescence HRP substrate, Millipore Corporation, Billerica, MA) and recorded on X-ray film (Amersham Hyperfilm ECL, GE Healthcare Limited, Japan). Optimally exposed autoradiographs were digitally scanned and analyzed using an Imaging Densitometer (Bio-Rad, GS670, Hercules, CA) and Quantity One-GS710 software (Bio-Rad).

Statistical Analysis. Results are presented as mean $\pm$ SE. Data were analyzed using one-way analysis of variance (ANOVA), followed by Dunn or Tukey tests when appropriate. Results for glucose uptake rate with and without insulin were compared by unpaired t test. The level of statistical significance was defined as $P < 0.05$.

Results

We first analyzed the concentration-dependent effects of glucose on glucose transport (Fig. 1A). An increase in glucose concentration from 10 mM to 20 mM did not change the glucose uptake rate, which could be observed when extracellular glucose was further increased to 30 mM. The time-dependent effect of 30 mM glucose on glucose uptake is presented in Figure 1B. Increased glucose uptake under HG conditions (30 mM) was an early event and could be observed after 1 hr of HG stimulation. Glucose uptake remained increased up to 24 hrs when it probably started to decrease, since the values found after 36 hrs and 48 hrs were intermediary between the control (time 0) and 24-hr groups. After 60 hrs and 72 hrs, glucose uptake was normalized and similar to the control levels.

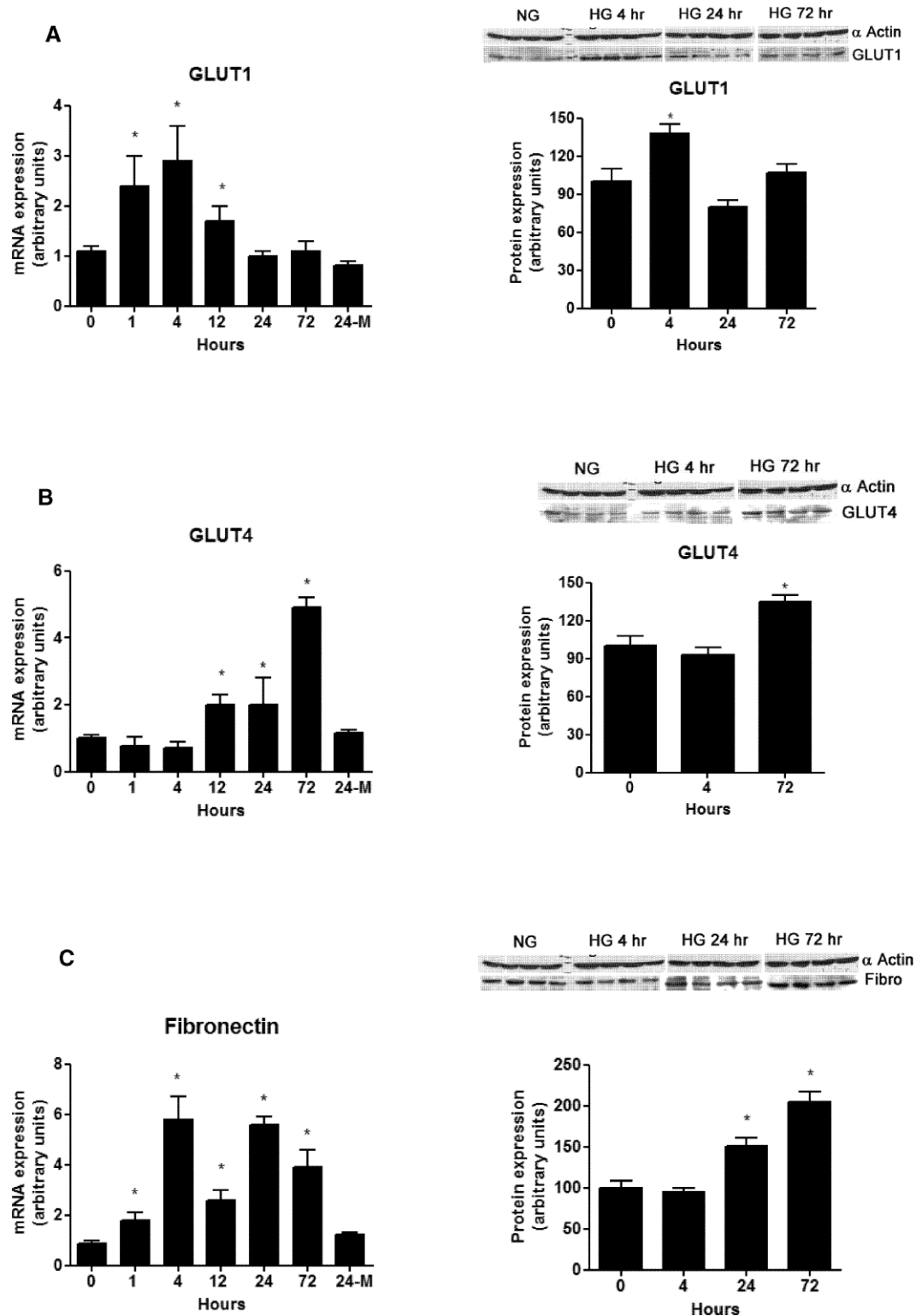


Figure 2. Effects of the incubation time under high glucose (30 mM) milieu on the expression levels of mRNA (left side) and protein (right side). GLUT1 levels are in panel A, GLUT 4 in panel B, and fibronectin in panel C. Protein levels were estimated by densitometry analysis of the bands shown in the blots. $P < 0.05$ * vs. NG (time 0). The osmotic control for HG was accomplished by using 20 mM of mannitol instead of glucose (24-M). MCs were exposed to high mannitol (M) concentration during 24 hrs. Results were obtained from 5–7 cultures for each period.

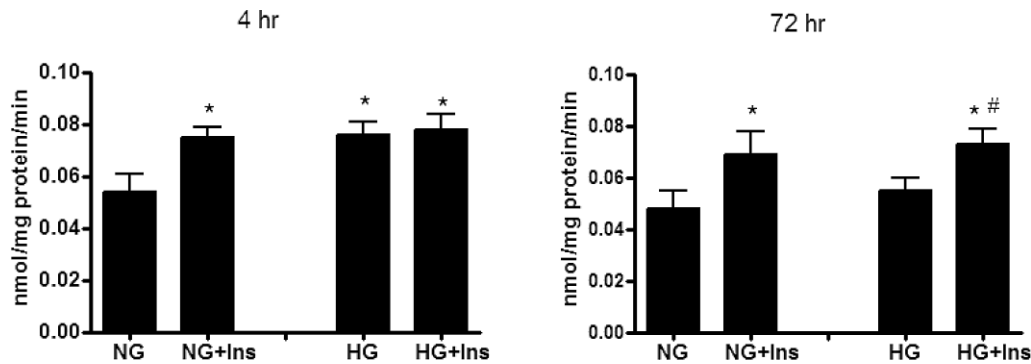


Figure 3. Effect of insulin on glucose uptake. MCs were stimulated by HG medium during 4 hrs (A) or 72 hrs (B) and the rate of glucose uptake was determined in the absence and presence of 0.8 units of insulin. $P < 0.05$: * vs. NG; # vs. HG. Results were obtained in triplicate from 8–13 cultures for each measurement.

An HG environment induced time-dependent changes in the glucose transporters GLUT1 and GLUT4. GLUT1 mRNA expression exhibited an early upregulation (1, 4, and 12 hrs) followed by a normalization in the gene expression after 24 hrs, and remained near the NG levels up to 72 hrs (Fig. 2A, left panel). In contrast, HG induced a later upregulation initiated after 12 hrs in the insulin-dependent GLUT4 compared to GLUT1, which remained elevated up to 72 hrs later (Fig. 2B). The protein expression profile for both glucose transporters was similar to the mRNA expression, as shown in the right panel; however, there was a difference of magnitude between mRNA expression and protein levels, probably indicating an additional posttranscriptional control of these glucose transporters. Fibronectin gene transcription was stimulated as soon as 1 hr after HG stimulation and remained upregulated during all periods analyzed, although the protein levels were significantly higher only after 72 hrs (Fig. 2C). Exposure of MC to 20 mM mannitol during 24 hrs did not change the mRNA expression levels of GLUT1, GLUT4, and fibronectin.

Although MCs can spontaneously uptake glucose through GLUT1, we found that the glucose transport capacity was increased by insulin in MCs kept in standard NG conditions (Fig. 3). As shown in Figure 3A, HG exposure for a short period (4 hrs) induced a 40% rise in the glucose uptake rate, which was unaffected by insulin treatment. In contrast, in cells exposed to HG for 72 hrs, glucose uptake was increased only in the presence of insulin (Fig. 3B), indicating a role for GLUT4 in glucose uptake after more prolonged periods of HG stimulation.

Considering the beneficial effects of RAS inhibition in retarding the progression of DN and also that the high rate of glucose uptake may be involved in MC dysfunction, we next evaluated the effect of Ang II receptor blockade on glucose uptake in MCs treated with HG medium. We observed that losartan did not affect the glucose transport in NG cells, but it significantly reduced the glucose uptake rate in the HG group (Fig. 4). This losartan-mediated reduction in glucose transport in HG cells probably occurred due to its effects on the glucose transporters, since the expression

levels of GLUT1, GLUT4, and fibronectin were reduced by losartan (Fig. 5).

Discussion

It is well established that functional alterations in MCs in response to high extracellular glucose levels have been involved in the pathophysiology of DN. Increased glucose uptake and metabolism appears to be one of the initial steps triggering MC abnormalities (19). The insulin-independent glucose transporter GLUT1 has been recognized as the main pathway for glucose uptake in MCs, and since it appears to be regulated by the extracellular glucose concentration, these cells are potentially susceptible to the deleterious effects induced by an HG environment (3, 4, 20). In fact, it was observed that increased extracellular glucose was followed by a spontaneous (absence of insulin) increase in the rate of glucose uptake, as previously observed by Heilig and collaborators (4) in MCs exposed to an HG milieu for up to 3 months. In the present study, we attempted to analyze the more precocious responses of MCs to elevated extracellular glucose from 1 to 72 hrs, and we found that 1 hr of HG stimulation was enough to significantly increase glucose uptake. This response persisted for 24 hrs, when it started to decrease and returned to control levels after 72 hrs, when glucose transport was increased only in the presence of

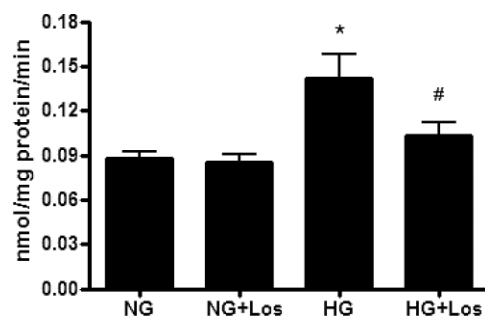


Figure 4. Effect of losartan on glucose uptake. MCs were maintained under NG or HG glucose conditions in the absence or presence of 100 mM losartan during 24 hrs. $P < 0.05$: * vs. NG; # vs. HG. Results were obtained in triplicate from 12 cultures for each measurement.

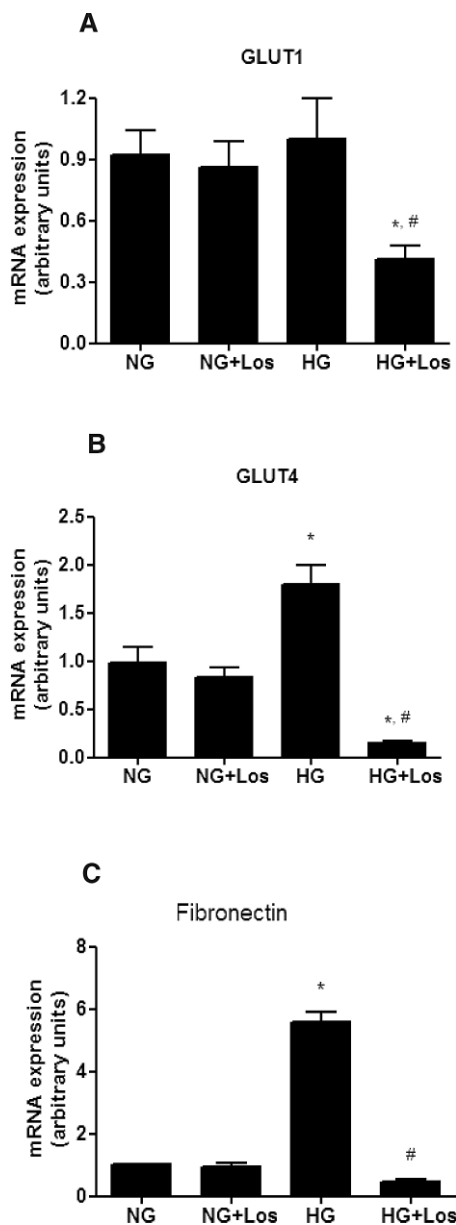


Figure 5. Effect of losartan on the mRNA expression levels of GLUT1 (A), GLUT4 (B), and fibronectin (C). MCs were maintained under NG or HG glucose conditions in the absence or presence of 100 mM losartan during 24 hrs. $P < 0.05$: * vs. NG; # vs. HG. Results were obtained from 5–8 cultures for each period.

insulin, suggesting a desensitization of the GLUT1. As expected, the early elevation in glucose uptake was coincident with the upregulation of GLUT1 and it was not further stimulated by insulin, indicating a predominance of GLUT1-mediated glucose transport. This correlation persisted after 72 hrs, when a decrease in both glucose uptake and GLUT1 expression was observed. In the Heilig study (4), it was also observed that glucose uptake was increased in MCs stimulated with 20 mM glucose after 3 months, but not after 3 days. This was attributed to the low affinity of GLUT1 transporter since they found that GLUT1 expression was significantly increased after 3 days. The differences in

GLUT1 expression observed between the two studies could be due to different cell lines and or to cell culture conditions, but both results point to a reduced glucose uptake during 3 days of HG stimulation compared to more prolonged (3 months) exposure (4). Our results suggest that although MCs initially take up more glucose in an HG environment, they may be able to compensate for the excess of extracellular glucose by reducing GLUT1-mediated glucose uptake. However, this ability appears to be lost when the HG stimulus persists for more prolonged periods, as demonstrated in the Heilig study, which found significantly increased in glucose uptake after 3 months of HG stimulation.

Although MCs cultured under standard glucose conditions express a small amount of the insulin-dependent glucose transporter GLUT4 (21), the role of GLUT4 in the MC response to hyperglycemia has been much less explored. Interestingly, we found that in contrast to the early upregulation of GLUT1, GLUT4 expression remained in the low range during the first hours, but progressively increased after 12 hrs of HG exposure, reaching the highest level after 72 hrs of HG stimulation. This result was coincident with observations of increased insulin-stimulated glucose uptake after 72 hrs, but not during shorter periods of HG exposure. The significance of this late upregulation of GLUT4 under HG stimulation in MCs is not known at this moment, but the increase in glucose transport in the presence of insulin may constitute an interesting link between hyperglycemia and insulin needs in certain patients. In those with poor glycemic control, insulin treatment may induce additional glucose uptake mediated by GLUT4, which could contribute to a state of the glucotoxicity for the MCs after a hyperglycemic episode. The increased intracellular glucose concentration results in activation of several pathophysiologic mechanisms, including the metabolic polyol pathway, increased synthesis of TGF- β 1, and intracellular production of advanced glycation end products; generation of reactive oxygen species also increases the local formation of Ang II. All of these effects contribute to the increased synthesis of the mesangial matrix, fibrogenesis components such as fibronectin and collagen, leading to mesangial expansion and glomerular sclerosis (22).

The production of fibronectin (mRNA and protein) increased after 1 hr and remained significantly elevated during all periods of HG exposure, independent of GLUT expression profiles and the glucose uptake rate, suggesting that the initial increase in glucose flux was enough to trigger cellular mechanisms that culminate with mesangial matrix overproduction. We hypothesized that one of these mechanisms would be mediated by locally synthesized Ang II. Previous studies have demonstrated that Ang II treatment induces an increase in the glucose transport mediated by GLUT1 in vascular smooth muscle cells (23, 24) and in MCs (25). In the present study, however, we evaluated the role of endogenously generated Ang II, since MCs in culture are able to synthesize and secrete Ang II, which is strongly

stimulated by the HG environment (11, 12). We found that the AT1 receptor blocker losartan reduced the HG-stimulated glucose uptake to near control levels, in spite of a decrease in both GLUT1 and GLUT4 mRNA expression to levels even lower than in NG cells, suggesting a possibility that other glucose transporters, such as Na-glucose cotransporter, may be involved. Losartan did not change fibronectin, GLUT1, and GLUT4 expression levels in NG cells, and since Ang II levels are relatively low under standard culture conditions, it probably has no relevant role in the glucose transporters gene transcription in control cells. It is well known that Ang II has many physiologic and pathophysiologic effects on MCs, including cell contraction, proliferation/growth, gene transcription of cytokines, and the profibrotic TGF- β . The present results suggest that in addition to these actions, endogenous Ang II produced in response to an HG environment can also upregulate the glucose transporters and thus, glucose uptake, contributing to the deleterious effects of the excess of extracellular glucose. The mechanism by which Ang II stimulates glucose uptake in MCs exposed to an HG environment is unknown, but one exciting possibility includes a secondary effect involving growth activities induced by Ang II in many cell types, including MCs, which could increase cellular demands for energy, as previously proposed (24). Although this hypothesis needs further investigation, it constitutes an interesting link between glucose utilization and hormonal/cytokine activation mediated by Ang II and, thereby, to the renal dysfunction observed in DN.

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