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Angiotensin II type 1 receptor-mediated increase in cytosolic Ca^{2+} and proliferation in mesothelial cells

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Abstract

We investigated the Ca^{2+} signaling pathways of the response to angiotensin II in pleural mesothelial cells and the role of these Ca^{2+} signaling pathways in mesothelial cell proliferation. Rat pleural mesothelial cells were maintained in vitro, and the Ca^{2+} movement to angiotensin II was evaluated using the fluorescent Ca^{2+} indicator fura 2. Furthermore, proliferation of mesothelial cells was assessed using a spectrophotometric 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl-2*H*-tetrasodium bromide (MTT) assay. Angiotensin II (1 pM–100 μM) induced in mesothelial cells a biphasic elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) that consisted of a transient initial component, followed by a sustained component. Neither removal of extracellular Ca^{2+} nor inhibition of Ca^{2+} influx by 1 μM nifedipine affected the angiotensin II-induced initial transient elevation of $[Ca^{2+}]_i$ in mesothelial cells. Nifedipine did not block angiotensin II-induced sustained elevation of $[Ca^{2+}]_i$. Angiotensin II (1 pM–100 μM) had a proliferative effect on mesothelial cells in a dose-dependent manner. Angiotensin II type 1 (AT_1) receptor antagonist ([Sar^1, Ile^8]angiotensin II) inhibited both angiotensin II-induced elevation of $[Ca^{2+}]_i$ and proliferation of mesothelial cells. Pertussis toxin did not affect angiotensin II-induced responses. These results suggest that angiotensin II-induced responses to mesothelial cells are extremely dependent on the angiotensin AT_1 receptor coupled with pertussis toxin-insensitive G protein.

Keywords: Angiotensin II; Angiotensin II type 1 receptor; Ca^{2+}, cytosolic; Mesothelial cell; Pleura

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; Owens and Grisham, 1993; Ito et al., 1995; Kuwahara and Kuwahara, 1998). However, the intracellular signaling mechanisms governing mesothelial cell growth, regeneration and differentiation remain essentially undefined. The peptide hormone angiotensin II evokes diverse physiological responses including cell proliferation in many cell types (Timmermans et al., 1993). Therefore, it could be of considerable interest in defining its signaling pathways that mediate the growth response of mesothelial cells. Pharmacological evidence has defined at least two subtypes of angiotensin II receptors, designated angiotensin II type 1 (AT_1) and type 2 (AT_2) receptor subtypes (Murphy et al., 1991; Sasaki et al., 1991; Timmermans et al., 1993). Angiotensin AT_1 receptor activation by angiotensin II is initiated by stimulation of a phosphatidylinositol-specific phospholipase C, leading to the generation of inositol trisphosphate (IP_3) and diacylglycerol in cultured rat vascular smooth muscle cells (Griendling et al., 1986). These reactions are involved in intracellular Ca^{2+} mobilization (Berridge, 1987) and protein kinase C activation (Nishizuka, 1988). Previously, we have clarified the mechanisms of cytosolic Ca^{2+} mobilization in the mesothelial cells (Ito et al., 1995). Therefore, we examined the Ca^{2+} signaling pathways of the response to angiotensin II in mesothelial cells with the use of the fluorescent Ca^{2+} indicator fura 2 (Grynkiewicz et al, 1985). Furthermore, to confirm the role of these Ca^{2+} signaling pathways in mesothelial cell growth, prolifera-

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tion of mesothelial cells was assessed using a spectrophotometric 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl-2*H*-tetrasodium bromide (MTT) assay (Mosmann, 1983).

2. Materials and methods

2.1. Reagents, media and buffers

For the Ca^{2+} measurements we used a *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer containing (in mM) 10 HEPES, 136.9 NaCl, 5.4 KCl, 1.0 $MgCl_2$, 1.5 $CaCl_2$, 0.001 EDTA and 5.5 glucose (HEPES-buffered solution). In Ca^{2+}-free experiments, the same solution was used except that 1.5 mM $CaCl_2$ was omitted and 0.5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid (EGTA) was added. Dulbecco's modified eagles medium (DMEM), Hanks' balanced salt solution (HBSS), fetal bovine serum, and trypsin-EDTA were purchased from GIBCO, Grand Island, NY. Angiotensin II, [Sar^1, Ile^8]angiotensin II, ionomycin, nifedipine, pertussis toxin, thapsigargin and 1,2-bis(2-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) were purchased from Sigma, St. Louis, MO. Fura 2-acetoxymethyl ester (fura 2-AM) was obtained from Dojindo Laboratories, Kumamoto, Japan. Other materials and chemicals were obtained from commercial sources.

2.2. 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 exanguination from a severed abdominal aorta. The complete thoracic wall was removed under sterile conditions and immersed in petridishes for 20 min in 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 DMEM with 10% fetal bovine serum, 10^5 U/l penicillin, and 100 mg/l streptomycin, at 37°C in 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).

2.3. Measurement of $[Ca^{2+}]_i$

Changes in $[Ca^{2+}]_i$ were determined as previously reported (Ito et al., 1995). Pleural mesothelial cells were incubated on 25-mm glass coverslips (Matsunami, Tokyo, Japan) in DMEM with 10% fetal bovine serum. After reaching confluence, the fetal bovine serum in culture medium was depleted for 12 h, and then the mesothelial cell monolayers were loaded with fura 2 by incubating them with 2 μM fura 2-AM for 30 min at 37°C in HEPES-buffered solution. Loaded cells were washed in HEPES-buffered solution and maintained in this solution for 20 min at room temperature to allow for complete hydrolysis of fura 2-AM to the acid form.

The glass coverslip was placed horizontally in a temperature-controlled (37°C) bath that was mounted on an equipment for fluorescence measurements (CAF-100, Japan Spectroscopic, Tokyo, Japan). Fluorescence excitation was obtained from a xenon high-pressure lamp (150 W). Ultraviolet light of alternating 340 and 380 nm (10 nm bandwidth) was obtained with a monochromator equipped with a chopping wheel (400 Hz) placed in front of the monochromator. The dichroic mirror was used as a beam splitter to transmit emitted fluorescence (500 nm) into the photomultiplier. The fluorescence signals (340 and 380 nm) and their ratio (340:380) were continuously recorded on a chart recorder. At the end of experimental run, background autofluorescence (the inherent fluorescence emitted from cells, coverslip, and bath at 340 and 380 nm) was obtained by the method of Hallam et al. (1988).

After autofluorescence was subtracted, the changes in $[Ca^{2+}]_i$ were determined quantitatively by using the following equation: $[Ca^{2+}]_i = K_d[(R - R_{min})/(R_{max} - R)](Sf_2/Sb_2)$ where the dissociation constant K_d has a value of 224 nM (Grynkiewicz et al., 1985), R is the fluorescence ratio within the cells, R_{max} is the maximal fluorescence ratio after addition of 40 μM ionomycin in the presence of 1.5 mM $CaCl_2$, R_{min} is the minimal ratio determined by the subsequent addition of 5 mM EGTA, and Sf_2/Sb_2 is the ratio of fluorescence values at 380-nm excitation determined at R_{min} and R_{max}, respectively.

2.4. Cell proliferation

Mesothelial cells were sparsely seeded at a density of 2.5×10^3 cells/well in 96-well plates in DMEM with 10% fetal bovine serum and allowed to adhere overnight. Subconfluent conditions were chosen to allow detection for maximal growth response. The media was changed to DMEM without fetal bovine serum for 24 h to induce quiescence. Some blocking agents were added 5 min before angiotensin II treatment. Angiotensin II concentrations diluted in DMEM ranging from 1 pM to 1 μM were added to each well for 24 h, and cell replication was assessed using a spectrophotometric MTT assay (Mosmann, 1983). The MTT solution (5 mg/ml) was added directly to the assay plates at a rate of 50 μl of cell culture medium and then the cells were incubated for another 1 h at 37°C. The purple formazan crystals formed were dissolved by the addition of dimethyl-sulphoxide (DMSO) followed by thorough mixing. The plates were subsequently read on a spectrophotometer at 570 nm.

2.5. Statistics

Results were expressed as the mean ± S.E.M. of five separate experiments for each category. For some studies, statistical comparisons were made by using a one-way analysis of variance (ANOVA).

3. Results

3.1. Angiotensin II-induced increase in $[Ca^{2+}]_i$ in mesothelial cells

Angiotensin II induced a biphasic elevation of $[Ca^{2+}]_i$ in mesothelial cells, which consisted of a transient initial component, followed by a sustained component in the presence of 1.5 mM extracellular Ca^{2+} (Fig. 1). The initial component peaked within 30 s after angiotensin II stimulation. Angiotensin II (1 pM–100 μM) elicited an elevation of $[Ca^{2+}]_i$ in a dose-dependent manner. Concentration–response curves were obtained from the peak values both initial transient and sustained component of the $[Ca^{2+}]_i$ response as shown in Fig. 2. The half-maximal effective concentration (EC_{50}) and maximum effect (E_{max}) of transient peak values for angiotensin II were 10 and 1240 nM in mesothelial cells, respectively. The sustained values of $[Ca^{2+}]_i$ reached plateau for more than 100 nM angiotensin II at 250 nM.

3.2. Response to angiotensin II in the presence of a Ca^{2+}-entry blocker, in the absence of extracellular Ca^{2+} and in the depletion of Ca^{2+} stores

As shown in Fig. 3, both the angiotensin II-induced initial transient elevation of $[Ca^{2+}]_i$ and the sustained component were still present after nifedipine treatment (Fig. 3A). In the absence of extracellular Ca^{2+} with 0.5 mM EGTA, angiotensin II induced only a transient elevation of $[Ca^{2+}]_i$, whereas the sustained component was eliminated (Fig. 3B). Pretreatment of thapsigargin attenuated the initial transient elevation of $[Ca^{2+}]_i$. These result suggest that angiotensin II-induced elevation of $[Ca^{2+}]_i$ in mesothelial cells was not dependent on dihydropyridine-sensitive, voltage-dependent Ca^{2+} channels. Moreover, an-

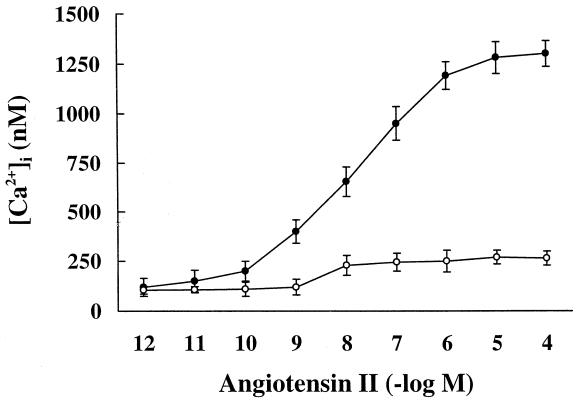

Fig. 2. Dose–response curve showing magnitude of Angiotensin II-induced initial transient (●) and sustained component (○) of the $[Ca^{2+}]_i$ in mesothelial cells. Each point represents mean ± S.E.M. of five separate experiments.

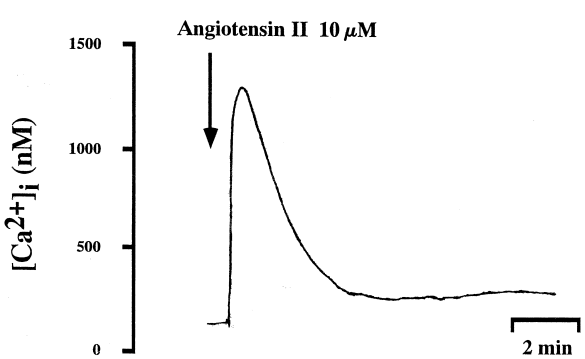

Fig. 1. Tracing showing effect of 10 μM Angiotensin II on cytosolic Ca^{2+} concentration level ($[Ca^{2+}]_i$) of mesothelial cells in presence of 1.5 mM extracellular Ca^{2+}. Addition of Angiotensin II induced biphasic elevation of $[Ca^{2+}]_i$: initial transient component followed by sustained component in fura 2-loaded pericardial mesothelial cells. Tracing is representative of five independent experiments.

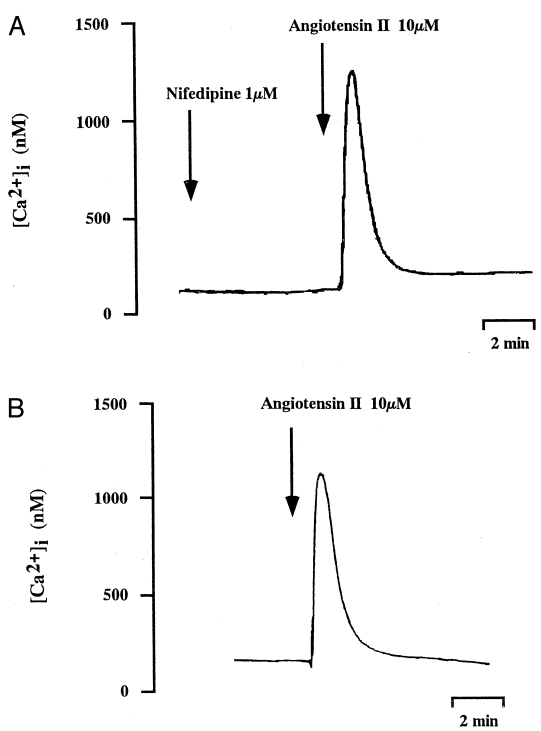

Fig. 3. Effects of 1 μM nifedipine (A) and absence of extracellular Ca^{2+} with 0.5 mM EGTA (B) on 10 μM Angiotensin II-induced elevation of $[Ca^{2+}]_i$ in mesothelial cells. Nifedipine was added 5 min before angiotensin II treatment. Nifedipine did not affect Angiotensin II-induced elevation of $[Ca^{2+}]_i$. Sustained component was not observed in absence of extracellular Ca^{2+}. Each tracing is representative of five independent experiments.

giotensin II-induced transient elevation of $[Ca^{2+}]_i$ originated from intracellular Ca^{2+} stores.

3.3. Effects of the angiotensin AT_1 receptor antagonist on angiotensin II-induced $[Ca^{2+}]_i$ response

An angiotensin AT_1 receptor antagonist, [Sar[1], Ile[8]]angiotensin II (1 μM), was used as pretreatment to identify the angiotensin receptor subtype mediating the increased $[Ca^{2+}]_i$ in mesothelial cells. The angiotensin AT_1 receptor antagonist almost abolished the angiotensin II-induced elevation of $[Ca^{2+}]_i$ in the mesothelial cells (Fig. 4). This result suggested that angiotensin II-induced elevation of $[Ca^{2+}]_i$ in the mesothelial cells was extremely dependent on angiotensin AT_1 receptor.

3.4. Effects of pertussis toxin treatment on angiotensin II-induced $[Ca^{2+}]_i$ response

As shown in Fig. 4, angiotensin II-induced elevation of $[Ca^{2+}]_i$ in the mesothelial cells was still present after pertussis toxin (100 ng/ml) treatment. This result suggested that angiotensin II-induced elevation of $[Ca^{2+}]_i$ in the mesothelial cells was not dependent on pertussis toxin-sensitive G protein (G_s) coupled receptor.

3.5. Angiotensin II-induced mesothelial cell proliferation

The proliferative effects of angiotensin II on mesothelial cells are shown in Fig. 5. Angiotensin II (1 pM–100 μM) apparently has a replicating effect on mesothelial cells in a dose-dependent manner. Angiotensin II (10 μM) was about 80% increase in mesothelial proliferation comparison with no treatment. The proliferative effect of angiotensin II (10 μM) was significantly decreased to less than 20% in the presence of BAPTA (10 mM) and [Sar[1], Ile[8]]angiotensin II (1 μM), but nifedipine (1 μM) and pertussis toxin (100 ng/ml) did not affect angiotensin II-induced proliferation (Fig. 6). Thapsigargin (1 μM) also significantly reduced the proliferative effect of angiotensin II, although the degree of inhibitory effect was less than BAPTA treatment. These results suggest that Ca^{2+} influx may play a very important role on angiotensin II-induced proliferation, although the transient elevation of $[Ca^{2+}]_i$ may be in part responsible of the proliferative response in mesothelial cells. Moreover, these observations provide further evidence that pleural mesothelial cells have the angiotensin AT_1 receptor coupled with pertussis toxin-insensitive G protein.

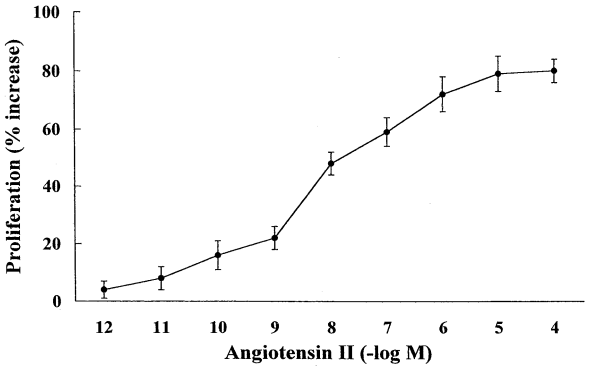

Fig. 5. Dose–response curves showing % increase of Angiotensin II-induced mesothelial cell proliferation. Each point represents mean ± S.E.M. of five separate experiments.

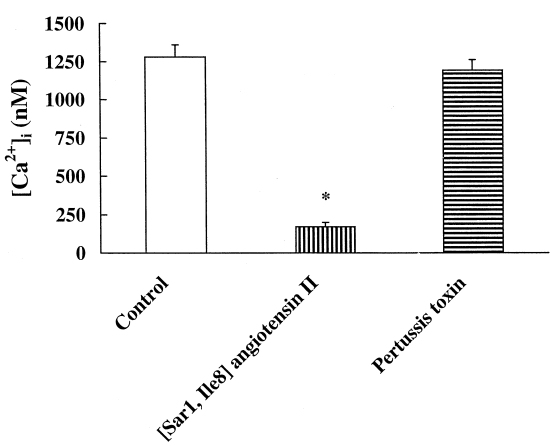

Fig. 4. Effects of 1 μM [Sar[1], Ile[8]]angiotensin II and 100 ng/ml pertussis toxin on 10 μM Angiotensin II-induced elevation of $[Ca^{2+}]_i$ in mesothelial cells. These blockades were added 5 min before angiotensin II treatment. Angiotensin II-induced elevation of $[Ca^{2+}]_i$ was almost abolished by [Sar[1], Ile[8]]angiotensin II, but pertussis toxin did not affect Angiotensin II-induced elevation of $[Ca^{2+}]_i$. Each tracing is representative of five independent experiments. Each bar represents mean ± S.E.M. of five separate experiments. *: $P < 0.05$, significant difference from control.

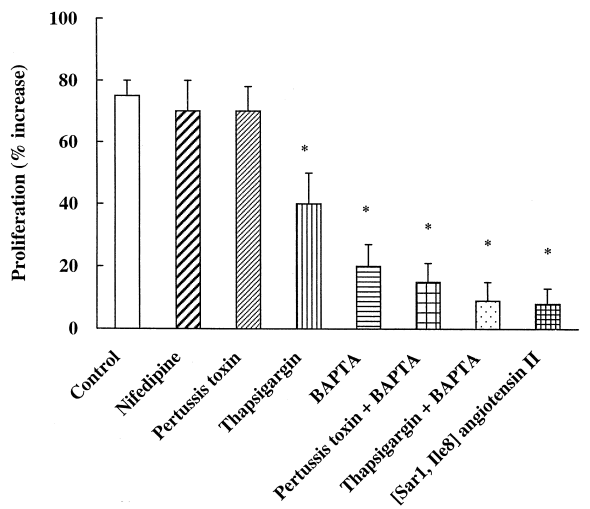

Fig. 6. Effects of some agents on mesothelial cell proliferation. These agents were added 5 min before angiotensin II treatment and mesothelial cells were incubated for 24 h. Nifedipine (1 μM) and pertussis toxin (100 ng/ml) did not affect Angiotensin II-induced proliferation. BAPTA (10 mM) and [Sar[1], Ile[8]]angiotensin II (1 μM) inhibited Angiotensin II-induced proliferation. Each bar represents mean ± S.E.M. of five separate experiments. *: $P < 0.05$, significant difference from control.

4. Discussion

This study has shown that cultured pleural mesothelial cells respond to angiotensin II through elevation of $[Ca^{2+}]_i$. Moreover, angiotensin II has a proliferative effect on pleural mesothelial cells. An angiotensin AT_1 receptor antagonist ([Sar^1, Ile^8]angiotensin II) inhibits both angiotensin II-induced elevation of $[Ca^{2+}]_i$ and proliferation of mesothelial cells. Pertussis toxin does not affect angiotensin II-induced responses. These results suggest that angiotensin II-induced responses to mesothelial cells are extremely dependent on the angiotensin AT_1 receptor coupled with pertussis toxin-insensitive G protein.

Angiotensin II receptors have been classified into the 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 (Murphy et al., 1991; Sasaki et al., 1991; Kakar et al., 1992; 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 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.

There are some possibilities that angiotensin II-induced elevation of $[Ca^{2+}]_i$ may depend on both the nucleus and the cytosol, because angiotensin II receptors are found not only cell surface but also nucleus membranes (Haller et al., 1996). Angiotensin II increased $[Ca^{2+}]_i$ in the cytosol rather than in the nucleus using confocal microscopy with fluo 3 loaded mesothelial cells (unpublished observation). Moreover, the angiotensin AT_1 receptor antagonist almost abolished the angiotensin II-induced elevation of $[Ca^{2+}]_i$ in the mesothelial cells. Therefore, the elevation of $[Ca^{2+}]_i$ might be induced via cell surface membrane angiotensin AT_1 receptors.

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 PKC, respectively. In contrast with the angiotensin AT_1 receptor, much less is known about the physiological actions and signal transduction pathways associated with the angiotensin AT_2 receptor. However, there is some evidence that they mediate effects through a pertusis toxin-sensitive G_i protein (Nahmias and Strosberg, 1995), and their localization is mainly in the brain (Millan et al., 1991). The angiotensin AT_1 receptor antagonist ([Sar^1, Ile^8]angiotensin II) inhibits both angiotensin II-induced elevation of $[Ca^{2+}]_i$ and proliferation of mesothelial cells. Pertussis toxin does not affect angiotensin II-induced responses. Although it is reported that phospholipase C-γ has been shown to be coupled to the angiotensin AT_1 receptor in a G protein-independent manner in smooth muscle cells (Marrero et al., 1994), the present study has obtained almost the same results in earlier studies on other cell types. Therefore, it seems that angiotensin II-induced responses to mesothelial cells may be dependent on the angiotensin AT_1 receptor coupled with pertussis toxin-insensitive G protein.

Angiotensin II receptors are present in many tissues and angiotensin II evokes diverse physiological responses including vasoconstriction, aldosterone secretion, and cell growth and proliferation in many cell types (Peach and Dostal, 1990). One function of Ca^{2+} signal is to activate the immediate early genes responsible for introducing resting cells (G_0) to re-enter the cell cycle. Angiotensin II induces a rapid increase in expression of these prot-oncogenes, c-*fos*, c-*Jun*, and c-*myc*, in vascular smooth muscle cells (Naftilan et al, 1989; Taubman et al., 1989). The Ca^{2+} signal may relate to this pathway in angiotensin II-induced mesothelial cell proliferation. To clarify the role of Ca^{2+} on angiotensin II-induced mesothelial proliferation, we used nifedipine, BAPTA, and thapsigargin. Because BAPTA strongly inhibit angiotensin II-induced mesothelial proliferation, it seems that Ca^{2+} influx through non L-type Ca^{2+} channels may play a very important role on this response. The transient initial elevation of $[Ca^{2+}]_i$ may also be partly responsible of the proliferative response in mesothelial cells, because thapsigargin reduce the mesothelial proliferation. Furthermore, angiotensin II stimulates mitogen-activated protein kinases (MAPKs) (Duff et al., 1992; Tsuda et al., 1992; Molloy et al., 1993). Earlier reports proposed a dominant role of PKC in the mechanism of angiotensin II-mediated MAPKs activation in vascular smooth muscle cells (Tsuda et al., 1992; Molloy et al., 1993), whereas more recent studies have indicated that Ca^{2+} signals rather than PKC are critical for MAPKs activation by angiotensin II in cardiac cells (Booz et al., 1994; Sadoshima et al., 1995). Therefore, MAPKs pathway may also be of relevance to the angiotensin II-mediated mesothelial proliferation. However, further studies will be needed to define signal transduction cascades leading to MAPKs activation by the angiotensin II in mesothelial cells.

Previously, we have shown that mesothelial cells have three pathways for increasing $[Ca^{2+}]_i$: release from intracellular Ca^{2+} stores, Ca^{2+} influx through L-type voltage-dependent channels, and receptor-operated Ca^{2+} channels (Ito et al., 1995). Angiotensin II-induced elevation of $[Ca^{2+}]_i$ in mesothelial cells was not dependent on L-type Ca^{2+} channels, and was dependent on intracellular Ca^{2+} stores and Ca^{2+} influx through non L-type Ca^{2+} channels. For L-type independent Ca^{2+} influx in a sustained component, Ca^{2+} release activated channels and R-type Ca^{2+}

channels have been observed in some cell types (Bkaily et al., 1997). However, there is currently no data about these channels in mesothelial cells. Therefore, further studies will be needed to clarify the receptor agonist induced mechanisms in a sustained elevation of $[Ca^{2+}]_i$.

Many investigators have failed to show receptors and a signal transduction system for angiotensin II in endothelial cells (André et al., 1992; Burnier et al., 1994). Moreover, although we have clarified voltage-dependent Ca^{2+} channels in mesothelial cells (Ito et al., 1995), some studies have failed to show the existence of voltage-dependent Ca^{2+} channels in endothelial cells (Hallam and Pearson, 1986; Colder-Stanfield et al., 1987). Mesothelial and endothelial cells are both mesodermally derived cells arising from splanchnic mesoderm. However, because of close similarities, it is practically impossible to distinguish one from the other by morphology alone. Therefore, the characteristics of Ca^{2+} signaling in mesothelial cells may be useful in identifying and differentiating mesothelial from endothelial cells.

In the thoracic cavity, pleural mesothelial cells are the first cells to encounter foreign bodies, cells, and other substances entering the pleural space. A variety of pulmonary diseases, such as tuberculosis, malignancy, mineral dust exposure, and trauma, may be associated with pleural inflammation and injury. It has been reported in various organs that high-density angiotensin converting enzyme and angiotensin II receptor binding were anatomically coincident with sites of fibrous tissue formation and that this contrasted to non-injured tissue where respective binding densities were low (Weber et al., 1997). Angiotensin II involvement in tissue repair and fibrogenesis that follows inflammation can be inferred form these studies. We have shown that angiotensin II has a proliferative effect on pleural mesothelial cells. Therefore, it seems that pleural mesothelial cells may respond to various stimuli on pleura in healthy and diseased states.

5. Conclusion

We have shown that cultured pleural mesothelial cells respond to angiotensin II through the angiotensin AT_1 receptor coupled with pertussis toxin-insensitive G protein. Angiotensin II has a proliferative effect on pleural mesothelial cells. The Ca^{2+} signal may relate to angiotensin II-induced mesothelial cell proliferation. These results suggest that angiotensin II may play an important role in the pleural cavity in healthy and diseased states.

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References

André, P., Michel, M., Schott, C., Stoclet, J.C., 1992. Characterization of cultured rat aortic endothelial cells. J. Physiol. (Paris) 86, 177–184.

Berridge, M.J., 1987. Inositol triphosphate and diacylglycerol: two interacting second messengers. Annu. Rev. Biochem. 56, 59–193.

Bkaily, G., Jaalouk, D., Jacques, D., Economos, D., Hassan, G., Simaan, M., Pothier, P., 1997. Bradykinin activates R-, T-, and L-type Ca^{2+} channels and induces a sustained increase of nuclear Ca^{2+} in aortic vascular smooth muscle cells. Can. J. Physiol. Pharmacol. 75, 652–660.

Booz, G.W., Dostal, D.E., Singer, H.A., Baker, K.M., 1994. Involvement of protein kinase C and Ca^{2+} in angiotensin II-induced mitogenesis of cardiac fibroblasts. Am. J. Physiol. 267, C1308–C0318.

Boylan, A.M., Ruegg, C., Kim, K.J., Hebert, C.A., Hoeffel, J.M., Pytela, R., Sheppard, D., Goldstein, I.M., Broaddus, V.C., 1992. Evidence of a role for mesothelial cell-derived interleukin 8 in the pathogenesis of asbestos-induced pleurisy in rabbits. J. Clin. Invest. 89, 1257–1267.

Burnier, M., Centreno, G., Burki, E., Brunner, H.R., 1994. Confocal microscopy to analyze cytosolic and nuclear calcium in cultured vascular cells. Am. J. Physiol. 266, C1118–C1127.

Colder-Stanfield, M., Schilling, W.P., Ritchie, A.K., Eskin, S.G., Navarro, L.T., Kunze, D.L., 1987. Bradykinin-induced increases in cytosolic calcium and ionic currents in cultured bovine aortic endothelial cells. Circ. Res. 61, 632–640.

Duff, J.L., Berk, B.C., Corson, M.A., 1992. Angiotensin II stimulates the pp44 and pp42 mitogen-activated protein kinases in cultured rat aortic smooth muscle cells. Biochem. Biophys. Res. Commun. 188, 257–264.

Griendling, K.K., Rittenhouse, S.E., Brock, T.A., Ekstein, L.S., Gimbrone, M.A., Alexander, R.W., 1986. Sustained diacylglycerol formation from inositol phospholipids in angiotensin II-stimulated vascular smooth muscle cells. J. Biol. Chem. 261, 5901–5906.

Grynkiewicz, G., Poenie, M., Tsien, R.Y., 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. 260, 3440–3450.

Hallam, T.J., Pearson, J.D., 1986. Exogenous ATP raises cytoplasmic free calcium in fura-2 loaded piglet aortic endothelial cells. FEBS Lett. 207, 95–99.

Hallam, T.J., Pearson, J.D., Needham, L.A., 1988. Thrombin stimulated elevation of endothelial cell cytoplasmic free calcium concentration causes prostacyclin production. Biochem. J. 251, 243–249.

Haller, H., Lindschau, C., Erdmann, B., Quass, P., Luft, F.C., 1996. Effects of intracellular angiotension II in vascular smooth muscle cells. Circ. Res. 79, 765–772.

Hott, J.W., Sparks, J.A., Godbey, S.W., Antony, V.B., 1992. Mesothelial cell response to pleural injury: thrombin-induced proliferation and chemotaxis of rat pleural mesothelial cell. Am. J. Respir. Cell Mol. Biol. 6, 421–425.

Ito, K., Kuwahara, M., Sugano, S., Kuwahara, M., 1995. Role of intra- and extracellular calcium stores in mesothelial cell response to histamine. Am. J. Physiol. 268, L63–L70.

Kakar, S.S., Sellers, J.C., Devor, D.C., Musgrove, L.C., Neill, J.D., 1992. Angiotensin II type-1 receptor subtype cDNAs: differential tissue expression and hormonal regulation. Biochem. Biophys. Res. Commun. 183, 1090–1096.

Kambayashi, Y., Bardhan, S., Takahashi, K., Tsuzuki, S., Inui, H., Hamakubo, T., Inagami, T., 1993. Molecular cloning of a novel angiotensin II receptor isoform involved in phosphotyrosine phosphatase inhibition. J. Biol. Chem. 268, 24543–24546.

Kuwahara, M., Kagan, E., 1995. The mesothelial cell and its role in asbestos-induced pleural injury. Int. J. Exp. Pathol. 76, 163–170.

Kuwahara, M., Kuwahara, M., 1998. Pericardial mesothelial cells produce endothelin-1 and possess functional endothelin ET_B receptors. Eur. J. Pharmacol. 347, 329–335.

Kuwahara, M., Kuwahara, M., Bijwaard, K.E., Gersten, D.M., Diglio, C.A., Kagan, K., 1991. Mesothelial cells produce a chemoattractant

for lung fibroblasts: role of fibronectin. Am. J. Respir. Cell Mol. Biol. 5, 256–264.

Marrero, M.B., Paxton, W.G., Duff, J.L., Berk, B.C., Bernstein, K.E., 1994. Angiotensin II stimulates tyrosine phosphorylation of phospholipase C-gamma 1 in vascular smooth muscle cells. J. Biol. Chem. 269, 10935–10939.

Millan, M.A., Jacobowitz, D.M., Aguilera, G., Catt, K.J., 1991. Differential distribution of AT_1 and AT_2 angiotensin II receptor subtypes in the rat brain during development. Proc. Natl. Acad. Sci., USA 88, 11440–11444.

Molloy, C.J., Taylor, D.S., Weber, H., 1993. Angiotensin II stimulation of rapid protein tyrosine phosphorylation and protein kinase activation in rat aortic smooth muscle cells. J. Biol. Chem. 268, 7338–7345.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assay. J. Immunol. Methods 65, 55–63.

Mukoyama, M., Nakajima, M., Horiuchi, M., Sasamura, H., Prat, R.E., Dzau, V.J., 1993. Expression cloning of type 2 angiotensin II receptor reveals a unique class of seven-transmembrane receptors. J. Biol. Chem. 268, 24539–24542.

Murphy, T.J., Alexander, R.W., Griendling, K.K., Runge, M.S., Bernstein, K.E., 1991. Isolation of a cDNA encoding the vascular type-1 angiotensin II receptor. Nature 351, 233–236.

Naftilan, A.J., Pratt, R.E., Dzau, V.J., 1989. Induction of platelet-derived growth factor A-chain and c-*myc* gene expressions by angiotensin II in cultured rat vascular smooth muscle cells. J. Clin. Invest. 83, 1419–1424.

Nahmias, C., Strosberg, A.D., 1995. The angiotensin AT_2 receptor: searching for signal-transduction pathways and physiological function. Trends Pharmacol. Sci. 16, 223–225.

Nishizuka, Y., 1988. The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature 334, 661–665.

Owens, M.W., Grisham, M.B., 1993. Nitric oxide synthesis by rat pleural mesothelial cells: induction by cytokines and lipopolysaccharide. Am. J. Physiol. 265, L110–L116.

Peach, M.J., Dostal, D.E., 1990. The angiotensin II receptor and the actions of angiotensin II. J. Cardiovasc. Pharmacol. 16, S25–S30.

Sadoshima, J., Qiu, Z., Morgan, J.P., Izumo, S., 1995. Angiotensin II and other hypertrophic stimuli mediated by G protein-coupled receptors activate tyrosine kinase, mitogen-activated protein kinase, and 90-kD S6 kinase in cardiac myocytes. The critical role of Ca^{2+}-dependent signaling. Circ. Res. 76, 1–15.

Sasaki, K., Yamano, Y., Bardham, S., Iwai, N., Murray, J.J., Hasegawa, M., Matsuda, Y., Inagami, T., 1991. Cloning and expression of a complementary DNA encoding a bovine adrenal angiotensin II receptor. Nature 351, 230–233.

Sasamura, H., Hein, L., Krieger, J.E., Pratt, R.E., Kobilka, B.K., Dzau, V.J., 1992. Cloning, characterization, and expression of two angiotensin receptor (AT-1) isoforms from the mouse genome. Biochem. Biophys. Res. Commun. 185, 253–259.

Taubman, M.B., Berk, B.C., Izumo, S., Tsuda, T., Alexander, R.W., Nadal, G.B., 1989. Angiotensin II induces c-*fos* mRNA in aortic smooth muscle. Role of Ca^{2+} mobilization and protein kinase C activation. J. Biol. Chem. 264, 526–530.

Timmermans, P.B., Wong, P.C., Chiu, A.T., Herblin, W.F., Benfield, P., Carini, D.J., Lee, R.J., Wexler, R.R., Saye, J.A., Smith, R.D., 1993. Angiotensin II receptors and angiotensin II receptor antagonists. Pharmacol. Rev. 45, 205–251.

Tsuda, T., Kawahara, Y., Ishida, Y., Koide, M., Shii, K., Yokoyama, M., 1992. Angiotensin II stimulates two myelin basic protein/microtubule-associated protein 2 kinases in cultured vascular smooth muscle cells. Circ. Res. 71, 620–630.

Tsuzuki, S., Ichiki, T., Nakakubo, H., Kitami, Y., Guo, D.F., Shirai, H., Inagami, T., 1994. Molecular cloning and expression of the gene encoding human angiotensin II type 2 receptor. Biochem. Biophys. Res. Commun. 200, 1449–1454.

Wang, N.S., 1985. Mesothelial cells in situ. In: Chretien, J., Bignon, J., Hirsch, A. (Eds.), The Pleura in Health and Disease. Dekker, New York, pp. 23–42.

Weber, K.T., Sun, Y., Katwa, L.C., Cleutjens, J.P., 1997. Tissue repair and angiotensin II generated at sites of healing. Basic Res. Cardiol. 92, 75–78.