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STIMULATION OF PLATELET-DERIVED GROWTH FACTOR-INDUCED DNA SYNTHESIS BY ANGIOTENSIN II IN RABBIT VASCULAR SMOOTH MUSCLE CELLS[1]

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SUMMARY: In cultured rabbit vascular smooth muscle cells (VSMCs), angiotensin II by itself had little mitogenic effect even in the presence of cell-free plasma-derived serum (PDS), but markedly stimulated the platelet-derived growth factor (PDGF)-induced DNA synthesis in the presence of PDS. The maximal extent of DNA synthesis induced by PDGF plus angiotensin II was about twice that induced by PDGF alone. The stimulatory effect of angiotensin II was dose-dependent with the maximal response seen at 1 μM and was inhibited by the specific angiotensin II receptor antagonist, [Sar^1,Ile^8]angiotensin II. In VSMCs, both PDGF and angiotensin II induced expression of the c-fos gene in dose-dependent manners. In contrast to the synergistic effect of angiotensin II and PDGF on DNA synthesis, they induced expression of the c-fos gene in an additive manner. These results suggest that angiotensin II may act as a growth regulator for VSMCs in addition to acting as a vasoconstrictor.

VSMC proliferation has been recognized as central to the pathology of both major forms of vascular diseases: hypertension and atherosclerosis (for a review, see Ref. 1). Thus, it is important to understand the cellular mechanisms involved in the control of VSMC proliferation. Tissue culture studies with VSMCs have identified several growth factors that stimulate VSMC proliferation: these include PDGF (2), EGF (3), fibroblast growth

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The abbreviations used are: VSMC, vascular smooth muscle cell; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; PDS, cell-free plasma-derived serum.

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factor (3) and a growth factor released from endothelial cells (4). Among them, PDGF released not only from platelets but also from macrophages, endothelial cells and VSMCs is considered to play a central role in VSMC proliferation in atherosclerotic lesions (for a review, see Ref. 5). Previous reports from our laboratories have shown that PDGF induces the phospholipase C-mediated hydrolysis of phosphoinositides in rabbit VSMCs and that protein kinase C is involved at least partly in the mitogenic action of PDGF for this cell type (6,7).

Angiotensin II is a potent vasoconstrictor which plays a crucial role in the pathogenesis of some forms of hypertension (for a review, see Ref. 8). It has been well demonstrated that angiotensin II induces the phospholipase C-mediated hydrolysis of phosphoinositides followed by the protein kinase C activation and the intracellular Ca^{2+} mobilization (9-12). Recently, we and others have shown in rat VSMCs that angiotensin II induces expression of a proto-oncogene, c-*fos*, through the protein kinase C activation and the intracellular Ca^{2+} mobilization (13,14). This gene is implicated in the regulation of cell proliferation (for a review, see Ref. 15). Moreover, interesting evidence has been presented that PDGF and EGF induce the contraction of vascular strips (16,17). These observations have promptly raised the question as to whether angiotensin II can also act as a growth factor for VSMCs.

In the present studies, we have addressed the possible role of angiotensin II in the proliferation of VSMCs and found that this vasoconstrictor markedly stimulates the PDGF-induced DNA synthesis in rabbit VSMCs although it has little mitogenic effect by itself. This paper also provides the first definitive demonstration of the PDGF-induced expression of the c-*fos* gene in VSMCs.

EXPERIMENTAL PROCEDURES

Materials and Chemicals – The method of VSMC isolation from the aortae of male Japanese White rabbits has been described previously (6). Recombinant PDGF, a homodimer of B-chains, was obtained from Amersham Japan, Tokyo, Japan. This preparation of PDGF exhibited a purity of at least 95% based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis followed by silver staining. Angiotensin II and [Sar^1,Ile^8]angiotensin II were generous gifts from Ciba-Geigy Ltd., Basle, Switzerland and Daiichi Seiyaku Co., Tokyo, Japan, respectively. [methyl-^{3}H]Thymidine and [α-^{32}P]dCTP were from Amersham Japan, Tokyo, Japan. Cell-free PDS was prepared as described previously (6). A pBR322 plasmid containing the v-*fos* gene (p*fos*-1) was a generous gift from Drs. T. Sugiyama (Kyoto University School of Medicine, Kyoto, Japan) and R.

Takahashi (Kobe Uiversity School of Medicine, Kobe, Japan) who originally obtained it from Dr. I.M. Verma (Molecular Biology and Virology Laboratory, The Salk Institute, San Diego, USA). Other materials and chemicals were obtained from commercial sources.

Assays — DNA synthesis was assayed by measuring the incorporation of [^{3}H]thymidine into acid-insoluble materials or autoradiography of the labeled nuclei as described previously (18). The c-fos mRNA levels were analyzed by dot blotting or Northern blotting as described previously (13). The amount of the probe hybridized was proportional to the amount of RNA on the filter at a range from 1 to 8 μg.

RESULTS

Incubation of quiescent cultures of VSMCs with various doses of PDGF in the presence of 10% PDS induced DNA synthesis in a dose-dependent manner as shown in Fig. 1. Angiotensin II markedly stimulated the PDGF-induced DNA synthesis at any PDGF concentration in the presence of PDS. The maximal level of DNA synthesis induced by PDGF plus angiotensin II was about twice that induced by

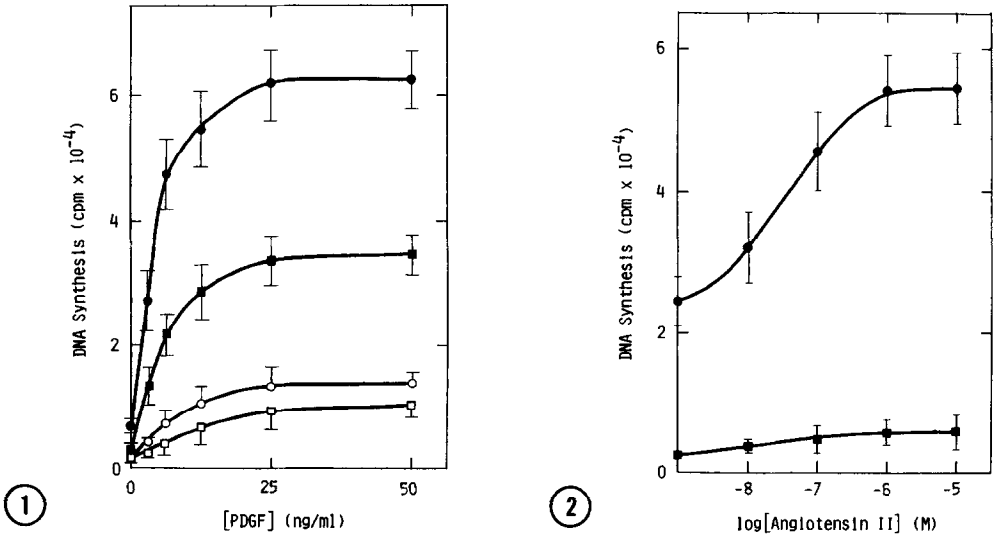


Fig. 1. Stimulation by angiotensin II of the PDGF-induced DNA synthesis. Quiescent cultures of VSMCs were stimulated by various doses of PDGF with (●,○) or without (■,□) 10 μM angiotensin II in the presence (●,■) or absence (○,□) of 10% PDS. After 24 h, DNA synthesis was assayed by measuring the incorporation of [^{3}H]-thymidine into acid-insoluble materials. Results are presented as the mean ± SE of at least five separate experiments.

Fig. 2. Dose-dependent effect of angiotensin II on the PDGF-induced DNA synthesis. Quiescent cultures of VSMCs were stimulated by various doses of angiotensin II with (●) or without (■) 25 ng/ml of PDGF in the presence of 10% PDS. After 24 h, DNA synthesis was assayed by measuring the incorporation of [^{3}H]-thymidine into acid-insoluble materials. Results are presented as the mean ± SE of three separate experiments.

PDGF alone. Angiotensin II alone had little mitogenic activity even in the presence of PDS. In the absence of PDS, PDGF induced DNA synthesis to a small extent and angiotensin II had little effect on the PDGF-induced DNA synthesis. Figure 2 shows the effect of various doses of angiotensin II with or without PDGF on DNA synthesis in the presence of PDS. Angiotensin II stimulated the PDGF-induced DNA synthesis in a dose-dependent manner and the maximal response was seen at 1 μM.

In Figs. 1 and 2, DNA synthesis was assayed by measuring the incorporation of [^{3}H]thymidine into acid-insoluble materials. When it was assayed by autoradiography of the labeled nuclei, 24.2 ± 2.0% (mean ± SE) of the cells were labeled after treatment with 25 ng/ml of PDGF in the presence of PDS. The labeled cells increased to 47.5 ± 4.1% after treatment with 25 ng/ml of PDGF plus 10 μM angiotensin II in the presence of PDS. The control cells treated with PDS alone had an extremely low level of [^{3}H]thymidine labeling, and the labeled cells did not increase significantly

Table 1

Receptor-dependent effect of angiotensin II on the PDGF-induced DNA synthesis

Additions	DNA synthesis (cpm x 10^{-4})
PDS	0.3 ± 0.1
PDS + PDGF	3.5 ± 0.3
PDS + PDGF + angiotensin II	6.3 ± 0.5
PDS + PDGF + angiotensin II + [Sar^1,Ile^8]angiotensin II	3.8 ± 0.4
PDS + PDGF + [Sar^1,Ile^8]angiotensin II	3.6 ± 0.3

Quiescent cultures of VSMCs were preincubated for 10 min with or without 10 μM [Sar^1,Ile^8]angiotensin II, and then stimulated by 25 ng/ml of PDGF with or without 10 μM angiotensin II in the presence of 10% PDS. After 24 h, DNA synthesis was assayed by measuring the incorporation of [^{3}H]thymidine into acid-insoluble materials. Results are presented as the mean ± SE of four separate experiments.

after treatment with angiotensin II alone even in the presence of PDS (data not shown).

Using a specific angiotensin II receptor antagonist, [Sar^1,Ile^8]angiotensin II, we examined whether the stimulatory effect of angiotensin II on the PDGF-induced DNA synthesis was receptor-mediated. As shown in Table 1, an equimolar concentration of [Sar^1,Ile^8]angiotensin II markedly attenuated the stimulatory effect of angiotensin II. This antagonist by itself did not exert any effect on DNA synthesis. These results indicate that angiotensin II stimulates the PDGF-induced DNA synthesis *via* its specific receptor.

Kindy and Sonenshein (19) have described that expression of the c-*fos* gene may be involved in the fetal calf serum-induced proliferation of calf VSMCs. However, it has not yet been demonstrated whether PDGF induces expression of the c-*fos* gene in VSMCs. Figure 3 shows that PDGF as well as angiotensin II induces a transient increase in the c-*fos* mRNA level as estimated by Northern blot analysis. The time courses of the increase in the c-*fos* mRNA level were similar to each other and the maximal level was obtained at 30 min after the addition of either PDGF or angiotensin II. However, the maximal level of the c-*fos* mRNA induced by PDGF was significantly higher than that induced by angiotensin II. The dose-response curves of PDGF and angiotensin II for the increase in the c-*fos* mRNA level are shown in Fig. 4. The doses of PDGF and angiotensin II necessary for the increase in

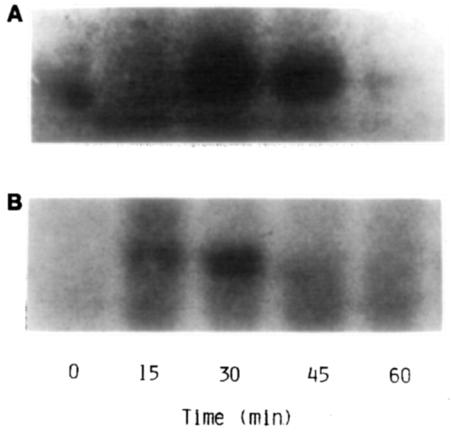
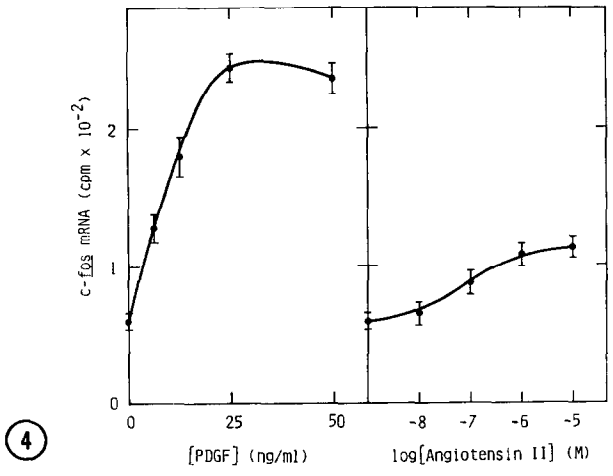


Fig. 3. Increase in the c-*fos* mRNA level induced by PDGF or angiotensin II. Quiescent cultures of VSMCs were stimulated by 25 ng/ml of PDGF (A) or 10 μM angiotensin II (B) for various periods of time. The c-*fos* mRNA levels were analyzed by Northern blotting.



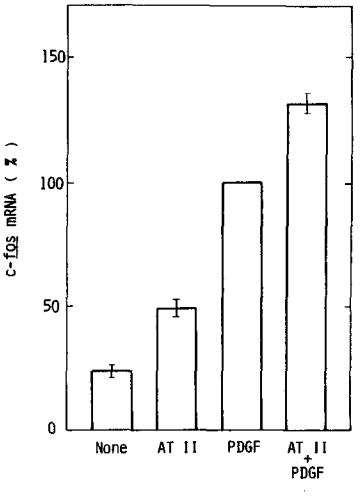


Fig. 4. Dose-response curves of PDGF and angiotensin II for the increase in the c-fos mRNA level. Quiescent cultures of VSMCs were stimulated by various doses of PDGF (left panel) or angiotensin II (right panel) for 30 min. The c-fos mRNA levels were analyzed by dot blotting. Results are presented as the mean ± SE of three separate experiments.

Fig. 5. Additive effect of PDGF and angiotensin II on the increase in the c-fos mRNA level. Quiescent cultures of VSMCs were stimulated by 10 μM angiotensin II alone, 25 ng/ml of PDGF alone or 10 μM angiotensin II plus 25 ng/ml of PDGF for 30 min. The c-fos mRNA levels were analyzed by dot blotting. The c-fos mRNA levels were expressed as a percentage of the level after the incubation with PDGF alone. Results are presented as the mean ± SE of three separate experiments. AT II indicates angiotensin II.

the c-fos mRNA level were similar to those necessary for DNA synthesis (Figs. 1, 2 and 4).

In order to investigate the intracellular mechanisms of the stimulatory effect of angiotensin II on the PDGF-induced DNA synthesis, we examined expression of the c-fos gene during the simultaneous action of PDGF and angiotensin II. Figure 5 shows a quantitative analysis of the c-fos mRNA level during the action of angiotensin II alone, PDGF alone, and angiotensin II plus PDGF. The time course of the increase in the c-fos mRNA level during the action of angiotensin II plus PDGF was similar to that during the action of either of them, and the maximal level was obtained at 30 min after the addition of angiotensin II plus PDGF (data not shown). In contrast to the synergistic effect of angiotensin II and PDGF on DNA synthesis, they increased the c-fos mRNA level in an additive manner.

DISCUSSION

In the present studies, we have explored the possibility that angiotensin II can act as a growth factor for VSMCs and shown that

this vasoconstrictor markedly stimulates the PDGF-induced DNA synthesis *via* its specific receptor although it shows little mitogenic activity by itself for VSMCs. We and others have previously shown that angiotensin II as well as PDGF induces the phospholipase C-mediated hydrolysis of phosphoinositides in VSMCs (7,9-12). However, in contrast to PDGF, angiotensin II by itself shows little mitogenic activity for VSMCs. These results suggest that the signaling pathway mediated by the phospholipase C reactions alone is not sufficient and that other signaling pathway(s) is(are) also necessary for DNA synthesis in VSMCs. It has been described that PDGF stimulates the tyrosine kinase activity of PDGF receptors (20). Tyrosine kinase activity is characteristic of several other growth factor receptors (21). Tyrosine phosphorylation has not been well studied in VSMCs, but it is possible that, in addition to the phospholipase C-mediated pathway, the tyrosine kinase-mediated pathway is also necessary for the mitogenic action of PDGF in VSMCs.

In Swiss 3T3 cells, PDGF induces the phospholipase C-mediated hydrolysis of phosphoinositides and activates the c-*fos* gene through the protein kinase C activation and the intracellular Ca^{2+} mobilization (22-24). However, it has not yet been demonstrated whether PDGF induces expression of the c-*fos* gene in VSMCs, one of the most important target cells for this growth factor *in vivo*. In the present studies, we have also shown that PDGF indeed induces expression of the c-*fos* gene in VSMCs. In contrast to the synergistic effect of angiotensin II and PDGF on DNA synthesis, they induce the increase in the c-*fos* mRNA level in an additive manner and the c-*fos* mRNA level does not increase in proportion to the increase in DNA synthesis. These observations suggest that the stimulatory effect of angiotensin II on the PDGF-induced DNA synthesis cannot be explained simply by the stimulatory effect of this vasoconstrictor on expression of the c-*fos* gene. Further studies are required to clarify the intracellular mechanisms of the synergistic effect of angiotensin II and PDGF on DNA synthesis of VSMCs.

Hypertension is well recognized as a major risk factor for the development of atherosclerosis, but the mechanism whereby it exerts this effect is unknown. It has been suggested that the association between hypertension and atherosclerosis relates to the accelerated proliferation of VSMCs which is a common feature in both diseases (25). Our present results that angiotensin II markedly stimulates the PDGF-induced DNA synthesis of VSMCs may explain the relation-

ship between some forms of hypertension and accelerated atherosclerosis.

REFERENCES

1. Ross, R., and Glomset, J.A. (1976) N. Engl. J. Med. 295, 369-377, 420-425.
2. Ross, R., Glomset, J., Kariya, B., and Harker, L. (1974) Proc. Natl. Acad. Sci. USA 71, 1207-1210.
3. Gospodarowicz, D., Moran, J.S., and Braun, D.L. (1977) J. Cell. Physiol. 91, 377-385.
4. Gajdusek, C., DiCorleto, P., Ross, R., and Schwartz, S.M. (1980) J. Cell Biol. 85, 467-472.
5. Ross, R. (1986) N. Engl. J. Med. 314, 488-500.
6. Kariya, K., Kawahara, Y., Tsuda, T., Fukuzaki, H., and Takai, Y. (1987) Atherosclerosis 63, 251-255.
7. Kawahara, Y., Kariya, K., Araki, S., Fukuzaki, H., and Takai, Y. (1988) Biochem. Biophys. Res. Commun. 156, 846-854.
8. Kaplan, N.M. (1982) Clinical Hypertension, (N.M. Kaplan, Ed.) pp. 210-232, Williams & Wilkins, Baltimore.
9. Smith, J.B., Smith, L., Brown, E.R., Barnes, D., Sabir, M.A., Davis, J.S., and Farese, R.V. (1984) Proc. Natl. Acad. Sci. USA 81, 7812-7816.
10. Nabika, T., Velletri, P.A., Lovenberg, W., and Beaven, M.A. (1985) J. Biol. Chem. 260, 4661-4670.
11. Alexander, R.W., Brock, T.A., Gimbrone, M.A.,Jr., and Rittenhouse, S.E. (1985) Hypertension 7, 447-451.
12. Griendling, K.K., Rittenhouse, S.E., Brock, T.A., Ekstein, L.S., Gimbrone, M.A.,Jr., and Alexander, R.W. (1986) J. Biol. Chem. 261, 5901-5906.
13. Kawahara, Y., Sunako, M., Tsuda, T., Fukuzaki, H., Fukumoto, Y., and Takai, Y. (1988) Biochem. Biophys. Res. Commun. 150, 52-59.
14. Taubman, M.B., Berk, B.C., Izumo, S., Tsuda, T., Alexander, R. W., and Nadal-Ginard, B. (1989) J. Biol. Chem. 264, 526-530.
15. Callahan, M., Cochran, G.H., and Stiles, C.D. (1985) Growth Factors in Biology and Medicine, Ciba Foundation Symposium 116, (D. Evered, J. Nugent, and J. Whelan, Eds.) pp. 87-96, Pitman, London.
16. Berk, B.C., Brock, T.A., Webb, R.C., Taubman, M.B., Atkinson, W.J., Gimbrone, M.A.,Jr., and Alexander, R.W. (1985) J. Clin. Invest. 75, 1083-1086.
17. Berk, B.C., Alexander, R.W., Brock, T.A., Gimbrone, M.A.,Jr., and Webb, R.C. (1986) Science 232, 87-90.
18. Kariya, K., Fukumoto, Y., Tsuda, T., Yamamoto, T., Kawahara, Y., Fukuzaki, H., and Takai, Y. (1987) Exp. Cell Res. 173, 504-514.
19. Kindy, M.S., and Sonenshein, G.E. (1986) J. Biol. Chem. 261, 12865-12868.
20. Ek, B., and Heldin, C.-H. (1982) J. Biol. Chem. 257, 10486-10491.
21. Hunter, T., and Cooper, J.A. (1985) Ann. Rev. Biochem. 54, 897-930.
22. Habenicht, A.J.R., Glomset, J.A., King, W.C., Nist, C., Mitchell, C.D., and Ross, R. (1981) J. Biol. Chem. 256, 12329-12335.
23. Berridge, M.J., Heslop, J.P., Irvine, R.F., and Brown, K.D. (1984) Biochem. J. 222, 195-201.
24. Tsuda, T., Hamamori, Y., Yamashita, T., Fukumoto, Y., and Takai, Y. (1986) FEBS Lett. 208, 39-42.
25. Schwartz, S.M., Campbell, G.R., and Campbell, J.H. (1986) Circ. Res. 58, 427-444.