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European Journal of Pharmacology 436 (2002) 15–21

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Involvement of Rho and tyrosine kinase in angiotensin II-induced actin reorganization in mesothelial cells

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Received 23 July 2001; received in revised form 5 December 2001; accepted 7 December 2001

Abstract

We investigated the role of angiotensin II type 1 (AT_1) receptors in angiotensin II-induced actin reorganization and the signaling pathways of the response in pleural mesothelial cells. The effects of angiotensin II on actin reorganization in pleural mesothelial cells were evaluated by dual fluorescence labeling of filamentous (F) and monomeric (G) actin with fluorescein isothiocyanate (FITC)-labeled phalloidin and Texas Red-labeled DNase I, respectively. Angiotensin II (10 μM) induced actin reorganization in the presence and the absence of extracellular Ca^{2+}. An angiotensin AT_1 receptor antagonist ([Sar^1,Ile^8]angiotensin II) inhibited angiotensin II-induced actin reorganization. Pretreatment with C3 exoenzyme or tyrosine kinase inhibitors significantly reduced angiotensin II-induced actin reorganization. However, pertussis toxin, phosphatidylinositol-3-kinase and protein kinase C inhibitors had no effect on these responses. These results suggest that angiotensin II-induced actin reorganization in pleural mesothelial cells is extremely dependent on the angiotensin AT_1 receptor coupled with pertussis toxin-insensitive heterotrimeric G proteins, Rho GTPases and tyrosine phosphorylation pathways.

Keywords: Actin; Angiotensin II; Angiotensin AT_1 receptor; Mesothelial cell; Pleura; Pertussis toxin; Protein kinase C; Tyrosine kinase

1. Introduction

Mesothelial cells form a sheet on the surface of ever-moving organs (Wang, 1985) and have many functions involving production of cytokines, growth factors and extracellular matrix constituents (Kuwahara and Kagan, 1995). In turn, mesothelial cells also respond to some agents including cytokines and growth factors (Boylan et al., 1992; Hott et al., 1992; Ito et al., 1995; Kuwahara and Kuwahara, 1998; Owens and Grisham, 1993). However, the intracellular signaling mechanisms governing mesothelial cell growth, regeneration and differentiation remain essentially undefined. The peptide hormone, angiotensin II, evokes diverse physiological responses in many cell types (Timmermans et al., 1993). Recently, we have shown that pleural mesothelial cells respond to angiotensin II through the angiotensin II type 1 (AT_1) receptor coupled with pertussis toxin-insensitive G protein (Kuwahara et al., 2000). Angiotensin II has a proliferative effect on pleural mesothelial cells. G protein-coupled receptor-mediated mitogenic signaling via the Ras superfamily is involved in diverse biological processes, such as cell growth and differentiation, cell motility and reorganization of the actin cytoskeleton. Actin is a cellular protein essential for the motility of both non-muscle and muscle cells. In non-muscle cells, dynamic actin polymerization and depolymerization is the basic mechanism of cell motility (Stossel, 1989). In smooth muscle cells, the cytoskeleton is a filamentous network consisting largely of filamentous actin (F actin), which provides a scaffold on which motor proteins such as myosin translocate to generate internal stress and alter the mechanical properties of cells. Rho GTPases, which are a subfamily of the Ras superfamily of monomeric 20–30-kDa GTP-binding proteins, are involved in the signaling pathways mediating cytoskeleton reorganization (Chardin et al., 1989; Hirshman and Emala, 1999; Janmey, 1998; Nobes and Hall, 1995; Stossel, 1989; Togashi et al., 1998). The major intermediates upstream of Rho GTPases are heterotrimeric G proteins. The signaling pathways by which the heterotrimeric G proteins couple to Rho GTPases are known to be

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0014-2999/02/$ - see front matter
PII: S0014-2999(01)01591-6

cell specific and little is known about the angiotensin II-mediated reorganization of actin in pleural mesothelial cells. Therefore, we investigated the role of angiotensin AT_1 receptors in angiotensin II-induced actin reorganization and the signaling pathways of the response in pleural mesothelial cells.

2. Materials and methods

2.1. Rat pleural mesothelial cell cultures

Rat pleural mesothelial cells were obtained and established in culture as described previously (Kuwahara et al., 1991). Briefly, rats were anesthetized with sodium pentobarbital (40 mg/kg i.p.) and were immediately killed by exsanguination from a severed abdominal aorta. The complete thoracic wall was removed under sterile conditions and immersed in petri dishes for 20 min in Hanks' balanced salt solution (HBSS). The parietal pleural surfaces were scraped repeatedly with cell scrapers. The cells were then seeded into culture dishes. The cultures were maintained for up to 10 passages in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 10^5 U/l penicillin and 100 mg/l streptomycin at 37 °C in a humidified environment containing 5% CO_2. The cultured cells exhibited the characteristic features of mesothelial cells: a polyhedral, cobblestone morphologic pattern and positive immunohistochemical staining for cytokeratin and vimentin (Kuwahara et al., 1991).

2.2. Confocal microscopy

Actin localization was observed by confocal microscopy as described previously (Saegusa et al., 2001). Cells were fixed in 3% phosphate buffered saline (PBS)-formalin for 10 min and permeabilized with 1% Triton X-100 in PBS for 10 min at room temperature. After two washes with PBS, the cells were stained with fluorescein isothiocyanate (FITC)-labeled phalloidin (5 U/ml in PBS) to localize F actin and Texas Red-labeled DNase I (10 μg/ml in PBS) to localize G actin for 20 min in a dark room at room temperature (Knowles and McCulloch, 1992). The cells were washed with PBS twice and maintained in PBS. Dishes were mounted on the stage of a Leica TCS NT confocal laser scanning microscope equipped with an Ar–Kr laser. The excitation and emission wavelengths for FITC-phalloidin were 490 and 525 nm, whereas the excitation and emission wavelengths for Texas Red-DNase I were 596 and 615 nm. To standardize the fluorescence intensity measurements among experiments, the time of image capture, the image intensity gain, the image enhancement and the image black level in both channels were optimally adjusted at the outset and kept constant for all experiments. The F-to-G actin staining ratio was calculated with quantification software (Leica TCS NT confocal laser scanning microscope system) in at least 10 cells from three fields for each treatment. At least four separate experiments were performed and these values were averaged for a single data point. An increase in the F to G actin ratio indicated an increase in actin reorganization. Almost the same method was used to investigate the F-to-G actin ratio by other studies (Togashi et al., 1998; Hirshman and Emala, 1999).

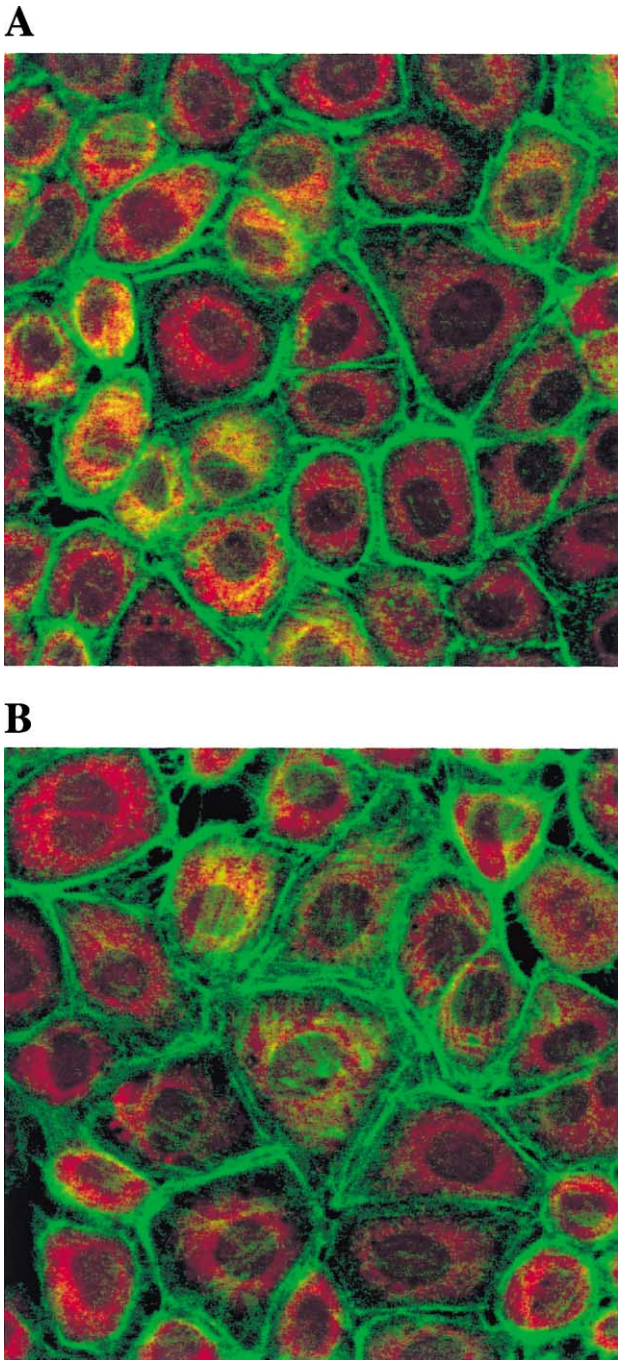

Fig. 1. Rat pleural mesothelial cells stained with FITC-phalloidin (green) and Texas Red-DNase I (red) to illustrate F and G actin, respectively. Stimulation with angiotensin II (10 μM) for 5 min induced an increase in F actin and a decrease in G actin (B) in comparison with that in untreated cells (A). The images are from at least four separate experiments.

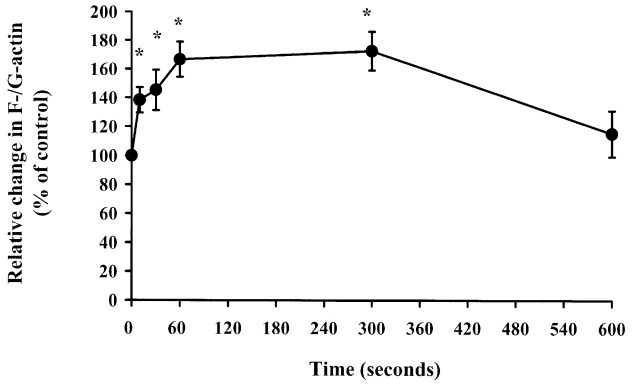

Fig. 2. Time course of the F-to-G actin fluorescence intensity ratio after angiotensin II stimulation. The cells were stimulated with 10 μM angiotensin II for different periods of time (10, 30, 60, 300 and 600 s). Values are expressed as percentages of control and are means ± S.E.M. from four separate experiments. * $P < 0.01$, significant difference from untreated cells.

2.3. Pretreatment with C3 exoenzyme, pertussis toxin and kinase inhibitors

To determine whether the angiotensin II-induced increase in stress fiber formation involved Rho proteins, cells were incubated with 10 μg/ml C3 exoenzyme for 72 h, after which the cells were left untreated or treated with angiotensin II (10 μM) for 5 min.

To determine whether the angiotensin II-induced increase in stress fiber formation involved G_i proteins, cells were incubated with 100 ng/ml pertussis toxin for 4 h, after which the cells were left untreated or treated with angiotensin II (10 μM) for 5 min.

To determine whether phosphatidylinositol-3-kinase, protein kinase C and tyrosine kinase were involved in the angiotensin II-induced increase in stress fiber formation, each cell was incubated with 500 nM wortmannin, 100 nM GF-109203X(3-[*N*-(dimethyl-amino)propyl-3-indolyl]-4-[3-indolyl]maleimide) or 20 μM genistein for 20 min, after which the cells were left untreated or treated with angiotensin II (10 μM) for 5 min.

Dosages of angiotensin II was selected according to results of previous study (Kuwahara et al., 2000). Moreover, dosages of inhibitors were selected according to our recent study using almost the same method (Saegusa et al., 2001).

2.4. Statistical analysis

All data are presented as means ± S.E.M. Statistical analysis was performed using an analysis of variance (ANOVA). A value of $P < 0.05$ was considered significant.

2.5. Drugs

Angiotensin II, [Sar^1,Ile^8]angiotensin II and pertussis toxin were purchased from Sigma, St. Louis, MO. GF-109203X, wortmannin, genistein and C3 exoenzyme were purchased from BIOMOL Research Laboratories, Plymouth Meeting, PA. FITC-phalloidin and Texas Red-

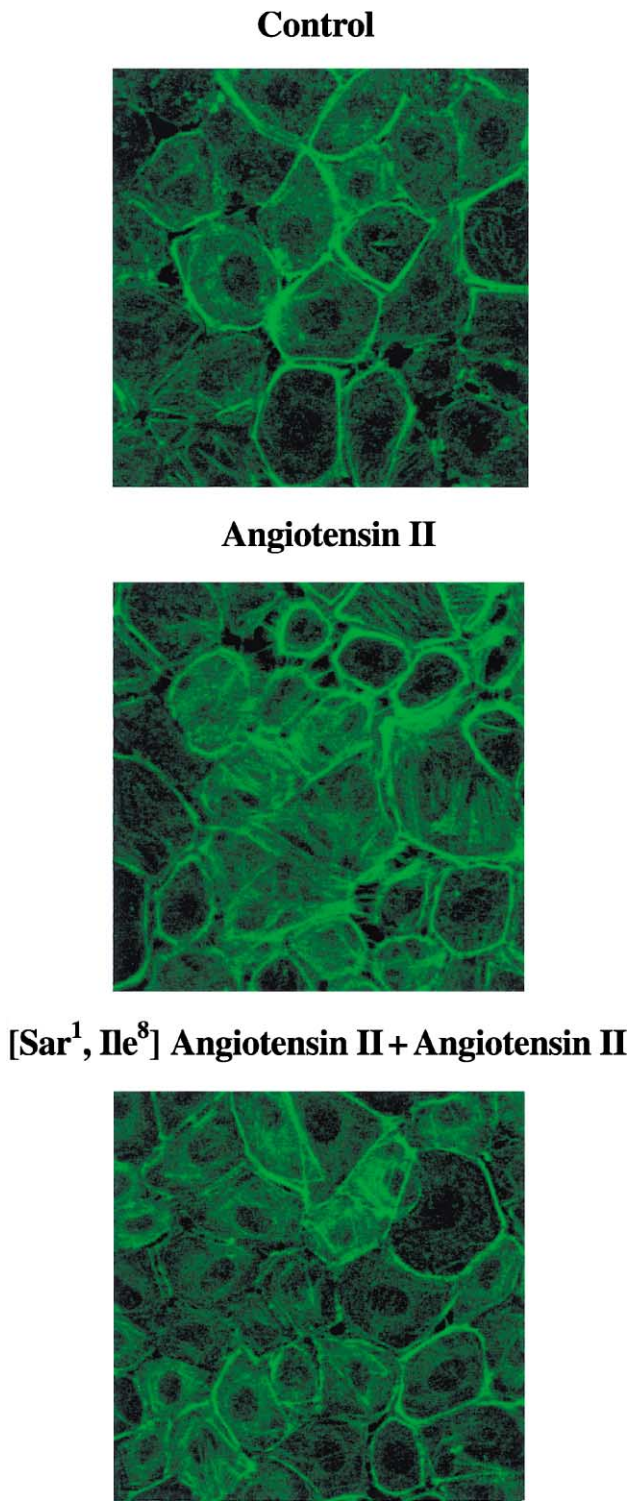

Fig. 3. Effect of the angiotensin AT_1 receptor antagonist, [Sar^1,Ile^8]angiotensin II, on angiotensin II-induced F actin reorganization. Pretreatment with [Sar^1,Ile^8]angiotensin II (1 μM) for 20 min inhibited the angiotensin II-induced increase in F actin reorganization. The images are from at least four separate experiments.

DNase I were purchased from Molecular Probes, Eugene, OR.

3. Results

3.1. Actin reorganization induced by angiotensin II

Exposure of pleural mesothelial cells to 10 μM angiotensin II for 5 min resulted in an increase in the FITC-phalloidin staining intensity of F actin and a decrease in the Texas Red-DNase I staining intensity of G actin compared with that in the untreated cells (Fig. 1). The F-to-G actin fluorescence-staining ratio, which is indicative of actin fiber reorganization, increased significantly to 172.4 ± 13.5% of that of untreated cells.

The cells were stimulated with 10 μM angiotensin II for different periods of time. Fig. 2 shows the percentage change in the F-to-G actin staining ratio following stimulation with 10 μM angiotensin II. The F-to-G actin staining ratio increased rapidly (138.1 ± 8.1% after 10 s) and this increase lasted at least 5 min. After 10 min, it returned to 115.1 ± 15.8% of that of untreated cells.

3.2. Effect of the angiotensin AT1 receptor antagonist on actin reorganization induced by angiotensin II

Treatment with the angiotensin AT_1 receptor antagonist, [Sar^1,Ile^8]angiotensin II (1 μM), alone for 20 min did not affect either F actin staining or the F-to-G actin ratio (105.6 ± 9.6%). A 20-min preincubation with the angiotensin AT_1 receptor antagonist (10 μM) reduced the increase in F actin staining (Fig. 3) and in the F-to-G actin ratio induced by angiotensin II from 172.4 ± 13.5% to 103.2 ± 12.7% ($P < 0.05$, Fig. 4).

3.3. Inhibition of angiotensin II-induced actin reorganization by C3 exoenzyme and genistein

To determine whether Rho proteins and/or tyrosine phosphorylation are intermediates in the signaling pathway in the actin reorganization induced by angiotensin II, the cells were pretreated with C3 exoenzyme (10 μg/ml) for 72 h or with the tyrosine kinase inhibitor, genistein (20 μM) for 20 min.

C3 exoenzyme or genistein pretreatment alone had no effect on F and G actin staining (Fig. 5) or on the F-to-G actin ratio (103.7 ± 6.0%) (Fig. 4). In the cells pretreated with C3 exoenzyme or genistein and then challenged with angiotensin II, F actin staining did not increase (Fig. 5). Moreover, the increase in the F-to-G actin ratio induced by angiotensin II was reduced by C3 exoenzyme and genistein pretreatment to 95.8 ± 8.9% and 93.8 ± 7.3%, respectively ($P < 0.05$, Fig. 4).

3.4. Effects of pertussis toxin, wortmannin and GF-109203X on actin reorganization induced by angiotensin II

To determine whether pertussis toxin sensitive heterotrimeric G protein (G_i), phosphatidylinositol-3-kinase or protein kinase C is required for the actin reorganization induced by angiotensin II, the cells were pretreated with pertussis toxin (100 ng/ml) for 4 h, the phosphatidylinositol-3-kinase inhibitor, wortmannin (500 nM) or the protein kinase C inhibitor, GF-109203X (100 nM), for 20 min before stimulation with 10 μM angiotensin II. The inhibitors alone had no effect on F and G actin staining or on the F-to-G actin ratio. Pretreatment with pertussis toxin, wortmannin or GF-

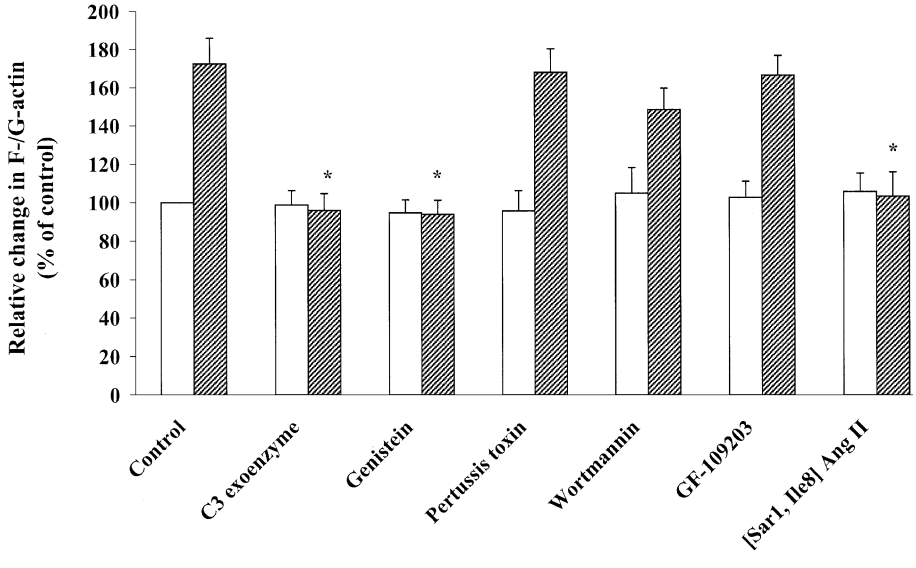

Fig. 4. Effects of C3 exoenzyme, genistein, pertussis toxin, wortmannin, GF-109203 and [Sar^1,Ile^8]angiotensin II on the F-to-G actin fluorescence intensity ratio in the absence (open bars) or the presence (hatched bars) of 10 μM angiotensin II. Values are expressed as percentages of control and are means ± S.E.M. from four separate experiments. * $P < 0.05$, significant difference from each control.

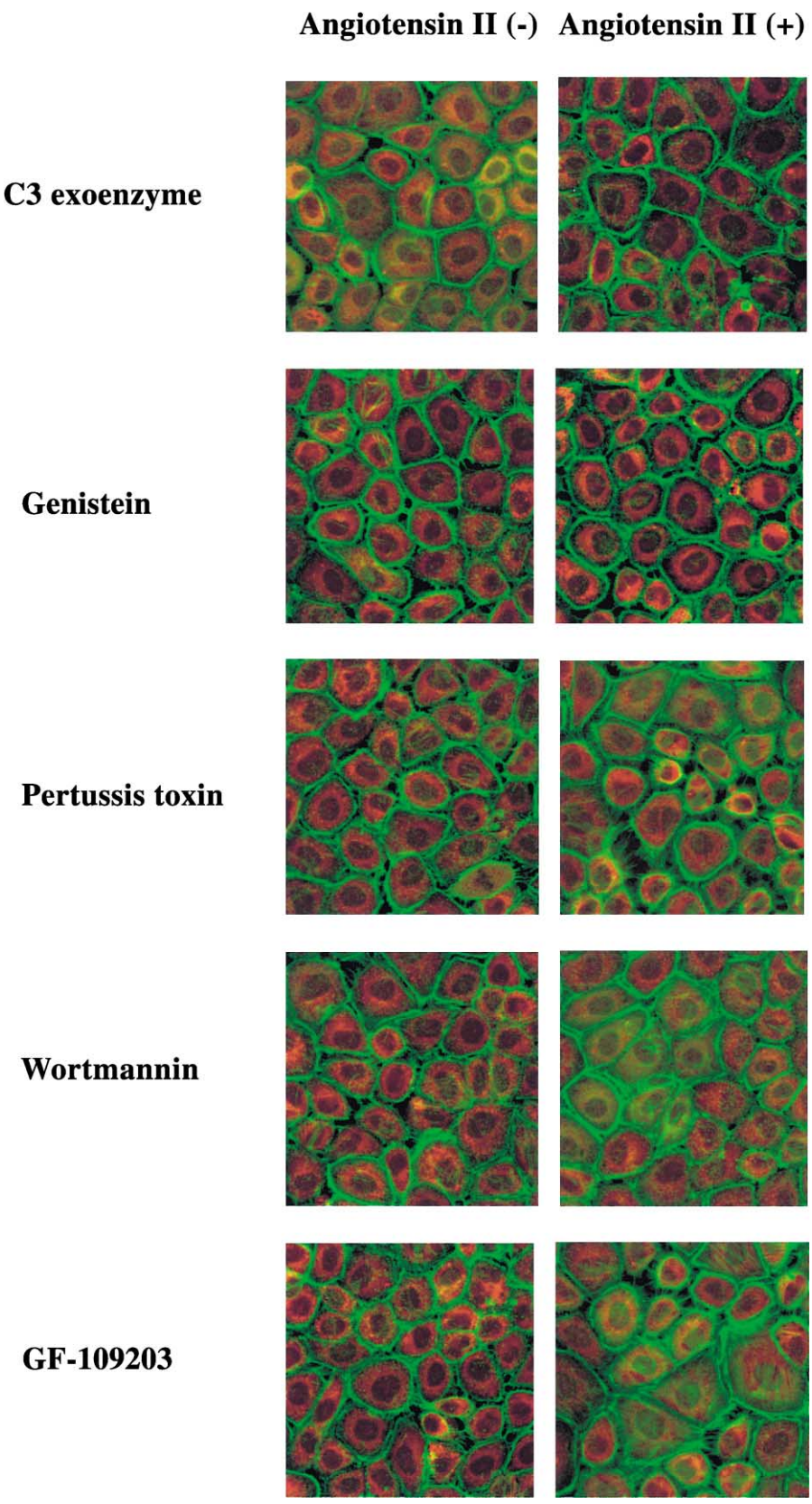

Fig. 5. Effects of pretreatment with 10 μg/ml C3 exoenzyme, 20 μM genistein, 100 ng/ml pertussis toxin, 500 nM wortmannin or 100 nM GF-109203 on angiotensin II-induced F and G actin staining. Untreated cells are shown in the left panel and 10 μM angiotensin II-treated cells are shown in the right panel. Pretreatment with C3 exoenzyme and genistein blocked angiotensin II-induced increases in F actin staining. The images are from at least four separate experiments.

109203X did not significantly inhibit the increase in F actin staining (Fig. 5) or that in the F-to-G actin ratio (Fig. 4). In the cells pretreated with pertussis toxin, the F-to-G actin ratio was increased by angiotensin II to 167.7 ± 12.3%. Moreover, in the cells pretreated with wortmannin and GF-109203X, the F-to-G actin ratio was increased by angiotensin II to 148.3 ± 11.1% and 166.1 ± 10.3%, respectively.

4. Discussion

This study demonstrated that activation of the angiotensin AT_1 receptor with angiotensin II led to a rapid and sustained reorganization of the actin cytoskeleton in pleural mesothelial cells. We determined the intermediate proteins involved in this signaling pathway. Pretreatment with C3 exoenzyme or genistein inhibited the angiotensin AT_1 receptor activation to induce stress fiber formation. However, pertussis toxin and inhibitors of phosphatidylinositol-3-kinase and protein kinase C had no effect. These results indicate that pertussis toxin-insensitive heterotrimeric G proteins and Rho GTPases are involved in this pathway. Further, tyrosine phosphorylation may be important in the actin reorganization induced by angiotensin II in pleural mesothelial cells.

Angiotensin II receptors have been classified into angiotensin AT_1 and AT_2 receptor subtypes (Timmermans et al., 1993). An angiotensin AT_1 receptor has been cloned from a variety of species and tissues (Kakar et al., 1992; Murphy et al., 1991; Sasaki et al., 1991; Sasamura et al., 1992). All consist of a single polypeptide, 359 amino acids in length, arranged with topography comprising seven α-helical transmembrane regions, typical of the G protein-coupled receptor family. They display a high degree of sequence identity at the amino acid level (over 94% identical between all mammalian species) (Kakar et al., 1992; Sasamura et al., 1992). An angiotensin AT_2 receptor has been cloned and is also a member of the G protein-coupled receptor family (Kambayashi et al., 1993; Mukoyama et al., 1993; Tsuzuki et al., 1994). Its consists of 363 amino acids and shares only 34% sequence identity at the amino acid level to the angiotensin AT_1 receptor.

In many cells, activation of angiotensin AT_1 receptors, which are coupled to the phospolipase C signal transduction pathway through a pertussis toxin-insensitive G protein (G_q or G_{11}), results in the generation of the second messengers, IP_3 and diacylglycerol, which in turn mobilize Ca^{2+} from intracellular stores and activate protein kinase C, respectively. Besides this classical pathway, recent studies indicate that angiotensin II activates both non-receptor-type and receptor-type tyrosine kinases, which are typically activated by cytokine and epidermal growth factor receptor stimulation (Sadoshima, 1998). Activation of tyrosine kinases by angiotensin II is of great interest, because tyrosine kinases mediate activation of small GTP binding proteins and mitogen-activated protein kinases. These GTPases act as molecular switches to regulate cellular functions. Recently, it has been reported that activation of the angiotensin AT_1 receptor is known to activate Rho in cardiac myocytes and vascular smooth muscle cells (Aoki et al., 1998; Yamakawa et al., 2000).

Activation of angiotensin AT_1 receptors with angiotensin II leads to reorganization of the actin cytoskeleton in pleural mesothelial cells. A signaling role for RhoA in cytoskeletal organization has been suggested since the early 1990s (Paterson et al., 1990; Ridley and Hall, 1992, 1994). Activated RhoA induced stress fiber formation in serum-starved Swiss 3T3 fibroblasts (Paterson et al., 1990; Ridley and Hall, 1992). In human airway smooth muscle cells, actin reorganization induced by carbachol and leukotriene D_4 is linked to Rho GTPases via a pertussis toxin-sensitive G protein (G_i) (Togashi et al., 1998; Saegusa et al., 2001) and that induced by endothelin-1 and lysophosphatidic acid is linked to Rho GTPases via G_q and G_{i-2} activation (Hirshman and Emala, 1999). Our results indicate that angiotensin II-induced actin reorganization involves Rho via a pertussis toxin-insensitive G protein. Further, tyrosine phosphorylation may be important in the actin reorganization induced by angiotensin II in pleural mesothelial cells. However, phosphatidylinositol-3-kinase and protein kinase C are not involved in angiotensin II-induced actin reorganization. These results suggest that angiotensin II-induced actin reorganization in pleural mesothelial cells may be mediated by the tyrosine kinases-mediated activation of the Rho GTPases pathway rather than the classical heterotrimeric G_q protein pathway.

Recently, the angiotensin AT_1 receptor has been shown to directly associate with intracellular signaling molecules. The putative Stat5 binding motif (YXXL) has been found on intracellular loop 1 and on the carboxyl tail of the angiotensin AT_1 receptors (McWhinney et al., 1998). Small GTP binding proteins have been shown to interact with the amino acid sequence containing NPXXY (amino acids 298–302) in the seventh transmembrane domain of the angiotensin AT_1 receptor in an angiotensin II-dependent manner in rat anterior pituitary cells (Mitchell et al., 1998). Moreover, the NPXXY motif is not found in all G_q-coupled receptors. Therefore, it seems that these findings may also support our observations about the pathway in angiotensin AT_1 receptor mediated actin reorganization in pleural mesothelial cells.

In conclusion, this study provided the first evidence that Rho GTPases may play an important role in angiotensin II-induced actin reorganization in pleural mesothelial cells. However, further study will be needed to investigate the detailed mechanisms involved in angiotensin AT_1 receptor-mediated events in pleural mesothelial cells.

Acknowledgements

This work was supported in part by a Grant-in-Aid from the scientific Research Fund of the Ministry of Education, Science, Sports and Culture, Japan.

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