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Vasoconstrictor-induced protein-tyrosine phosphorylation in cultured vascular smooth muscle cells

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In cultured rat aortic smooth muscle cells, angiotensin II induced tyrosine phosphorylation of at least 9 proteins with molecular masses of 190, 117, 105, 82, 79, 77, 73, 45 and 40 kDa in time- and dose-dependent manners. Other vasoconstrictors such as [Arg]vasopressin, 5-hydroxytryptamine and norepinephrine induced the tyrosine phosphorylation of the same set of proteins as angiotensin II. The tyrosine phosphorylation of these proteins was mimicked by the protein kinase C-activating phorbol ester, phorbol 12 myristate 13-acetate, and the Ca^{2+} ionophore, ionomycin. These results demonstrate that the vasoconstrictors stimulate the tyrosine phosphorylation of several proteins in vascular smooth muscle cells and suggest that the tyrosine phosphorylation reactions are the events distal to the activation of protein kinase C and Ca^{2+} mobilization in the intracellular signalling pathways of the vasoconstrictors.

Vasoconstrictor; Tyrosine phosphorylation; Protein kinase C; Calcium; Vascular smooth muscle cell

1. INTRODUCTION

Stimulation of VSMC with vasoconstrictors such as ang II, AVP, 5HT and NE causes a rapid phospholipase C-mediated hydrolysis of inositol phospholipids resulting in the generation of two second messengers, diacylglycerol and inositol trisphosphate [1–4]. Diacylglycerol activates protein kinase C [5] whereas inositol trisphosphate mobilizes Ca^{2+} from intracellular Ca^{2+} stores [6] followed by the activation of Ca^{2+}/calmodulin-dependent protein kinases. Ca^{2+}/calmodulin-dependent activation of myosin light chain kinase induces myosin light chain phosphorylation which initiates vascular smooth muscle contraction [7]. However, little is known as to the molecular mechanisms by which these signalling pathways lead to the sustained contraction and growth of VSMC.

Protein-tyrosine phosphorylation has been considered to be involved in proliferation and transformation of cells because several viral transforming gene products and a number of growth factor receptors possess a tyrosine kinase activity [8,9]. Recently, there is increasing evidence that protein-tyrosine phosphorylation is involved not only in proliferation and transformation but also in other cellular responses such as secretion from various cell types [10–15]. However, it has not yet been reported whether protein-tyrosine phosphorylation is related to the vasoconstrictor actions in VSMC.

In the present study, we investigated the possible involvement of protein-tyrosine phosphorylation in the intracellular signalling pathways of vasoconstrictors in VSMC. The data reported here demonstrate that several vasoconstrictors including ang II, AVP, 5HT and NE induce the tyrosine phosphorylation of several proteins in cultured VSMC. These reactions are mimicked by the protein kinase C-activating phorbol esters and the Ca^{2+} ionophore, suggesting that these vasoconstrictors induce the protein-tyrosine phosphorylation through the activation of protein kinase C and Ca^{2+} mobilization.

Abbreviations: VSMC, vascular smooth muscle cells; ang II, angiotensin II; AVP, [Arg]vasopressin; 5HT, 5-hydroxytryptamine; NE, norepinephrine; PMA, phorbol 12-myristate 13-acetate; PDBu, phorbol 12,13-dibutyrate; 4α-PDD, 4α-phorbol 12,13-didecanoate; PDGF, platelet-derived growth factor; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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2. MATERIALS AND METHODS

2.1. *Materials*

VSMC were isolated from rat thoracic aorta by enzymatic dissociation as described previously [16]. Mouse monoclonal anti-phosphotyrosine antibody (IgM) was developed by immunizing with v-*abl*-expressing bacteria as described [17]. Growth media of hybridoma cells were purified by phosphotyrosine affinity column chromatography. Ang II, 5HT, prazosin, (±)-propranolol, PMA, PDBu, 4α-PDD and phosphoamino acids were purchased from Sigma (St. Louis, MO, USA). AVP, (±)-NE, ionomycin and recombinant human PDGF-BB were from Wako Pure Chemical Industries

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(Osaka, Japan), Aldrich (Milwaukee, WI, USA), Calbiochem (San Diego, CA, USA) and Collaborative Research (Bedford, MA, USA), respectively. Goat anti-mouse IgM conjugated with peroxidase was from Cappel (West Chester, PA, USA). Polyclonal rabbit anti-PDGF receptor β subunit antibody was kindly supplied by Dr. T. Matsui (Kobe University, Kobe, Japan). Other materials and chemicals were obtained from commercial sources.

2.2. *Assay for protein-tyrosine phosphorylation*

Confluent VSMC (passage levels 9–18) were cultured in serum-free Dulbecco's modified Eagle's medium for 48 h and stimulated with various stimuli. The reactions were terminated by chilling the dishes on ice and washing twice with ice-cold phosphate-buffered saline. The cells were lysed in the buffer containing 50 mM Tris-HCl, pH 7.5, 1 mM sodium vanadate, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride and 100 kallikrein-inactivating U/ml of aprotinin, and scraped from the dishes. Cell lysates were centrifuged for 15 min at 15 000 rpm using Beckman Microfuge E and the supernatants (150 μg of protein) were subjected to SDS-PAGE (8–16% gradient gel) using the buffer system of Laemmli [18]. The separated proteins were electrophoretically transferred to nitrocellulose membranes. Nitrocellulose blots were incubated with the mouse monoclonal anti-phosphotyrosine antibody and then with the peroxidase-labeled goat anti-mouse IgM. Peroxidase-labeled proteins were visualized by incubation with peroxidase color development reagents containing the enzyme substrate 3,3'-diaminobenzidine.

3. RESULTS

To detect phosphotyrosine-containing proteins, detergent extracts of VSMC were prepared in the presence of a phosphotyrosine phosphatase inhibitor, sodium vanadate, and subjected to SDS-PAGE followed by immunoblot analysis using the monoclonal anti-phosphotyrosine antibody. To test the reliability of this method, we first examined the effect of PDGF on tyrosine phosphorylation in VSMC since PDGF is well known to induce protein-tyrosine phosphorylation [19,20]. Upon stimulation with PDGF, immunoreactivity of several proteins was increased which comprised the most heavily stained protein at about 200 kDa, at least 3 proteins above 200 kDa, and lower molecular mass proteins at about 190, 158, 117, 105, 82, 79, 77, 73, 45 and 40 kDa (Fig. 1). These protein bands were specifically recognized on phosphotyrosine by the antibody because these protein bands were completely abolished when the blots were incubated with the antibody in the presence of 3 mM phosphotyrosine, but not in the presence of the same concentration of phosphoserine or phosphothreonine (Fig. 2). The 200-kDa protein was recognized by anti-PDGF receptor β subunit antibody (data not shown). These results are consistent with the previous observations that PDGF induces tyrosine phosphorylation of several proteins including PDGF receptors in various cell types [19,20]. Under the same conditions, ang II also increased the im-

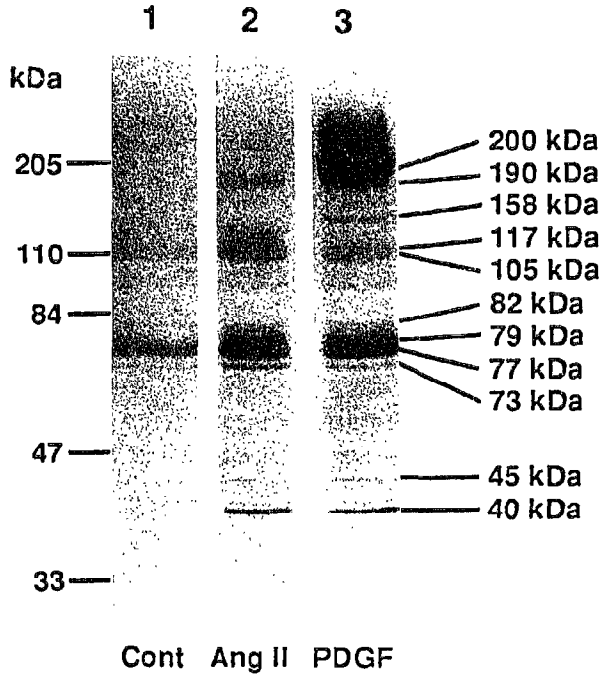

Fig. 1. Anti-phosphotyrosine immunoblot of VSMC stimulated with ang II and PDGF. Cultured rat aortic VSMC were stimulated with 100 nM and II for 2 min or with 50 ng/ml PDGF for 5 min. Lane 1, control (Cont); lane 2, ang II; lane 3, PDGF. The positions of ang II- and PDGF-responsive proteins are indicated at right. The experiment shown respresents one of six independent trials which gave nearly identical results.

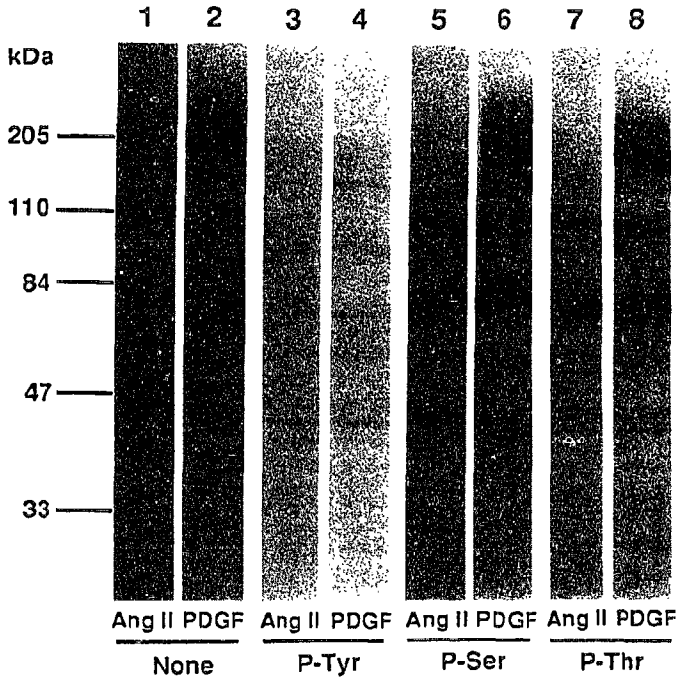

Fig. 2. Effects of phosphoamino acids on anti-phosphotyrosine immunoblot. Cultured rat aortic VSMC were stimulated with 100 nM ang II for 2 min or with 50 ng/ml PDGF for 5 min. Immunoblot analysis was carried out in the absence or the presence of either 3 mM phosphotyrosine, phosphoserine or phosphothreonine during the incubation with the anti-phosphotyrosine antibody. Lanes 1, 3, 5 and 7, stimulated with ang II; lanes 2, 4, 6 and 8, stimulated with PDGF. Lanes 1 and 2, in the absence of phosphoamino acids (None); lanes 3 and 4, phosphotyrosine (P-Tyr); lanes 5 and 6, phosphoserine (P-Ser); lanes 7 and 8, phosphothreonine (P-Thr). The experiment shown represents one of three independent trials which gave nearly identical results.

munoreactivity to the anti-phosphotyrosine antibody of at least 9 proteins at about 190, 117, 105, 82, 79, 77, 73, 45 and 40 kDa (Fig. 1). All these protein bands migrated at the same positions on SDS-PAGE as those of PDGF-responsive 190-, 117-, 105-, 82-, 79-, 77-, 73-, 45- and 40-kDa proteins. The appearance of these protein bands was also specifically blocked by the presence of phosphotyrosine (Fig. 2), indicating that these proteins were tyrosine-phosphorylated in response to ang II. The relative intensities of these protein bands were somewhat varied from experiment to experiment but the 79-, 77-, 73-, 45- and 40-kDa proteins were prominently phosphorylated by ang II in the repeated experiments.

Fig. 3 shows the time course of ang II-induced protein-tyrosine phosphorylation. The tyrosine phosphorylation of the 73-kDa protein peaked around 1 min after ang II stimulation followed by a gradual decline to nearly the basal level within 30 min. The tyrosine phosphorylation of the 45- and 40-kDa proteins peaked around 2 min and was declined to nearly the basal level within 15 min after ang II stimulation. Whereas the tyrosine phosphorylation of the 79- and 77-kDa proteins peaked around 5 min and was sustained above the basal level for at least 30 min after ang II stimulation. Fig. 4 shows the dose-response relationship of ang II-induced protein-tyrosine phosphorylation. The maximal level of tyrosine phosphorylation of the 73-kDa protein was obtained at as low as 0.1–1 nM, while the maximal levels of the 45- and 40-kDa proteins, and the 79- and 77-kDa proteins were obtained at 1–10 nM and 10–100 nM ang II, respectively.

As shown in Fig. 5, AVP, 5HT and NE induced the tyrosine phosphorylation of the same set of proteins as ang II. The effect of NE was markedly inhibited by

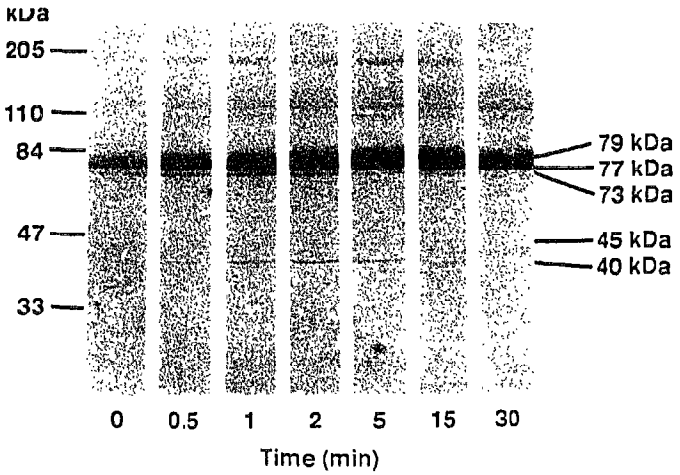

Fig. 3. Time course of ang II-induced tyrosine phosphorylation. Cultured rat aortic VSMC were stimulated with 100 nM and ang II for various periods of time as indicated. The positions of the 79-, 77-, 73-, 45- and 40-kDa proteins are indicated at right. The experiment shown represents one of four independent trials which gave nearly identical results.

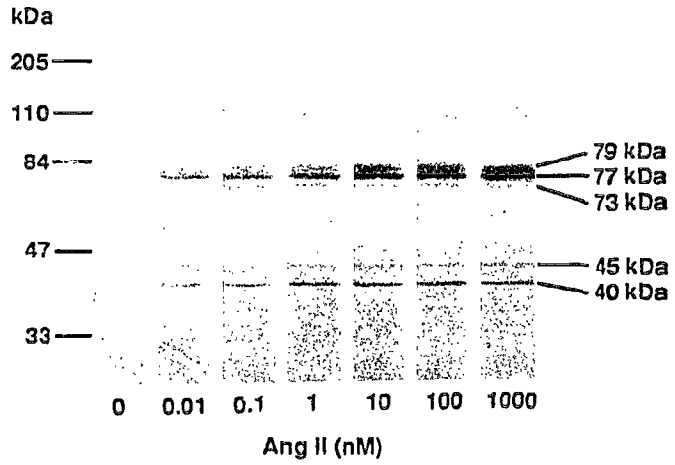

Fig. 4. Dose-response relationship of ang II-induced tyrosine phosphorylation. Cultured rat aortic VSMC were stimulated for 2 min with various concentrations of ang II as indicated. The positions of the 79-, 77-, 73-, 45- and 40-kDa proteins are indicated at right. The experiment shown represents one of three independent trials which gave nearly identical results.

equimolar concentration of the α_1-adrenergic receptor antagonist, prazosin, but not by the β-adrenergic receptor antagonist, propranolol, indicating that the effect of NE is mediated via α_1-adrenergic receptors.

All the vasoconstrictors described above induce the phospholipase C-mediated hydrolysis of inositol phospholipids resulting in the activation of two signal-

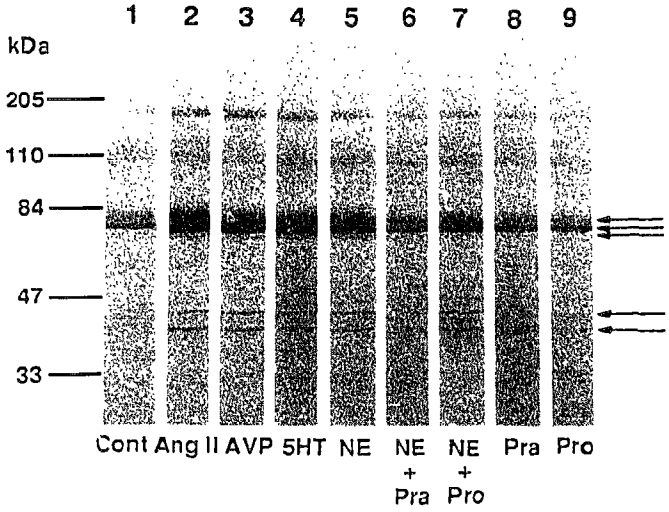

Fig. 5. Effects of various vasoconstrictors on tyrosine phosphorylation. Cultured rat aortic VSMC were stimulated for 2 min with 100 nM ang II, 100 nM AVP or 100 nM 5HT, or with 10 μM NE in the absence or presence of 10 μM prazosin or 10 μM propranolol. Lane 1, control (Cont); lane 2, ang II; lane 3, AVP; lane 4, 5HT; lane 5, NE; lane 6, NE in the presence of prazosin (Pra); lane 7, NE in the presence of propranolol (Pro); lane 8, prazosin; lane 9, propranolol. Arrows indicate the positions of the 79-, 77-, 73-, 45- and 40-kDa proteins. The experiment shown represents one of three independent trials which gave nearly identical results.

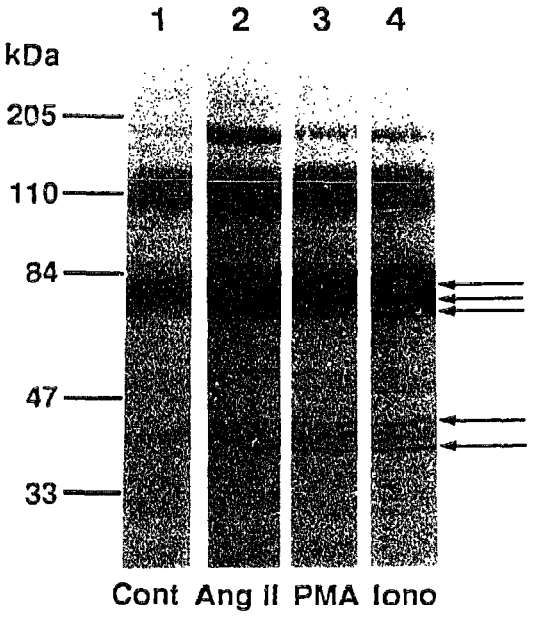

Fig. 6. Effects of PMA and ionomycin on tyrosine phosphorylation. Cultured rat aortic VSMC were stimulated with 100 nM ang II for 2 min, 100 nM PMA for 5 min or 2 μM ionomycin for 1 min. Lane 1, control (Cont); lane 2, ang II; lane 3, PMA; lane 4, ionomycin (Iono). Arrows indicate the positions of the 79-, 77-, 73-, 45- and 40-kDa proteins. The experiment shown represents one of three independent trials which gave nearly identical results.

ling pathways, diacylglycerol-protein kinase C and inositol trisphosphate-Ca^{2+} [1–4]. In the last set of experiments, we examined the relationships between these signalling pathways and the protein-tyrosine phosphorylation. Protein kinase C-activating phorbol ester, PMA, induced the tyrosine phosphorylation of the same set of proteins as the vasoconstrictors (Fig. 6). Another active phorbol ester, PDBu, also induced these reactions whereas 4α-PDD which does not activate protein kinase C failed to induce the reactions (data not shown). Fig. 6 also shows that the vasoconstrictor-induced protein-tyrosine phosphorylation was mimicked by the Ca^{2+} ionophore, ionomycin.

4. DISCUSSION

We showed in the present study that ang II and other vasoconstrictors such as AVP, 5HT and NE stimulated protein-tyrosine phosphorylation of the same set of proteins in VSMC. These observations could reflect either the activation of tyrosine kinases or the inhibition of phosphotyrosine phosphatases during the action of these vasoconstrictors. The differences in the time courses and dose-response relationships of ang II-induced tyrosine phosphorylation of the individual proteins suggest that several distinct tyrosine kinases and/or phosphotyrosine phosphatases are involved in these reactions. Although molecular mechanisms by which the vasoconstrictors activate tyrosine kinases or inhibit phosphotyrosine phosphatases are uncertain, the results of the present study provide the first demonstration of vasoconstrictor-induced protein-tyrosine phosphorylation in VSMC.

We also demonstrated that the protein kinase C-activating phorbol esters and the Ca^{2+} ionophore stimulated the tyrosine phosphorylation of the same set of proteins as the vasoconstrictors. Since all the vasoconstrictors described here activate two signalling pathways, diacylglycerol-protein kinase C and inositol trisphosphate-Ca^{2+} in VSMC [1–4], these observations strongly suggest that the vasoconstrictors induce the protein-tyrosine phosphorylation through the activation of protein kinase C and Ca^{2+} mobilization. Consistent with this idea, current studies have shown that various protein kinase C-activating and Ca^{2+}-mobilizing stimuli induce protein-tyrosine phosphorylation in their respective target cells such as thrombin and collagen in platelets [10–12], carbachol in chromaffin cells [13] and crosslinking of membrane immunoglobulins in basophilic leukemia cells and B lymphocytes [15,21]. Recently, Huckle et al. [22] have reported that ang II stimulates protein-tyrosine phosphorylation in WB rat liver epithelial cells in a Ca^{2+}-dependent but protein kinase C-independent manner. The reason for the difference between our results and theirs is not clear at present, but it may be due to the difference in cell types.

The receptor-mediated tyrosine phosphorylation is essential for the mitogenic action of PDGF [23], but physiological roles of the vasoconstrictor-induced tyrosine phosphorylation in VSMC are at present entirely speculative. The results of the present study indicate that there are tyrosine-phosphorylated proteins common to both the vasoconstrictors and PDGF, and those specific for PDGF which include PDGF receptors. Since and II per se is not mitogenic for VSMC [24,25], these observations suggest that the tyrosine phosphorylation of the common substrates is not sufficient and the tyrosine phosphorylation of the PDGF-specific substrates is required for the mitogenic action for VSMC. Alternatively, the tyrosine phosphorylation of the common substrates may be responsible for the cellular responses common to the vasoconstrictors and PDGF such as contraction and protein synthesis [24–26]. Identification of the individual substrate proteins is absolutely necessary to elucidate the precise roles of the vasoconstrictor-induced tyrosine phosphorylation in VSMC functions.

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