Angiotensin II regulates migration in mouse cultured mesangial cells: evidence for the presence of receptor subtype-specific regulation

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Abstract

The biological effects of angiotensin II (AngII) are mediated by two major subtypes of AngII receptors, type 1 (AT1R) and type 2 (AT2R). In this study, we attempted to elucidate the role of AngII subtype receptor-specific regulation in migration and proliferation of mouse cultured mesangial (MSG) cells. We found that 100 nM AngII stimulated weak migration of MSG cells. Cell motility increased more in the presence of AT2R than in the presence of AT1R, and it was suppressed by guanylate cyclase inhibitors. On the other hand, the activation of AT1R resulted in increased cell numbers, while AT2R activation inhibited cell proliferation. Moreover, high concentrations of glucose (25 mM)

stimulated the expression of AT2R but not AT1R. These results indicate that there are receptor subtype-specific roles in MSG cells, and it is therefore possible that the activation of AT2R stimulates repair of glomerular tissue defect, by regulation of migration and proliferation of MSG cells. Taken together, these results suggest that the relative concentrations of AT1R and AT2R are important factors in the regulation of AngII function in glomerular tissue, and alterations in the concentrations of these receptors may contribute to progression of or protection from diabetic nephropathy.

Journal of Endocrinology (2006) **191**, 361–367

Introduction

Angiotensin II (AngII), a major component in the renin–angiotensin system (RAS), plays a primary role as a vasoconstrictor in controlling cardiovascular function and renal homeostasis. Besides its action as a potent hypertensive hormone, AngII has various biological effects on cell proliferation, migration, and invasion. Locally or systemically generated AngII influences vascular function in both physiological and pathological states via several mechanisms, such as induction of migration of vascular smooth muscle cells (Kohno *et al.* 2000), retinal microvascular pericytes (Nadal *et al.* 2002), and glomerular mesangial cells (Kohno *et al.* 1999). Available results suggest that AngII serves as a vascular remodeling modulator and also stimulates migration and growth of cells.

Overactivity of the RAS induces cardiovascular dysfunction and renal sclerosis (Matsusaka *et al.* 1996, Shibasaki *et al.* 2005). It has been reported that angiotensin converting enzyme inhibitors (ACEIs) and AngII receptor blockers (ARBs) have not only hypotensive but also renoprotective effects on the progression of several renal diseases, including diabetic nephropathy (Brenner *et al.* 2001, Lewis & Lewis 2003, Rossing *et al.* 2003, Izuhara *et al.* 2005, Burnier & Zanchi 2006). Both ACEIs and ARBs are known to prevent the progression of renal damage by inhibiting AngII-induced

glomerular capillary resistance and production of extracellular matrix (ECM).

Mesangial cells play important roles in the pathogenesis of glomerular diseases such as glomerular nephritis, glomerular sclerosis, and diabetic nephropathy. Previously, we confirmed that mouse cultured mesangial (MSG) cells express two subtypes of AngII receptors (ATRs), namely AT1R type 1 (AT1R) and type 2 (AT2R) (Lorenzo *et al.* 2002). It has been revealed that the stimulation of AT1R increases proliferation of mesangial cells and production of ECM, but the functional roles of AT2R in mesangial cells remain unclear. In this study, we attempted to ascertain the difference in functional roles between AT1R and AT2R in MSG cells, especially regarding cell migration.

Materials and Methods

Cell and chemicals

Mouse mesangial (MSG) cells were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal bovine serum (FBS) for 72 h before utilization. AngII, CGP-42112A (a potent AT2R agonist), PD123,319 (a potent and selective non-peptide AT2R antagonist), methylene blue (a guanylate cyclase inhibitor), and 6-anilino-5,8-quinolinequinone (a competitive inhibitor of soluble

guanylate cyclase inhibitor) were purchased from Sigma Chemical Co. RNH-6270 (olmesartan medoxamil, a highly selective AT1R antagonist) was kindly obtained from Sankyo Pharmaceutical Co. (Tokyo, Japan). Anti-AT1R rabbit polyclonal antibody (aAT1R) and anti-AT2R goat polyclonal antibody (aAT2R) were obtained from Santa Cruz (Santa Cruz, CA, USA) (Sahajpal & Ashton 2005). Anti- β -tubulin mouse monoclonal antibody (aTub) was obtained from Chemicon (Temecula, CA, USA).

Cell migration assay

Cell migration analysis was performed using a modified Boyden chamber method as previously described (Ikeo *et al.* 2001). MSG cells were trypsinized prior to experimentation. Cells were harvested with Ham's F12 media (GIBCO) plus 1% FBS, and the cell number was adjusted to 1×10^6 cells/ml. Two hundred microliters (2×10^5) of cells were placed onto 8.0 μ m pore cell culture inserts and incubated with 0.7 ml serum-free Ham's F12 with 3 μ g/ml type I collagen (Becton-Dickinson Franklin Lakes, NJ, USA) for 5 h in the presence or absence of test reagents. Cell migration was quantified by counting the number of cells that migrated through the pores. All non-migrated cells were removed from the inner surface of the insert membrane with a scraper, and migrated cells attached to the outer surface were fixed by drying, and stained with May-Grunwald and Giemsa stain solutions. The number of stained cells was counted with a microscope at a magnification of $200\times$.

Cell count

Cell count was performed for estimation of cell proliferation. Harvested MSG cells were suspended and placed into the wells containing DMEM plus 1% FBS in the presence of AngII (100 nM) with or without ATR antagonists. Media were changed every 12 h. After 72 h, the number of cells was counted with a microscope at a magnification of $200\times$.

AT1R and AT2R expression

MSG cells were grown to confluence in 24-multiwell culture plates. After 2 days, cells were incubated with DMEM containing 1% FBS and various concentrations of glucose. Media were changed every 3 h. After 9 h, the cells were lysed or used in the 125 I-AngII binding assay (as follows).

Immunoblotting

Immunoblotting was performed as previously described (Yamauchi *et al.* 2004). Whole-cell extracts were prepared by detergent solubilization of the cells in lysis buffer (20 mM Hepes (pH 7.4), 1% Triton X-100, 2 mM EDTA, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium

orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μ M leupeptin, 10 μ g/ml aprotinin, and 1.5 μ M pepstatin) for 1 h at 4 °C. For western blotting, 200 μ g protein of lysate was applied to each lane. Cell extracts were subjected to SDS-PAGE and western blotting using 1:1000 diluted aAT1R, aAT2R, or aTub primary antibodies and 1:20 000 diluted horseradish peroxidase-conjugated goat anti-rabbit IgG, rabbit anti-goat IgG, or goat anti-mouse IgG secondary antibody respectively and visualized with the Enhanced Chemical Luminescence detection system (Amersham Pharmacia Biotech).

AngII binding assay

Cells were incubated for 1 h at 22 °C in 500 μ l of a medium containing 50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 0.5 mM EDTA, 1 mM CaCl_2 , 0.1% BSA, and 0.6 nM 125 I-Ang II (Amersham Pharmacia Biotech) with or without AT1R selective antagonist, RNH-6270, and/or AT2R selective antagonist, PD123,319. Incubations were terminated by immersion of the whole-culture plates into three successive baths containing ice-cold PBS. Cells were then solubilized in 500 μ l NaOH (0.1 M) and the associated radioactivity was determined by spectrometry. Nonspecific binding was determined by the amount of 125 I-Ang II bound in the presence of unlabeled 50 nM AngII (Sahajpal & Ashton 2005).

Statistical analysis

Statistical analysis was carried out using ANOVA with Fisher's protected least-significant difference (StatView, SAS Institute Inc., Cary, NC, USA). A $P < 0.05$ value was considered statistically significant.

Results

Effect of AngII on migration of mesangial cells

AngII-dependent migration was observed in mesangial cells during cell culture. Levels of cell migration were low in the presence of 100 nM AngII (Fig. 1). As both AT1R and AT2R are known to deliver counteracting signals for each other (Dinh *et al.* 2001), we examined the effect of ATR subtype-specific modification. In the presence of a selective AT1R antagonist, RNH-6270 AngII markedly increased migration of MSG cells (Fig. 2). Additionally, CGP-42112A, a selective AT2R agonist, promoted cell migration more strongly than AngII alone in a dose-dependent manner (Fig. 3). On the other hand, AngII in the presence of PD123,319, a selective AT2R antagonist, did not cause cell migration (Fig. 4). These results suggest that AngII induces cell migration via AT2R but not via AT1R in MSG cells.

Figure 1 Dose-dependent effects of AngII on MSG cell migration. MSG cells were incubated with various concentrations of AngII for 5 h in a two-chamber system. Cells migrated slightly but significantly in a dose-dependent manner. Values are means $\pm$ S.E.M. from three independent experiments. Each experiment was performed in duplicate. NS, not significant; * P < 0.05 versus basal migration.

Figure 3 Dose-dependent effects of AngII and a selective AT2R agonist (CGP-42112A) on cell migration. MSG cells were incubated with various concentrations of CGP-42112A for 5 h. Cells significantly migrated in a dose-dependent manner. Values are means $\pm$ S.E.M. from three independent experiments. Each experiment was performed in duplicate. NS, not significant versus basal migration; * P < 0.05 versus basal migration; † P < 0.01 versus basal migration.

Effect of guanylate cyclase inhibitors on AT2R-mediated cell migration

Signal transduction of AT2R is at least partly dependent on cyclic guanine monophosphate (cGMP; Horiuchi 1996). We therefore tried to clarify the efficacy of methylene blue and 6-anilino-5,8-quinolinequinone, two potent inhibitors of

Figure 2 Effect of a selective AT1R antagonist (RNH-6270) on AngII-induced cell migration. Cells were incubated with or without 100 nM AngII for 5 h, in the presence or absence of RNH-6270. Presence of both the AngII and the AT1R agonist strongly stimulated cell migration. Values are means $\pm$ S.E.M. from three independent experiments. Each experiment was performed in triplicate. NS, not significant; * P < 0.05; † P < 0.01.

Figure 4 Effect of a selective AT2R antagonist (PD123,319) on AngII-induced cell migration. Cells were incubated with AngII or CGP-42112A in the presence or absence of PD123,319 for 5 h. The presence of an A2TR antagonist suppressed both AngII- and CGP-42112A-induced MSG cell migration. Values are means $\pm$ S.E.M. from three independent experiments. Each experiment was performed in triplicate. NS, not significant; * P < 0.05; † P < 0.01.

guanylate cyclase, on the migration via AT2R in mesangial cells. These reagents inhibited cell migration (Fig. 5), thereby indicating that mesangial cell migration is mediated by AT2R and is dependent on cyclic GMP production.

Effect of ATRs' activation on cell proliferation

AngII promotes not only cell migration but also cellular proliferation. We evaluated MSG cell proliferation by counting the number of cells. In the presence of 100 nM AngII, treatment of PD123,319 but not RNH-6270 increased cell number (Fig. 6). These data suggest that AngII induces cellular proliferation through AT1R.

Effect of glucose concentration on ATR expression

We observed the effect of glucose concentration on expression of ATRs in MSG cells. It was found that high concentrations of glucose stimulated the expression of AT2R. On the other hand, expression of AT1R was not influenced by glucose. As a repetitive glucose spike is considered to be an important factor in endothelial cell dysfunction (Ceriello 2004, Azuma *et al.* 2006), we explored the effect of glucose fluctuation on ATR expression in MSG cells, and found that continuous exposure to high glucose more effectively induced AT2R than glucose fluctuation (Fig. 7A).

Figure 5 Effect of guanylate cyclase inhibitors on AT2R-mediated cell migration. Cells were incubated with or without 100 nM CGP-42112A for 5 h, in the presence or absence of 100 µM methylene blue or 6-anilino-5,8-quinolinequinone. Both guanylate cyclase inhibitors suppressed AT2R agonist-induced cell migration. Values are means $\pm$ S.E.M. from three independent experiments. Each experiment was performed in triplicate. NS, not significant; * $P < 0.05$; † $P < 0.01$.

Figure 6 Effect of ATR subtype-specific stimulation on proliferation of MSG cells. Cells were incubated with 100 nM AngII in the presence or absence of ATR subtype-specific antagonists (RNH-6270 or CGP-42112A) for 5 h. Values are means $\pm$ S.E.M. from three independent experiments. Each experiment was performed in triplicate. * $P < 0.01$.

In order to confirm glucose-induced ATR expression, we observed the effect of glucose concentration on displacement of 125 I-AngII binding by ATR subtype-specific antagonists in MSG cells (Fig. 7B) and found that the binding assay demonstrated glucose-dependent alteration of binding capacities of ATRs. 125 I-AngII displacement by an AT2R antagonist in 25 mM glucose medium was significantly increased as compared with that in 5 mM glucose medium. Displacement by AT1R was about fourfold larger than that of AT2R, but displacement levels by AT1R did not change in different glucose concentrations. These data suggested that 25 mM glucose increased AT2R binding capacity of MSG cells but not AT1R. As binding capacity of ATR reflected ATR number (Sahajpal & Ashton 2005), these data are compatible with the immunoblotting data.

Discussion

The RAS is a humoral cascade that plays an integral role in cardiovascular homeostasis by influencing vascular tone, fluid and electrolyte balance, and the sympathetic nervous system. Additionally, the RAS has been implicated in the development of various cardiovascular diseases, such as hypertension, congestive heart failure, coronary ischemia, and renal insufficiency (Dzau 1994). Renal AngII is involved in regulating sodium and water reabsorption from the proximal tubules and inhibition of renin secretion from the macula

Figure 7 Effect of glucose on ATR subtype-specific expression in MSG cells. (A) Cells were incubated with indicated concentrations of glucose for 9 h. 25–2–25 mM glucose indicates that cells were initially incubated in medium with high glucose (25 mM) for 3 h, followed by a rapid reduction of glucose to 2 mM for the next 3 h; media was then changed to a concentration of 25 mM of glucose. 2–25–2 mM glucose indicates that concentrations were changed at 3 h intervals, but concentrations were initially 2, 25, and then 2 mM glucose medium. Whole-cell lysates were prepared as described in text and immunoblotted with an antibody directed against AT1R or AT2R. A typical immunoblot for the AT1R and AT2R in MSG cells is shown. The densitometry data are presented as relative density compared with density at 5.5 mM glucose concentration. Values are means $\pm$ S.E.M. from three independent experiments. * P < 0.05 versus 5.5 mM glucose condition; † P < 0.01 versus 5.5 mM glucose condition. (B) Cells were incubated in medium with normal concentration of glucose (5.5 mM) or high glucose (25 mM) for 9 h. Cells were then washed thrice with PBS. 125 I-AngII binding assay was performed as described in text. Each bar indicates count of 125 I-AngII displacement by co-incubation with RNH-6270 and/or PD123,319. Values are means $\pm$ S.E.M. from three independent experiments. Each experiment was performed in triplicate. * P < 0.05; † P < 0.01.

densa cells (Bell & Peti-Peterdi 1999). Virtually all of the known biological actions of AngII, including vasoconstriction, release of aldosterone, stimulation of sympathetic transmission, and cellular growth, are exclusively mediated by the AT1R (Timmermans *et al.* 1992, 1993). In contrast,

the functional role of AT2R is not fully understood. However, recent studies have ascribed a possible role for AT2R in the mediation of anti-proliferation, apoptosis, differentiation, and possibly vasodilatation (Horiuchi 1996, Csikos *et al.* 1998).

It has previously been reported that both AT1R and AT2R are expressed in mouse cultured mesangial cells (Lorenzo *et al.* 2002). We observed that MSG cells weakly migrated in the presence of AngII (100 nM). Considering this result, we anticipated that AngII might regulate migration of mesangial cells by receptor subtype-specific effects. AngII markedly increased migration of MSG cells in the presence of a selective AT1R antagonist (RNH-6270) in a concentration-dependent manner. Additionally, we demonstrated that a selective AT2R agonist also strongly induced migration of MSG cells. In contrast, AT2R antagonist, PD123,319, suppressed AngII-induced cell migration. These results suggest that AngII induces migration of MSG cells via AT2R but not via AT1R. Recently, various second messengers coupled to the AT2R have been described, including indirect negative coupling to guanylate cyclase (Bottari *et al.* 1992) and activation of potassium channels (Kang *et al.* 1993, 1994).

We then analyzed the signal transduction of AngII through AT2R by observation of the effect of methylene blue and 6-anilino-5,8-quinolinequinone, and the other guanylate cyclase inhibitors on mesangial cell migration. Both methylene blue and 6-anilino-5,8-quinolinequinone inhibited AngII-induced migration of MSG cells via AT2R, suggesting that guanylate cyclase participates in signal transduction of AngII through AT2R, thus promoting cell migration.

In the kidney, it has been reported that an excess of AngII induces proliferation of mesangial cells and ECM, both of which are important factors in the induction and progression of renal injury (Wolf & Neilson 1993). In fact, we confirmed that AngII causes cellular proliferation via AT1R. Recently, it has been reported that angiotensin converting enzyme inhibitors (ACEIs) and angiotensin II receptor blockers have renoprotective effects on the progression of several renal diseases, including diabetic nephropathy (Brenner *et al.* 2001, Lewis & Lewis 2003, Rossing *et al.* 2003, Izuhara *et al.* 2005, Burnier & Zanchi 2006). One possible explanation of these renoprotective effects is that these agents suppress proliferation of mesangial cells and inhibit production of mesangial matrix by blocking the effect of AngII through AT1R.

Importantly, the expression of AT2R is upregulated in pathological conditions, such as heart failure, renal failure, myocardial infarction, brain lesions, vascular injury, and wound healing (Kimura *et al.* 1992, Viswanathan *et al.* 1994, Nakajima *et al.* 1995, Nio *et al.* 1995, Ohkubo *et al.* 1997, Chung & Unger 1998). We examined the effect of high glucose concentration on the expression of ATRs in MSG cells, because it is well known that a continuous hyperglycemic state contributes to the development of glomerular injury that is prevalent in diabetic nephropathy. Our results demonstrate that continuous high glucose concentration induces the expression of AT2R but not AT1R.

Cell migration and cell proliferation are fundamental responses of mesangial cells to glomerular injury and contribute to the hypercellularity observed in a number of glomerular diseases. On the other hand, cell migration plays

an important role in repairing or remodeling damaged tissues. These findings suggest that the activation of AT2R contributes to repair or remodeling of injured kidney by induction of mesangial cell migration and suppression of MSG proliferation. Our data also suggest that expression of AT2R, induced by high glucose concentration, is a type of biological adaptation that promotes repair of injured glomerular tissue by enhancement of MSG cell migration.

In conclusion, we demonstrated that AngII induces mesangial cell migration through AT2R activation, and also that the nitric oxide (NO)-cGMP pathway might be involved in AT2R-mediated cell migration. Our data also showed that hyperglycemia induces AT2R expression in MSG cells. In injured glomerular tissue, the activation of AT2R in MSG cells may contribute to tissue repair by induction of migration and suppression of proliferation.

Acknowledgements

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received in final form 11 July 2006

Accepted 26 July 2006

Made available online as an Accepted Preprint

22 August 2006