Regulation of Glucose Transporter (GLUT1) Gene Expression by Angiotensin II in Mesangial Cells: Involvement of HB-EGF and EGF Receptor Transactivation

Atsuko NOSE, Yasukiyo MORI, Yoko UCHIYAMA-TANAKA, Noriko KISHIMOTO, Katsuya MARUYAMA, Hiroaki MATSUBARA, and Toshiji IWASAKA

In the development of diabetic nephropathy, angiotensin (Ang) II is thought to exert numerous actions on the glomerulus, and especially on the mesangium. However, the role(s) played by Ang II in the glucose metabolism per se in mesangial cells remains unclear. Ang II, at least via its type 1 receptor (AT1-R)-mediated effect, phosphorylates extracellular signal regulated kinase (ERK) by transactivation of epidermal growth factor receptors (EGF-Rs) via the Ca\(^{2+}\) or protein kinase C (PKC) pathways. Our objective in the present study was to assess the effect of Ang II on glucose transporter 1 (GLUT1) gene expression and to clarify the involvement of EGF-R in Ang II-mediated GLUT1 mRNA expression in glomerular mesangial cells. The results showed that Ang II upregulated GLUT1 mRNA accumulation in a time- and dose-dependent manner (peaking at 12 h; \(\sim 3.8\)-fold vs. control), and this upregulation was completely inhibited by the PKC inhibitor calphostin-C. The Ang II-induced GLUT1 expression was significantly inhibited by the EGF-R inhibitor AG1478 (\(\sim 80\%\) inhibition), by inactivation of ERK by PD98059, and by pretreatment with heparin and the metalloproteinase (MMP) inhibitor batimastat. On the other hand, phorbol ester markedly upregulated GLUT1 mRNA (\(\sim 8.6\)-fold). Batimostat and AG1478 significantly reduced the phorbol ester-induced GLUT1 mRNA expression (\(\sim 72\) and \(\sim 69\%\) inhibition, respectively). In conclusion, PKC-mediated heparin-binding (HB)-EGF/EGF transactivation followed by ERK activation plays a predominant role in the induction of GLUT1 expression by Ang II. (Hypertens Res 2003; 26: 67–73)

Key Words: angiotensin II, glucose transporter, epidermal growth factor, heparin-binding epidermal growth factor-like growth factor, mesangial cell

Introduction

End-stage renal disease (ESRD) continues to be a worldwide public health problem. Recent estimates indicate that diabetic nephropathy (DN) is a leading cause of ESRD in Western nations, accounting for over one-third of all patients beginning renal replacement therapy (1). Patients with type 2 diabetes comprise the largest and fastest growing group of patients requiring renal replacement therapy, and they account for all of the increasing number of patients with ESRD (2). Therefore, understanding the development of DN is crucial for developing a strategy to prevent chronic renal failure due to diabetes. Glomerular mesangial cells play a key role in maintaining the normal structure and physiological function of the glomerulus. DN is characterized by increased mesangial and tubular accumulation of extra-cellular matrix (ECM) components and progressive glomerulosclerosis, and these effects inevitably lead to renal failure (3). The high extracellular glucose concentration observed in association with dia-
betes is reported to stimulate mesangial ECM synthesis in vitro (4). Thus, facilitated glucose transport across the plasma membrane appears to be essential for the initiation of DN. It has also been strongly suggested that the renin-angiotensin system (RAS) plays a role, based on the observation in clinical trials that angiotensin converting enzyme (ACE) inhibitors or angiotensin receptor blockers (ARBs) can slow the progression of DN (5–7). In addition, experimental studies have suggested that activation of the intrarenal RAS is more critical for DN than the contribution of RAS is for systemic hypertension (8, 9). However, the pathogenic mechanism by which angiotensin II (Ang II) contributes to DN is unclear. Within the glomeruli of the kidney, glucose transporter 1 (GLUT1) is known to be the most important facilitative glucose transporter (10). Thus, we hypothesized that Ang II may contribute to DN by stimulating the expression of GLUT1. The goals of the present study were to determine the effects of Ang II on GLUT1 gene expression and its signaling mechanism and particularly the contribution of transactivation of epidermal growth factor receptor (EGF-R) to Ang II signaling in mesangial cells.

**Methods**

**Materials**

Heparin-binding (HB)-EGF, BAPTA-AM, TMB-8, genistein, PD98059, and AG1478 were provided from Calbiochem (San Diego, USA). Calphostin-C, phorbol 12-myristate 13-acetate (PMA) and heparin (porcine intestinal mucosa) were from Sigma Chemical (St. Louis, USA). Ang II and EGF were from Peptide Institute (Osaka, Japan) and Toyobo (Osaka, Japan), respectively. The metalloproteinase (MMP) inhibitor batimastat was from British Biotech (Cowley, Oxford, UK). The type 1 Ang II receptor (AT1-R) antagonist RNH6270 (active form of CS866) was provided by Sankyo (Tokyo, Japan).

**Cell Culture**

Mesangial cells were prepared from kidneys of 8-week-old male C57BL/6 mice by the explant method as described previously (11) and cultured with Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS). Subconfluent mesangial cells (passages 4 and 5) were serum-starved for 24 h and used for the experiments.

**Northern Blotting**

Total RNA was extracted from cultured mesangial cells using TRIZOL Reagent (Gibco BRL, Gaithersburg, USA). The GLUT1 cDNA fragment for Northern blotting was made by reverse transcription with oligo-dT as the primer (Takara Biochemical, Tokyo, Japan). A 1,479-bp GLUT1 fragment was amplified from 1 µg of total RNA by polymerase chain reaction (PCR); the sequences were 5’-ATGGATCCCAAGCAGAAGGTTGA-3’ and 5’-CAGACTTGGGAGTCGCCCCGAGA-3’. The PCR product was gel-purified and subcloned into the pGEM-T vector (CLONTECH, Palo Alto, USA) followed by verification of the sequence. Twenty micrograms of total RNA was fractionated on 1% agarose/formaldehyde gels and transferred to nylon membranes (13, 14). Blots were then hybridized with random-primed 32P-labeled cDNA probes for GLUT1 (388-bp-EcoRI fragment) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. Hybridization signals were quantified by scanning densitometry, and GLUT1 mRNA levels were arbitrarily normalized relative to the GAPDH mRNA levels.

**Statistical Analysis**

The results are shown as the means ± SE. Statistical analysis was done with one-way analysis of variance (ANOVA) followed by pairwise comparisons using Dunnett’s multiple comparisons test. Values of p < 0.05 were considered to indicate statistical significance.

**Results**

**Ang II Increases GLUT1 mRNA Accumulation**

Ang II (100 nmol/l) significantly increased GLUT1 mRNA accumulation after 6 h of incubation, reached the maximum increase (3.8-fold) after 12 h and thereafter maintained a near maximum level until 24 h (Fig. 1). Ang II induced GLUT1 mRNA accumulation in a dose-dependent fashion with an EC50 of ~45 nmol/l and the peak at 100 nmol/l; the increases were completely blocked by the AT1-R antagonist RNH6270 (1 µmol/l) (data not shown). Subsequent experiments were performed with 100 nmol/l Ang II stimulation for 12 h.

**Protein Kinase C (PKC) Rather than Ca2+ Is Involved in Ang II-Induced GLUT1 mRNA Accumulation**

To address the intracellular signaling of Ang II in GLUT1 expression, we investigated the role of PKC. Cultured mesangial cells were treated with the PKC inhibitor calphostin-C (100 nmol/l), resulting in the complete inhibition of Ang II-induced GLUT1 mRNA accumulation (Fig. 2). Ang II-mediated GLUT1 mRNA upregulation was partially inhibited by the intracellular Ca2+ chelators BAPTA-AM (50 µmol/l) and TMB-8 (100 µmol/l) (~20% and ~22% inhibition, Fig. 2), suggesting that the PKC pathway is more crucial than intracellular Ca2+ signaling is for the stimulating effect of Ang II on GLUT1 expression. Notably, genistein (200 µmol/l), which has a strong preference for tyrosine-specific kinases, inhibited Ang II-induced GLUT1 mRNA accumulation completely (Fig. 2). Since the processing of HB-
EGF is known to be stimulated by Ang II-mediated PKC signaling and the released HB-EGF transactivates EGF-R, which is activated by tyrosine kinase (14–16), we next examined the contribution of EGF-R transactivation to the Ang II effect on GLUT1 gene expression.

The Stimulating Effect of Ang II on GLUT1 mRNA Expression Is Regulated by EGF-R-Mediated Extracellular Signal-Regulated Kinase (ERK) Signaling

Interestingly, the EGF-R inhibitor AG1478 (1 µmol/l) significantly reduced Ang II-mediated GLUT1 mRNA expression (≈80% inhibition, Fig. 3). AG1478 alone did not affect the stimulating effect of Ang II (data not shown). The specificity of AG1478 as an inhibitor of EGF-R phosphorylation was well established by our previous studies of dominant negative mutants of EGF-R (16–18). Since EGF-R-mediated signals via AT1-R stimulation were reported to activate ERK in the induction of fibronectin mRNA expression in mesangial cells (11), cardiac fibroblasts (16) and vascular smooth muscle cells (19), we next examined whether these mitogen-activated protein kinases (MAPKs) are involved in induction of GLUT1 expression by Ang II. ERK inactivation by the MAP kinase (MEK) inhibitor PD98059 completely inhibited Ang II-mediated GLUT1 mRNA accumulation (Fig. 3). EGF (10 ng/ml) alone stimulated GLUT1 mRNA expression significantly (3.4-fold vs. control), thus verifying the inhibition by AG1478 (Fig. 3).

Processing of HB-EGF Is Involved in Upregulation of GLUT1 Gene Expression by Ang II

After detecting the involvement of EGF-R in the GLUT1 gene expression upregulated by Ang II, we examined whether the binding of HB-EGF to EGF-R is actually mediated by this signaling pathway. The addition of heparin (100 µg/ml), which competes with cell surface-associated heparin sulfate proteoglycans as a coreceptor for HB-EGF binding to EGF-R (20), significantly inhibited Ang II-mediated GLUT1 mRNA accumulation (≈88% inhibition, Fig. 4). Since MMPs mediate the proteolytic processing of HB-EGF prior to their release into the extracellular space (21), we tested the
effect of batimastat (10 \( \mu \text{mol/l} \)) on the Ang II effect. The addition of batimastat, which facilitates chelation of the zinc ion in the active site of membrane-bound zinc-endopeptidases including the interstitial collagenases, stromelysins 1-3, matrilysin, 92-kDa gelatinase B (MMP-9), and the 72-kDa gelatinase A (MMP-2) (21), significantly inhibited the stimulating effect of Ang II (\( \sim 84\% \) inhibition, Fig. 4). Finally, HB-EGF (2 ng/ml) was also found to stimulate \( \text{GLUT1} \) expression significantly (3.6-fold vs. control).

**HB-EGF/EGF Receptor Transactivation Is Involved in Phorbol Ester-Stimulated \( \text{GLUT1} \) mRNA Expression**

As shown in Fig. 3, the inhibitory effect of AG1478 on Ang II-induced \( \text{GLUT1} \) mRNA accumulation was not complete. Therefore, we examined the effects of batimastat and AG1478 on phorbol ester-induced \( \text{GLUT1} \) mRNA expression (Fig. 5). The treatment with PMA (100 nmol/l) for 12 h markedly stimulated \( \text{GLUT1} \) mRNA accumulation (8.6-fold vs. control), and this accumulation was completely inhibited by calphostin-C (100 nmol/l). The addition of batimastat (10 \( \mu \text{mol/l} \)) and AG1478 (1 \( \mu \text{mol/l} \)) significantly, but not completely, reduced the stimulating effect of PMA (\( \sim 72\% \) and \( \sim 69\% \) inhibition, respectively).

**Discussion**

The glucose transporters mediate the facilitative uptake of glucose into cells. Among the family of glucose transporters, \( \text{GLUT1} \) is the predominant form expressed in mesangial cells (22). *In vitro* experiments in mesangial studies have shown that increased \( \text{GLUT1} \) expression induces a positive feedback of greater \( \text{GLUT1} \) expression, increases aldose reductase expression, and activates PKC, leading to detrimental stimulation of ECM synthesis in mesangial cells (23). Thus, \( \text{GLUT1} \) may be the rate-limiting step for the use of glucose in mesangial cells. Interestingly, a recent report showed that a polymorphism in the \( \text{GLUT1} \) gene in humans is involved in the susceptibility to DN (24). Therefore, changes in \( \text{GLUT1} \) expression, translocation or activity that result in excess uptake of glucose may be required for the development of DN. The major new findings of the present study were that 1) Ang II upregulates \( \text{GLUT1} \) mRNA accumulation in mesangial cell; 2) the stimulating ef-
Effect of Ang II is mainly mediated by the processing and release of HB-EGF via activation of MMP; 3) HB-EGF released into the incubation medium binds EGF-R followed by activation of ERK to stimulate GLUT1 expression. Thus, the present results provide the first evidence that Ang II mediated by HB-EGF/EGF transactivation plays a pivotal role in GLUT1 expression in mesangial cells.

Daub et al. (17) originally reported that EGF-R signaling mediates Ras/ERK activation by G-protein-coupled receptors (GPCRs), and thereafter several groups, including ours, detected the involvement of HB-EGF in GPCR-mediated EGF-R transactivation (15, 18). Recently, we showed that ERK activation mediates the induction of fibronectin gene expression by Ang II in mesangial cells, and that ERK is phosphorylated downstream of EGF-R activated by HB-EGF binding (11). Cultured mouse mesangial cells, which we have used in our laboratory, have the AT1-R, which exerts most of the known effects of Ang II (25–28), indicating that the EGF-R transactivation is mediated by AT1-R. HB-EGF is proteolytically processed by MMPs in a PKC-dependent manner in mesangial cells (11). The stimulating effect of Ang II on GLUT1 expression is also significantly inhibited by pretreatment with an MMP inhibitor or a significant EGF-R inhibitor. Thus, the same mechanism at work in the induction of the fibronectin (FN) gene may also be involved in the GLUT1 expression induced by Ang II.

Treatment with phorbol ester, which strongly activates PKC, and the overexpression of ras or src oncogenes have been reported to enhance GLUT1 gene expression (29). Two homologous sequences of the tetradeoxyphophorbol-13-acetate-responsive element (TRE) have been identified in the mouse GLUT1 gene (30). However, it is unclear which signals downstream of PKC are involved in GLUT1 gene expression. In the present study, inhibition of the HB-EGF/EGF pathway by an MMP inhibitor or an EGF-R inhibitor also resulted in the significant reduction (70%) of PMA-induced GLUT1 expression. However, an HB-EGF/EGF-independent mechanism downstream of PKC activation contributed the other 30% of the PMA-induced GLUT1 expression. Therefore, there seem to be at least two different mechanisms, which together mediate a wide range of activities from PKC activation to GLUT1 expression. Nevertheless, the present study suggests that PKC-mediated HB-EGF/EGF transactivation followed by ERK activation is the dominant mechanism in the induction of GLUT1 expression by Ang II.

Singh et al. (31) showed that high glucose concentration in the medium stimulates the synthesis of Ang II in cultured rat mesangial cells. In addition, the content of Ang II in the kidney has been reported to increase in an animal model of DN (23). Ang II as well as high glucose per se are known to activate PKC in mesangial cells (8), leading to the upregulation of several growth factors such as TGF-β (32, 33) and vascular endothelial growth factor (34, 35). Interestingly, Inoki et al. reported that TGF-β by itself stimulates GLUT1 expression in rat mesangial cells (36). Thus, in DN a vicious cycle of high glucose and RAS system activation is involved in the upregulation of GLUT1, which in turn stimulates glucose transport. Alternatively, the increased GLUT1 may meet the increased metabolic demands of the growth processes and protein synthesis, including ECM, by Ang II.

Although there have been no reports regarding the roles of HB-EGF/EGF-R in DN, we have shown previously that HB-EGF/EGF-R transactivation regulates FN expression, which is increased in DN (37), by Ang II. In addition, HB-EGF protein and mRNA in the kidney are upregulated in several animal models of acute glomerular injury, such as puromycin aminonucleoside (PAN) (38) and passive Heymann nephritis (39), in the peri-infarct region of the remnant kidney (40), suggesting that HB-EGF is involved in the pathological changes in the glomerulus. Thus Ang II-induced stimulation of GLUT1 expression should be added to the list of mechanisms that play a role in HB-EGF/EGF-mediated glomerular injury.

In summary, we showed here that HB-EGF/EGF transactivation by AT1-R, which is mediated by PKC signals, plays an important role in the expression of GLUT1. Although in the clinical setting ACE inhibitors and ARBs are well-
known renoprotective agents against DN, the precise mechanisms of the ameliorative effects of these agents on the glomerulus, and particularly their nonhemodynamic actions, remain unknown. Our present results led us to construct a novel paradigm of cross-talk between Ang II and EGF-R signaling as one potential mechanism in the development of DN.

References


