Angiotensin II type 2 receptors mediate inhibition of mitogen-activated protein kinase cascade and functional activation of SHP-1 tyrosine phosphatase

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Angiotensin II type 2 (AT_2) receptors are involved in the inhibition of cell proliferation as well as in apoptosis and neuronal differentiation, through intracellular signalling pathways that remain poorly defined. The present study examines the effect of AT_2 -receptor stimulation on growth-factor-induced pathways leading to the activation of mitogen-activated protein (MAP) kinases. In N1E-115 neuroblastoma cells, AT_2 receptors inhibit the activity of MAP kinases induced by serum as well as by epidermal growth factor. The inhibitory effect of angiotensin II (Ang II) is rapid and transient, and affects both ERK1 and ERK2 (extracellular signal-related protein kinase) isoforms of the enzyme. AT_2 -mediated MAP kinase inactivation is not sensitive to pertussis toxin or okadaic acid, but involves a vanadate-sensitive protein tyrosine phosphatase (PTP).

Expression of MAP kinase phosphatase-1 (MKP-1) is not significantly modified upon AT_2 -receptor activation, and insensitivity to actinomycin D also rules out transcriptional induction of other MKPs as a possible mechanism for AT_2 -mediated inactivation of MAP kinases. In addition, we report here that both in N1E-115 cells and in Chinese hamster ovary cells expressing recombinant human AT_2 receptors, Ang II rapidly stimulates the catalytic activity of SHP-1, a soluble PTP that has been implicated in termination of signalling by cytokine and growth-factor receptors. These findings thus demonstrate functional negative cross-talk between heptahelical AT_2 receptors and receptor tyrosine kinases, and suggest that SHP-1 tyrosine phosphatase is an early transducer of the AT_2 receptor signalling pathway.

INTRODUCTION

In addition to being a major regulator of blood pressure, the octapeptide angiotensin II (Ang II) is a modulator of cellular growth. Interestingly, Ang II exerts positive or negative effects on cell growth depending on which subtype [Ang II type 1 (AT_1) receptor or Ang II type 2 (AT_2) receptor] of the seven-transmembrane-domain receptor this peptide binds to. Mitogenic and hypertrophic effects mediated by the AT_1 subtype are illustrated by the wide variety of phosphorylation cascades initiated by this receptor. Thus AT_1 receptor activation leads to phosphorylation of the platelet-derived growth factor receptor [1], and to stimulation of Ras-Raf-mitogen-activated protein (MAP) kinase [2], p70 S6 protein kinase [3] and Janus kinase (JAK) signalling pathways [4,5], usually associated with cytokine and growth factor receptors.

The AT_2 receptor subtype is much less well characterized, and its physiological role is not completely understood [6]. The emerging picture suggests that the AT_2 subtype may antagonize the *in vivo* effects of AT_1 on blood pressure [7,8] and renal natriuresis [9] and may participate in the regulation of vascular cell growth [10–12]. At the cellular level, AT_2 receptors mediate inhibition of cell proliferation in rat coronary endothelial cells [13] and in NIH3T3 fibroblasts stably expressing the rat AT_2 receptor cDNA [14]. AT_2 receptors have also recently been shown to induce apoptosis in rat pheochromocytoma PC12W cells [15] and neurite outgrowth in NG108-15 cells [16].

Little is known about intracellular AT_2 signalling in relation to cell growth and differentiation [6], although increasing evidence suggests the coupling of this receptor to protein phosphatases

[17–20]. In rat brain neuronal cultures, AT_2 receptors induce the opening of potassium channels and activation of the serine/threonine phosphatase called protein phosphatase 2A (PP2A) via coupling to a pertussis toxin (PTX)-sensitive G_i -protein [20]. In cultured cell lines such as PC12W [17,18] and NG108-15 [19], AT_2 receptors have been reported to activate a yet-unidentified protein tyrosine phosphatase (PTP) through coupling to PTX-insensitive G-proteins. We have recently shown that in neuroblastoma N1E-115 cells, AT_2 receptors induce decreased phosphorylation of several endogenous proteins on tyrosine residues [21]. To gain further insight into the mechanisms by which AT_2 receptors mediate cell-growth inhibition, we have investigated whether these heptahelical receptors could interfere with intracellular cascades induced by growth-factor-receptor tyrosine kinases.

In this study, we have focused our attention on the effect of AT_2 receptors on the activity of MAP kinases, known to play a key role in cell proliferation. We show that in N1E-115 neuroblastoma cells, AT_2 receptors rapidly block the activation of both ERK1 and ERK2 (extracellular signal-related protein kinase) isoforms of MAP kinases induced by serum as well as by epidermal growth factor (EGF). AT_2 -mediated inactivation of MAP kinases is insensitive to PTP and to okadaic acid and involves a vanadate-sensitive PTP. We provide evidence that this AT_2 signalling pathway does not involve transcriptional induction of genes corresponding to MAP kinase phosphatase-1 (MKP-1) or to other phosphatases of the MKP family. In addition, we identify here for the first time the PTP SHP-1, previously identified as a negative regulator of cell growth, as being directly activated upon AT_2 -receptor stimulation.

Abbreviations used: Ang II, angiotensin II; AT_1 receptor, Ang II type 1 receptor; CHO, Chinese hamster ovary; EGF, epidermal growth factor; ERK, extracellular signal-related protein kinase; FCS, fetal-calf serum, heat-inactivated; MAP, mitogen-activated protein; MBP, myelin basic protein; MKP, MAP kinase phosphatase; PP2A, protein phosphatase 2A; PTP, protein tyrosine phosphatase; PTX, pertussis toxin.

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EXPERIMENTAL

Reagents

Heat-inactivated fetal-calf serum (FCS) was purchased from Gibco (BRL) and was heat-inactivated at 56 °C for 40 min. The AT₂-selective agonist CGP 42112 was from Neosystem. Mouse EGF was from Collaborative Biomedical Products (TEBU). All other chemicals were purchased from Sigma unless otherwise specified.

Cell culture and transfections

N1E-115 neuroblastoma cells were cultured as previously described [21] and used at passages 39–50. N1E-115 cells were seeded at a density of 500 000 cells per 10 cm plate and allowed to grow for 24 h before they were growth-arrested by total serum deprivation for 18 h.

For transfection, N1E-115 cells (200 000 cells per 60 mm plate) were incubated with 5 µg of SHP-1 cDNA (kindly provided by Dr. M. Thomas, HHMI, St. Louis, MO, U.S.A.) in DOSPER Liposomal Transfection reagent (Boehringer Mannheim) for 6 h and allowed to recover for 24 h before they were serum-starved for 18 h.

Chinese hamster ovary (CHO) cells, deficient in dihydrofolate reductase, were transfected with a plasmid containing the entire coding region (2.5 kb *EcoRI*–*XbaI* fragment) of the human AT₂ receptor gene [22] and the dihydrofolate reductase gene as a selectable marker [23]. One stable transfectant expressing the dihydrofolate reductase gene and showing AT₂-receptor expression (designated here as 'CHO-hAT₂') was subcloned by limiting dilution in Ham's F12 medium (Gibco, BRL) supplemented with 10% (v/v) dialysed FCS and used at passages 10–20.

MAP kinase assay from cell lysates

Quiescent cells were treated at 37 °C as indicated, then they were rapidly rinsed, scraped in lysis buffer, sonicated and centrifuged as described elsewhere [24]. MAP kinase activity was assayed from supernatant fractions (4–8 µg of protein) using 1 µmol of synthetic peptide substrate (Ala-Pro-Arg-Thr-Pro-Gly-Gly-Arg-Arg), modelled after Thr-97 of myelin basic protein (MBP) (Thr corresponds to Thr-97) [25]. This peptide was synthesized and HPLC-purified by Dr. Troalen (IGR, Villejuif, France) or purchased from UBI (Lake Placid, NY, U.S.A.). Reactions were stopped by precipitation with 8% (w/v) trichloroacetic acid and supernatant fractions were spotted on Whatman P81 chromatography paper. After three washes in 175 mM phosphoric acid, radioactivity incorporated into substrate was measured by β -scintillation counting. The average radioactivity of two blank samples (500–1100 c.p.m.) containing no peptide substrate was subtracted from the result of each sample. Data are from two to four independent experiments performed in triplicate. MAP kinase activity in each cell lysate was assayed in duplicate.

Immunocomplex assay of ERK1 activity

Quiescent N1E-115 cells in 10 cm dishes were treated as indicated and the activity of ERK1 was analysed by immunocomplex kinase assay [26] after immunoprecipitation overnight at 4 °C with 5 µl of rabbit anti-ERK1 antiserum (kindly given by Dr. S. Meloche, University of Montreal, Canada). MBP kinase activity was assayed for 10 min at 30 °C and stopped by addition of 20% (v/v) formic acid. Supernatant fractions were spotted on to Whatman P81 paper and after three washes in 175 mM phosphoric acid, radioactivity incorporated into MBP was measured by scintillation counting.

Immunoblot analyses

Proteins (20–30 µg) from N1E-115 cell lysates were loaded on to SDS/10% (w/v)-polyacrylamide gels containing 0.4% (w/v) bisacrylamide, blotted to nitrocellulose membranes and incubated with monoclonal anti-ERK2 antibodies (UBI) or monoclonal anti-(SHP-1) antibodies (Transduction Laboratories, Lexington, KY, U.S.A.), overnight at 4 °C. Incubation with rabbit anti-mouse antibodies coupled to peroxidase and detection with enhanced chemiluminescence (Amersham) was performed as described [21].

Northern blot analysis

Poly(A)⁺ RNA (7 µg), extracted from N1E-115 cells by the SDS/Proteinase K method, were size-fractionated by electrophoresis on 1.2% (w/v) agarose-formaldehyde gel, transferred to Hybond N-fp membranes (Amersham) and hybridized [27] with 2 × 10⁶ c.p.m./ml of a random-primed ³²P-labelled (8 × 10⁸ c.p.m./µg) MKP-1 probe (full-length rat 3CH-134 cDNA, kindly provided by Dr. N. Tonks, Cold Spring Harbor, NY, U.S.A.) [28]. Final washes were in 1 × SSC (0.15 M NaCl/0.015 M sodium citrate)/0.1% (w/v) SDS at 45 °C. The same membranes were rehybridized with 0.2 × 10⁶ c.p.m./ml of a ³²P-labelled rat glyceraldehyde-3-phosphate dehydrogenase probe as described [22].

Immunoprecipitation and determination of SHP-1 protein phosphatase activity

N1E-115 cells (600 000 per 10 cm dishes), transfected N1E-115 cells (200 000 cells per 60 mm dishes) or CHO-hAT₂ cells (1 × 10⁶ cells per 10 cm dishes) were allowed to grow for 24 h before they were serum-starved for 18 h and treated with Ang II. After treatment, cells were rinsed with ice-cold PBS, then solubilized for 30 min at 4 °C in 50 mM Tris/HCl, pH 7.5, buffer containing 140 mM NaCl, 5 mM MgCl₂, 2.5% (w/v) CHAPS, 0.05% (w/v) soya bean trypsin inhibitor and 0.5 mg/ml cholesteryl hemisuccinate and centrifuged at 250 000 g for 10 min. The resulting supernatant (1–1.5 mg of proteins) was incubated for 2 h at 4 °C with 10 µl of polyclonal anti-(SHP-1) antibodies pre-bound to Sepharose-Protein A (Sigma). Immunocomplexes were washed three times in 50 mM Tris/HCl, pH 7.5, buffer containing 0.2% CHAPS, 0.05% soya bean trypsin inhibitor, 0.06 mg/ml cholesteryl hemisuccinate, 0.2% (w/v) BSA and assayed for PTP activity. Preimmune serum was used as a negative control. Anti-(SHP-1) antibodies [29] were raised in rabbits against a synthetic peptide (Glu-Lys-Val-Lys-Lys-Gln-Arg-Ser-Ala-Asp-Lys-Glu-Lys-Ser-Lys-Gly-Ser) corresponding to residues 575–591 of the human SHP-1 sequence. The specificity of polyclonal anti-(SHP-1) antibodies was assessed by means of immunoblotting. The efficiency of these antibodies in immunoprecipitating SHP-1 in its active form (i.e. incubation for 2 h at 4 °C) was estimated to be 32%.

PTP activity was measured as the release of [³³P]orthophosphate from phosphorylated poly(Glu,Tyr) as previously described [29].

RESULTS

AT₂ receptors inhibit MAP kinase activity induced by growth factors

Regulation of MAP kinase activity by AT₂ receptors was analysed in murine neuroblastoma N1E-115 cells that express significant

Figure 1 AT₂-receptor-mediated inhibition of FCS-induced MAP kinase activity

(A) Time-course of FCS-induced MAP kinase activation. Growth-arrested pre-confluent N1E-115 cells were incubated for the indicated periods of time with 10% FCS (●) or 1 nM Ang II (□). MAP kinase activity was measured from total cell lysates using a MBP peptide, as described in the Experimental section. Shown is one out of three independent experiments performed in triplicate. Results are presented as the percentage of control (i.e. basal levels of MAP kinase activity in untreated cells: 400 c.p.m.). (B) Time course of Ang II-stimulated inhibition of FCS-induced MAP kinase activity. Growth-arrested N1E-115 cells were preincubated with Ang II (1 nM) for various periods of time and FCS (10%) was added to the cells for 5 min. MAP kinase activity was measured from total cell lysates as in (A). Shown is one out of three independent experiments performed in triplicate. Results are presented as the percentage of maximal stimulation by FCS (i.e. levels of MAP kinase activity in cells treated with 10% FCS alone for 5 min). (C) MAP kinase inactivation by the AT₂-selective agonist CGP 42112. Quiescent N1E-115 cells were incubated with 0.1% BSA (–), Ang II (1 nM) or CGP 42112 (1 nM) for 10 min before 10% FCS was added to the cells for 5 min. MAP kinase activity was assayed from total cell lysates as in (A). Results are means $\pm$ S.E.M. of three independent experiments performed in triplicate. Data are expressed as the percentage of maximal stimulation by 10% FCS alone. *Different from control at $P < 0.05$ (Student's t test). (D) AT₂-mediated inactivation of ERK2. N1E-115 cells were either left untreated (–) or treated for 5 min with 10% FCS alone, or after preincubation with Ang II (1 nM) under the conditions described in (C). Total cell lysate proteins (20 μ g) were submitted to immunoblotting with anti-ERK2 monoclonal antibody. The presence of the activated, slower migrating form of ERK2 is indicated by an asterisk; both forms are indicated by arrows.

levels of the AT₂ receptor subtype but no AT₁ binding site [21]. Addition of 10% FCS to quiescent N1E-115 cells (Figure 1A) induced a rapid and transient peak of MAP kinase activity, which was maximal at 5 min and returned to basal levels by 15 min. In contrast, addition of Ang II to quiescent cells for 5–60 min had little effect on the basal activity of MAP kinases.

The effect of Ang II treatment on MAP kinase activity was subsequently analysed under conditions of maximal stimulation, i.e. with 10% FCS for 5 min. N1E-115 cells were preincubated with Ang II for various periods of time and FCS was added to the cells for the last 5 min. As shown in Figure 1(B), Ang II (1 nM) rapidly and transiently prevented activation of MAP kinases induced by FCS. Maximal inhibition ($52 \pm 6\%$, $n = 10$) was achieved when cells were preincubated with Ang II for 10 min. The effect of Ang II was observed in a concentration range of 0.1 nM to 1 μ M (results not shown). FCS-induced MAP kinase activity was also inhibited ($50 \pm 15\%$, $n = 3$) following 10 min pretreatment with the AT₂-selective agonist CGP 42112 (Figure 1C).

We then investigated whether both ERK1 (p44) and ERK2 (p42) isoforms of MAP kinases were inactivated following AT₂-receptor stimulation. ERK1 activity, measured by MBP kinase

Table 1 Effect of specific phosphatase inhibitors on AT₂-mediated inactivation of MAP kinases

Cells were pretreated with 10 nM okadaic acid for 1 h or with 0.1 mM sodium orthovanadate for 16 h, before adding 10% FCS for 5 min with or without Ang II (1 nM), under the conditions defined in Figure 1(C). Results are means $\pm$ S.E.M. of two independent experiments performed in triplicate and are expressed as the percentage of MAP kinase activity induced by 10% FCS for 5 min in the absence of an inhibitor.

	MAP kinase activity (% FCS stimulation)			
	Control		+ Inhibitor	
	–	Ang II	–	Ang II
Okadaic acid (10 nM)	100	55 $\pm$ 16	156 $\pm$ 12	106 $\pm$ 11
Orthovanadate (0.1 mM)	100	52 $\pm$ 4	148 $\pm$ 2	140 $\pm$ 6

assays on specific immunocomplexes, was indeed decreased by $51 \pm 8\%$ ($n = 2$) following treatment with Ang II (results not shown). Inactivation of ERK2 was visualized by immunoblotting with specific antibodies. As seen in Figure 1(D), Ang II could prevent the FCS-induced decrease in electrophoretic mobility of ERK2, known to reflect phosphorylation and activation of this enzyme.

To investigate possible coupling of AT₂ receptors to G_i/G_o regulatory proteins, N1E-115 cells were pretreated for 16 h with 100 ng/ml PTX. MAP kinase activation induced by FCS was significantly decreased in the presence of PTX ($55 \pm 6\%$ of control, $n = 2$), illustrating the complexity of serum-containing mitogenic agents that lead to activation of MAP kinases by several (PTX-sensitive and -insensitive) pathways. Under conditions of G_i/G_o blockade with PTX, Ang II was still able to inhibit ($75 \pm 3\%$ inhibition, $n = 2$) residual MAP kinase activity. Thus AT₂-mediated MAP kinase inactivation in N1E-115 cells mainly occurs through a PTX-insensitive pathway that does not involve coupling to G_i/G_o heterotrimeric G-proteins.

We then examined the consequences of AT₂-receptor stimulation on MAP kinase activity induced by a specific ligand such as EGF, acting through a defined receptor tyrosine kinase. MAP kinase activation induced by EGF (100 ng/ml) within 5 min, albeit lower ($68 \pm 3\%$, $n = 3$) than that induced by 10% FCS, was significantly inhibited ($54 \pm 3\%$ inhibition, $n = 2$) following AT₂-receptor stimulation. Thus AT₂ receptors also interfere with the MAP kinase activation cascade elicited by EGF.

AT₂ pathway involves a vanadate-sensitive PTP different from MKP-1

To address the role of protein phosphatases in AT₂-mediated inactivation of MAP kinases, we analysed the effects of specific inhibitors of serine/threonine or tyrosine phosphatases on this signalling pathway (Table 1). In cells treated with okadaic acid or sodium orthovanadate, FCS-induced MAP kinase activation was increased to $156 \pm 12\%$ ($n = 2$) and $148 \pm 2\%$ ($n = 2$) of control respectively, an effect that may be attributed to inhibition of phosphatases. In the presence of okadaic acid, at a dose (10 nM) that selectively blocks PP2A, Ang II was still able to inhibit FCS-induced MAP kinase activity ($45 \pm 3\%$ inhibition, $n = 2$), as compared with $45 \pm 16\%$ inhibition ($n = 2$) in control cells. This ruled out major participation of serine/threonine phosphatase PP2A in this AT₂ response, although these phosphatases have been reported to inactivate MAP kinases in other cell types [30]. In contrast, sodium orthovanadate (0.1 mM) completely abolished the inhibitory effect of AT₂ receptors on

Figure 2 AT_2 receptors do not induce expression of MKPs

(A) Northern blot analysis of MKP-1 transcripts. Poly(A)⁺ RNA (7 μ g) from N1E-115 cells untreated (0) or treated with Ang II (100 nM) or FCS (10%) for the indicated period of time (15' = 15 min, etc.) was submitted to Northern blot analysis and hybridized to specific MKP-1 or glyceraldehyde-3-phosphate dehydrogenase cDNA probes as described in the Experimental section. Membranes were exposed for 3.5 h at -70°C with two intensifying screens. (B) Effect of actinomycin D on AT_2 -mediated inactivation of MAP kinases. N1E-115 cells were treated for 30 min with 3 μ g/ml actinomycin D, before incubation with 10% FCS with or without Ang II (1 nM) as defined in Figure 1(C). Results are means $\pm$ S.E.M. of three experiments performed in triplicate and are expressed as the percentage of maximal FCS stimulation in the absence of actinomycin D. *Different from control at $P < 0.02$ (Student's *t* test).

MAP kinase activity ($5 \pm 3\%$ inhibition, $n = 2$, compared with $48 \pm 4\%$ inhibition, $n = 2$, in control cells; Table 1), therefore implicating a vanadate-sensitive PTP in the AT_2 pathway leading to MAP kinase inhibition.

Possible candidates for AT_2 -mediated MAP kinase inactivation may be found among members of the rapidly growing family of vanadate-sensitive MKPs [28,31–33], which are regulated at the transcriptional level and dephosphorylate MAP kinases at both threonine and tyrosine residues. MKP-1, the leader member of this family, is the product of an immediate early gene (3CH-134) strongly inducible within 20 min in response to growth factors [34]. We therefore investigated whether elevated expression of MKP-1 may be rapidly induced in response to Ang II and thus account for inactivation of MAP kinases by AT_2 receptors. Expression of MKP-1 phosphatase in N1E-115 cells was, however, too low to be detected by immunoblotting, even after treatment with Ang II or FCS (results not shown). Northern blot analyses performed on poly(A⁺) RNA confirmed the extremely low levels of MKP-1 expression in N1E-115 cells. As shown in Figure 2(A), MKP-1 mRNA levels were not significantly modified following treatment with Ang II, whereas FCS, added as a control, induced a 2.5-fold increase in MKP-1 mRNA levels after 1 h of treatment.

To investigate possible involvement of other transcriptionally regulated phosphatases in the AT_2 pathway, N1E-115 cells were pretreated with actinomycin D, a potent inhibitor of transcription. In the presence of 3 μ g/ml actinomycin D, Ang II was still able to inhibit by $57 \pm 11\%$ ($n = 3$) FCS-induced MAP kinase activity (Figure 2B), whereas induction of MKP-1 mRNA by FCS was completely abolished under these conditions (results not shown). Thus impairing transcription does not prevent AT_2 -mediated MAP kinase inactivation, indicating that the effect of

Figure 3 Ang II induces SHP-1 phosphatase activity

(A) Immunodetection of SHP-1. Cell lysates (30 μ g of protein) or immunocomplexes using anti-(SHP-1) polyclonal antibodies (Ab) were prepared from N1E-115 cells, N1E-115 cells overexpressing recombinant SHP-1 [N1E-115 (SHP-1)] or CHO-hAT₂ cells, and separated on SDS/10%-PAGE before immunoblotting (IP) with monoclonal anti-(SHP-1) antibody. Proteins (10 μ g) from Jurkat cell lysates were used as the positive control. Positions of molecular-mass standards (kDa) are shown on the left and of SHP-1 and IgG on the right. TL, Transduction Laboratories. (B) N1E-115, N1E-115 (SHP-1) and CHO-hAT₂ cells were incubated with 10 nM Ang II for various periods of time, before immunoprecipitation with polyclonal anti-(SHP-1) antibodies and subsequent measurement of ³²P-labelled poly(Glu,Tyr) dephosphorylation as described in the Experimental section. Results are means $\pm$ S.E.M. of three independent experiments performed in duplicate. Phosphatase activity was determined as released ³²P_{0.3}[−] in pmol $\cdot$ min^{−1} $\cdot$ mg^{−1} of protein. Basal activities were 0.57 ± 0.07 pmol $\cdot$ min^{−1} $\cdot$ mg^{−1} of protein, 0.95 ± 0.11 pmol $\cdot$ min^{−1} $\cdot$ mg^{−1} of protein and 1.02 ± 0.22 pmol $\cdot$ min^{−1} $\cdot$ mg^{−1} of protein in N1E-115 cells, N1E-115 (SHP-1) cells and CHO-hAT₂ cells respectively. ***Different from control at $P < 0.001$; ** $P < 0.01$ (Student's *t* test).

AT_2 receptors is not due to increased expression of MKPs, but rather involves activation of vanadate-sensitive PTP(s) through a direct pathway that does not require transcription.

AT_2 receptors activate SHP-1 PTP

The rapid and transient time course of Ang II-induced MAP kinase inactivation suggests that AT_2 receptors may recruit a vanadate-sensitive PTP already expressed within the cell. SHP-1 is a soluble PTP containing two N-terminal Src homology 2 (SH2) domains that allow binding to phosphotyrosines and participation in the regulation of receptor tyrosine kinase pathways. SHP-1 (formerly designated as HCP, SH-PTP1 or PTP1C [35]) was originally discovered as a major regulator of haematopoiesis in moth-eaten mice [36], and has recently been implicated in negative regulation of fibroblast cell growth [37] and in neuronal differentiation [38], leading us to investigate whether AT_2 receptors may activate SHP-1 tyrosine phosphatase in N1E-115 cells.

Immunoblotting and immunoprecipitation experiments were undertaken to examine expression of SHP-1 in the non-haematopoietic, N1E-115 neuroblastoma and CHO-hAT₂ epithelial-like cells. Whereas endogenous SHP-1 expressed in N1E-115 cells was barely detectable, a single band of apparent molecular mass 65 kDa was revealed by specific monoclonal anti-(SHP-1) antibodies in N1E-115 cells transfected with SHP-1 cDNA, as well as in CHO-hAT₂ cells (Figure 3A).

Regulation of SHP-1 catalytic activity by AT₂ receptors was analysed by immunoprecipitation and subsequent *in vitro* phosphatase assay. In N1E-115 cells, Ang II induced a rapid and transient stimulation of endogenous SHP-1 phosphatase activity, being maximal ($165 \pm 15\%$, $n = 3$) at 5 min and already decreasing at 10 min (Figure 3B). To further confirm that increased phosphatase activity could be attributed to SHP-1, phosphatase assays were also performed on N1E-115 cells overexpressing recombinant SHP-1 cDNA [N1E-115 (SHP-1)]. As expected, basal levels of SHP-1 phosphatase activity were higher in transfected (0.95 ± 0.11 pmol·min⁻¹·mg⁻¹ of protein) versus non-transfected (0.57 ± 0.07 pmol·min⁻¹·mg⁻¹ of protein) N1E-115 cells. In transfected cells, Ang II induced a similar time course of SHP-1 activation, with a maximum ($151 \pm 13\%$, $n = 3$) at 5 min (Figure 3B). Rapid induction of endogenous SHP-1 activity by Ang II was also observed in CHO-hAT₂ cells (maximal stimulation $162 \pm 8\%$, $n = 3$, at 3 min), providing evidence that SHP-1 can be directly activated upon stimulation of both murine and human AT₂ receptors in different cellular contexts.

DISCUSSION

The aim of the present study was to analyse the regulation of MAP kinase activity following AT₂ receptor activation and to investigate possible coupling of AT₂ receptors with PTPs in neuroblastoma N1E-115 cells. We show here that activation of ERK1 and ERK2 induced by growth factors such as FCS or EGF is prevented upon stimulation of AT₂ receptors (Figure 1). AT₂-mediated MAP kinase inactivation mainly occurs through a PTX-insensitive pathway that does not involve G_{i/o} regulatory proteins. The inhibitory effect of Ang II on MAP kinases is rapid and transient, and shows a maximum at 10 min (Figure 1B). These kinetics are similar to those previously determined for AT₂-mediated protein tyrosine dephosphorylation in N1E-115 cells [21].

Activation of MAP kinases requires phosphorylation of both Thr-183 and Tyr-185, and dephosphorylation of any of these residues leads to down-regulation of MAP kinase activity [39]. Protein phosphatases that dephosphorylate MAP kinases *in vivo* include the okadaic acid-sensitive serine/threonine phosphatase PP2A [30], the vanadate-sensitive dual-specificity (tyrosine/threonine) phosphatase MKP-1 [28], and a still-unidentified vanadate-sensitive tyrosine phosphatase that accounts for rapid inactivation of MAP kinases in several cell types [30]. In N1E-115 cells, inactivation of MAP kinases by AT₂ receptors is not sensitive to okadaic acid (Table 1) and therefore does not mainly involve PP2A. It is also unlikely that MKP-1 is a major phosphatase involved in the AT₂ pathway in N1E-115 cells, since expression of MKP-1 is not induced in response to Ang II (Figure 2A), although this phosphatase is known to be primarily regulated at the level of its synthesis [28]. In addition, actinomycin D does not affect the response to Ang II (Figure 2B), therefore ruling out transcriptional regulation of other MKPs as a possible mechanism for AT₂-mediated inactivation of MAP kinases in N1E-115 cells.

Two recent reports have described involvement of protein phosphatases in the AT₂ pathway leading to inactivation of MAP kinases in other cell types [15,40] but, interestingly, different conclusions have been drawn out from these studies. In PC12W cells, Yamada et al. [15] reported involvement of an okadaic acid-insensitive, vanadate-sensitive phosphatase in AT₂-mediated inhibition of MAP kinases and suggested, by antisense oligonucleotide strategy, that MKP-1 may play a role in this process. In contrast, Huang et al. [40] reported participation of PP2A in the AT₂ pathway leading to inhibition of MAP kinase activity in

neurons. Our present data in N1E-115 cells suggest yet another mechanism that does not involve MKP-1 or PP2A. These differences might be due to variation between individual cell types, and may reflect the complexity of networks involved in negative regulation of MAP kinases [30,39]. This raises the appealing hypothesis that AT₂ receptors may recruit various distinct intracellular pathways in different cellular contexts, to achieve the same final effect, e.g. inactivation of MAP kinases.

We show here that in both N1E-115 and CHO-hAT₂ cells, stimulation of AT₂ receptors leads to rapid activation of SHP-1 PTP (Figure 3). While there has been increasing evidence supporting a role for vanadate-sensitive PTPs in AT₂-mediated responses in several cell lines [17–19], these AT₂-associated PTPs have not yet been identified. Buisson et al. [19] have suggested that PTP activation in NG108-15 cells may be proximal to AT₂ receptor occupation, and it will now be of interest to investigate whether SHP-1 is also functionally coupled to AT₂-receptor activation in other cell types, including NG108-15 and PC12W cells. Our data do not, however, preclude that other cytoplasmic or membrane-bound PTPs may also participate in the AT₂ signalling pathway, either independently or together with SHP-1.

The rapid time course of SHP-1 activation (maximal at 5 min) precedes inhibition of EGF-stimulated MAP kinase activity (maximal at 10 min), suggesting that SHP-1 is an upstream component in the AT₂ intracellular cascade and a putative candidate for interrupting the growth-factor-induced MAP kinase pathway in N1E-115 cells. A link between AT₂-mediated activation of SHP-1 and inactivation of MAP kinases would need, however, to be firmly established. The lag time, albeit short, between maximal SHP-1 activation and MAP kinase inhibition argues against the hypothesis that MAP kinases may be direct cellular targets dephosphorylated by SHP-1, and would rather suggest that SHP-1 activation may directly or indirectly affect upstream intermediates of the growth-factor-induced MAP kinase pathway, possibly at the level of membrane tyrosine kinase receptors. Other groups have shown that SHP-1 physically interacts with the EGF receptor in human epithelial A431 cells [41] and with the NGF TrkA receptor in PC12 cells [38]. In addition, both dephosphorylation of the EGF receptor in A293 cells overexpressing SHP-1 [41], and inhibition of the EGF-induced MAP kinase cascade in 293 cells overexpressing mutant SHP-1 [42] have been reported. In haematopoietic cells, SHP-1 also forms complexes with a number of membrane receptors (c-Kit [43], the interleukin-3 receptor β -subunit [44], the erythropoietin receptor [45], the FcRIIB1 receptor [46], the interferon α receptor complex [47] and the T-cell receptor complex [48]), and appears to play a central role in termination of signalling by these receptors [49,50].

AT₂ receptors may possibly be representative of a novel functional subfamily of G-protein-coupled receptors, comprising dopamine [51] and somatostatin [52,53] receptors, which were also reported to activate membrane-associated PTPs and to inhibit cell proliferation. While PTPs associated with dopamine receptors are still not identified, recent data revealed physical interaction between somatostatin sst₂ receptors and SHP-1 in rat pancreatic acinar cells [54]. Translocation of SHP-1 from cytosol to membrane upon somatostatin-receptor stimulation in MCF-7 breast adenocarcinoma cells was also reported [55]. Dopamine and somatostatin receptors, however, function through coupling to PTX-sensitive G-proteins, and regulatory proteins associated with AT₂ receptors in N1E-115 cells still remain to be identified.

Switching off growth-factor-activation cascades is crucial to ensure control of cellular growth. Until now, analyses have mostly been directed at feed-back regulatory mechanisms elicited

in response to growth factors themselves. The present paper demonstrates negative regulation of the FCS- and EGF-induced MAP-kinase-activation cascade by the heptahelical AT₂ receptor, presumably through dephosphorylation of cellular targets which remain to be identified. Further dissection of the AT₂ receptor signalling pathway, and identification of cross-points with growth factor receptor intracellular cascades, may provide new perspectives for pharmacological targeting of human proliferative diseases. AT₂ receptors may thus function as physiological antagonists of receptor tyrosine kinases, providing a unique example of negative cross-talk between two major families of membrane receptors.

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