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PEPTIDE GROWTH FACTORS MARKEDLY DECREASE THE LIGAND BINDING OF ANGIOTENSIN II TYPE 2 RECEPTOR IN RAT CULTURED VASCULAR SMOOTH MUSCLE CELLS

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Of two major isoforms of angiotensin II receptors, AT_1 and AT_2, biological roles of AT_2 remain unclear. Using vascular smooth muscle cells, we investigated the regulation of expression of AT_2 by growth factors in comparison with that of AT_1. The cultured rat aorta smooth muscle cells had detectable AT_2 binding sites, which were reduced significantly by treatment with platelet derived growth factor-BB. On the other hand, AT_1 binding sites were increased under the same conditions. Other growth factors, such as epidermal growth factor and endothelin-1, also suppressed AT_2 receptors to varying extents. A negative correlation between DNA synthesis promoted by these growth factors and the binding capacity of AT_2 sites was observed. This study indicated that the expression of AT_2 is downregulated in cultured vascular smooth muscle cells by growth factors in contrast to that of AT_1, which was slightly upregulated.

At least two major isoforms of angiotensin II receptors have been identified to date. They are distinct from each other in their binding selectivity to isoform-specific ligands. Angiotensin II type 1 receptor (AT_1) has a high affinity to losartan, whereas type 2 receptor (AT_2) is defined as those that have a low affinity to losartan and high affinities to other angiotensin II receptor ligands, such as PD 123319 and CGP 42112A (1-4). The AT_1 receptors have been cloned from bovine and rat tissues in our and other laboratories (5, 6) and has been characterized as the G-protein-coupled receptors with putative seven trasmembrane domains. On the other hand, the molecular nature and regulatory mechanism of AT_2 have not yet been clarified inspite of extensive tissue distribution studies in the brain, uterus, adrenal, aorta and the fetus (1, 4, 7-13).

In vascular smooth muscle cells, transition from a quiescent to proliferative state is considered as a critical turning point in the development of atheromatous plaques or neointimal formaton. Some peptide growth factors, such as platelet derived growth factor (PDGF), are involved in this process as a trigger for migration and growth of vascular smooth muscle cells (14, 15). The abundant and extensive expression of AT_2 receptor in fetal tissues suggests that AT_2 might play some roles in the development and differentiation of the fetal tissues (7, 8, 16). We investigated possible regulation of AT_2 in vascular

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smooth muscle cells by growth factors with the objective of delineating the possible role of AT_2 in remodeling or differentiation of vascular vessels.

We have found evidence that the vascular AT_2 is down-regulated by PDGF-BB, a typical growth factor for vascular smooth muscle cells, and that the down-regulation is correlated with [^{3}H]thymidine uptake elicited by various growth factors.

METHODS AND MATERIALS

Materials: Human platelet derived growth factor (PDGF)-BB and epidermal growth factor (EGF) were purchased from Upstate Biotechnology Incorporation (Lake Placid, NY). Human endothelin-1 (ET-1) was from Pennisula Laboratories (Belmont, CA). [^{125}I]NaI and [^{3}H]thymidine were purchased from New England Nuclear (Boston, MA). Other reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Dup753 and CGP42112A were gifts of DuPont (Wilmington, DE) and Ciba Gigey (Basel, Switzerland), respectively.

Cells: Rat vascular smooth muscle cells were obtained by the enzymatic dispersion method from the thorathic aorta of Wister Kyoto rats (17, 18) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and gentamycin (50 mg/l) under the atomosphere of 5 % CO_2 and 95 % O_2. The cells were used for experiments after 48 h serum deprivation maintained in 5 mg/l insulin, 5 mg/l transferrin, and 5 mg/l sodium selenite, unless stated otherwise.

Iodination of ligands: [^{125}I]CGP 42112A and [^{125}I]Sarile ([Sar^1, Ile^8]-angiotensin II), were prepared by the conventional lactoperoxidase method (19). The products were purified by reverse phase HPLC.

Binding experiments: The number of AT_1 binding sites was estimated with [^{125}I]Sarile in the presence and absence of losartan (1 μM), an AT_1 specific antagonist. The number of AT_2 binding sites was estimated by the binding of [^{125}I]CGP 42112A, a specific ligand for AT_2. Vascular smooth muscle cells grown in 6-well plates were washed once with Hank's buffered saline solution (HBSS) and incubated for 90 - 120 min at an ambient temperature with an iodinated ligand (0.5 nM) in DMEM including 0.2 % bovine serum albumin. After the binding, free ligand was washed out with ice-cold HBSS and the cells were solubilized in 0.2N NaOH, and the cell-bound radioactivity was counted by a γ-counter. Protein concentration was determined using a Bio-Rad protein assay kit.

[^{3}H]Thymidine incorporation: Cells were treated with growth factors for 1 h unless stated otherwise. Sixteen hours later they were incubated with 2 μCi [^{3}H]thymidine for 4 h and were successively washed with HBSS, 10 % trichloroacetic acid and ethanol-ether (2:1), then solubilized in 1.5 ml of 0.2 N NaOH and mixed with 10 ml of scintilant BCN, (Amersham, Arlington Heights, IL) for counting.

Statistics: Comparison between groups were made by two-factor ANOVA. Differences between individual mean values were tested by t-test.

RESULTS

Effects of PDGF and heparin on expression of AT_2 in vascular smooth muscle cells.

PDGF-BB is a typical growth factor for vascular smooth muscle cells (13, 20) and heparin is known as a growth inhibitor (21). In order to see how proliferation of the cells affects the expression of AT_2, we examined the effects of these growth factor and inhibitor on the expression of AT_1 and AT_2 receptors. As shown in Fig. 1A, 48 h incubation with 25 ng/ml PDGF-BB decreased the AT_2 binding sites by 88 ± 5 %, whereas 50 units/ml heparin slightly (36 ± 25 %) increased it. By contrast, AT_1 binding sites were significantly increased by 27 ± 5 % by the same treatment with PDGF-BB (Fig. 1B).

As PDGF is known to phosphorylate various cellular proteins, the down-regulation of AT_2 by PDGF could be caused by the reduction of affinity of the receptor to ligand due to

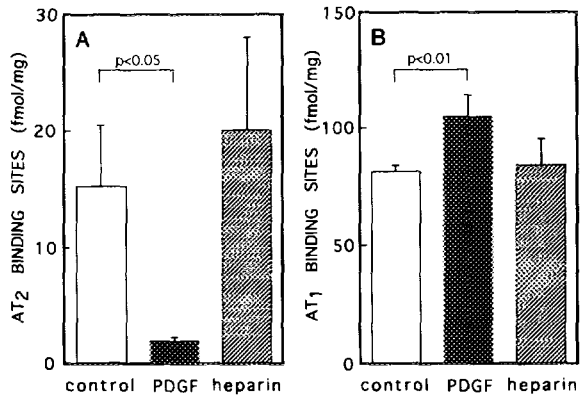

Fig. 1. Effects of PDGF-BB on expression of angiotensin II receptors.
Vascular smooth muscle cells which had been deprived of serum for 48 h were treated with 50 units/ml heparin or 25 ng/ml PDGF-BB for 48 h. AT_2 sites were estimated using 0.5 nM [^{125}I]CGP 42112A (**A**) and AT_1 sites were estimated using 0.5 nM [^{125}I]Sarile (**B**). Non-specific binding was determined using 1 μM CGP 42112A and losartan, respectively.

possible modification of the receptor molecule. In order to rule out this possibility, Scatchard analysis for [^{125}I]CGP 42112A binding was performed for cells treated with PDGF-BB or heparin. As shown in Fig. 2, the change in binding is solely attributable to the change in the number of maximum binding sites.

Effects of other growth factors on expression of AT_2 in vascular smooth muscle cells.

To see whether the reduction of AT_2 binding sites is specific to PDGF-BB, we also carried out similar experiments for other growth factors, such as EGF and ET-1. The

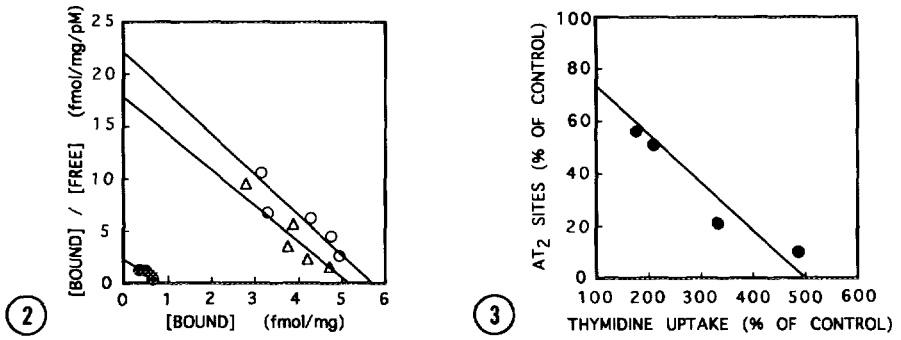

Fig. 2. Scatchard analysis for angiotensin II type 2 receptor under various conditions.
Vascular smooth muscle cells were cultured for 48 h without (○) or with 50 units/ml heparin (△) or 25ng/ml PDGF-BB (●). After binding experiments using various concentrations of CGP 42112A, [Bound] / [Free] was plotted against [Bound]. The slopes and intercepts on the abscissa were determined after optimization by linear regression analysis.

Fig. 3. Relationship between down-regulation of angiotensin II type 2 receptor and DNA synthesis stimulated by growth factors.
Vascular smooth muscle cells were treated with PDGF-BB (1h or 16h), ET-1 (16 h) or EGF (1 h). Binding and thymidine uptake experiments were carried out 16 h after the challenge by growth factors. The data are mean values from two to five experiments.

treatment with 300 nM ET-1 for 16 h decreased the AT_2 sites by 45 ± 21 %. [^{3}H]Thymidine incorporation was increased under the same conditions by 94 ± 50 %. Time course studies for the PDGF-induced down-regulation indicated that 1 and 16 hour-exposures to PDGF-BB (25 ng/ml) caused 70 % and 85 % reduction in AT_2 receptor number, respectively. Thus the 48 hour treatment employed here seems to elicit maximum decrease in AT_2 receptor. Only 1 hour treatment is known to be sufficient for PDGF to exert its proliferative effects. The present data indicate a short term exposure is sufficient also for bringing about the down-regulation, suggesting that the early cellular responses stimulated by PDGF-BB trigger the cellular process. Likewise a short-term exposure was effective for EGF (100 ng/ml) stimulation; CGP 42112A binding decreased by 43 ± 6 % and [^{3}H]thymidine uptake was increased by 75 ± 16 % in 1 hour. For all these experiments, the percent change in CGP 42112A binding was plotted versus the one in [^{3}H]thymidine uptake (Fig. 3). There was a significant negative correlation between AT_2 receptor number and the mitogenic activity of the growth factors ($\gamma^2 = 0.934$, $p < 0.05$). These results indicate that the down-regulation of AT_2 is not specific to PDGF-BB but related to cellular responses leading to cell proliferation.

DISCUSSION

We found differential regulation of angiotensin II type 1 receptor (AT_1) and type 2 receptor (AT_2) by using the combination of the subtype specific ligand losartan (AT_1), [^{125}I]CGP 42112A (AT_2) and non-specific ligand [^{125}I]Sarile. AT_2 was down-regulated by growth factors such as PDGF-BB or EGF, whereas AT_1 is upregulated under the same conditions. We also demonstrated that the down-regulation of AT_2 is related to the DNA synthesis caused by these growth factors.

It is well-known that growth factors, such as PDGF, activate transcription of immediate early genes such as *c-fos* and *c-jun*, which in turn activates transcription of other genes (22). On the other hand, it was recently reported that the transcription of myosin heavy chain, a muscle cell specific protein, was negatively regulated by basic fibroblast growth factor through a protein kinase C - mediated mechanism, which subsequently inactivated the transcriptional activity of myogenin (23). Taken together, the differential regulation of AT_1 (upregulation) and AT_2 (down-regulation) could be two different ways of expression mediated by a common growth factor - stimulated mechanism at least in early cellular responses. A similar differential regulation of receptor isoforms was reported for PDGF receptors (24). PDGF-receptor α-subunit was down regulated in quiescent 3T3 cells in response to various growth factors and β-subunit was not affected under the same conditions. The underlying mechanisms and the physiological significance of the differential regulation are not yet clear.

AT_1 was shown to be involved in the progression of vascular hypertrophy and neointimal thickening after angioplasty (25, 26). The present finding of the negative correlation of AT_2 receptor expression to cell growth suggests that AT_2 might counteract the action of AT_1. Recently it was demonstrated that AT_2 in PC12W cells modulated protein

tyrosin phosphatase activity(27), which is presumably involved in regulation of cell growth and differentiation (28). Thus the vascular AT_2 might be involved in the differentiation of vascular vessels through the modulation of protein tyrosin phosphatase activity.

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