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CV-11974, the Active Metabolite of TCV-116 (Candesarten), Inhibits the Synergistic or Additive Effect of Different Growth Factors on Angiotensin II-Induced Proliferation of Vascular Smooth Muscle Cells

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ABSTRACT. Many previous studies have demonstrated that angiotensin II (AII) type 1 (AT1) receptor antagonists remarkably reduced intimal lesions in rats following balloon injury. Using vascular smooth muscle cells (VSMC) in culture, we tested the hypothesis that other classical growth factors may enhance AII effects on VSMC growth, and AT1 receptor antagonists may inhibit these effects. AII, platelet-derived growth factor-BB (PDGF-BB), and epidermal growth factor (EGF) caused a 3426 ± 262%, 277 ± 69%, and 1568 ± 62% increase in [^{3}H]thymidine incorporation in VSMC (mean ± SD, n = 3), respectively. The exposure of the cells to AIIin combination with PDGF-BB or EGF resulted in an approximately 2-fold or 1.5-fold elevation of the AII-dependent effect, respectively. 2-ethoxy-1-[[2′-(1H-tetrazol-5-yl)biphenyl-4-yl]methyl]-1H-benzimidazole-7-carboxylic acid (CV-11974), the active metabolite of the specific nonpeptide AT_1 receptor antagonist (±)-1-cyclohexyloxycarbonyloxy)ethyl 2-ethoxy-1-[[2′-(1H-tetrazol-5-yl)biphenyl-4-yl]methyl]-1H-benzimidazole-7-carboxylate (TCV-116, Candesartan), suppressed the effect of AII down to basal values, as well as reducing the synergistic effect of PDGF or the additive effect of EGF on AII-induced [^{3}H]thymidine incorporation. AII and PDGF-BB *per se* induced 57 ± 19 and 70 ± 14% increase in VSMC number. Combination of both agonists resulted in a 2-fold increase of the AII effect on cell number. Again, CV-11974 blocked the effect of AII, as well as the additive effect of PDGF-BB on cell number. From these findings, it may be concluded that AT1 receptor antagonists may reduce or prevent the development of intimal lesions following vascular injury through inhibition of direct and indirect growth-promoting effects of AII in VSMC. BIOCHEM PHARMACOL 52;1:123–126, 1996.

KEY WORDS. CV-11974; angiotensin II; VSMC growth

VSMC† proliferation plays an important pathophysiological role in the development of both major forms of vascular diseases, hypertension and atherosclerosis [1, 2]. It has been suggested that intimal lesions occur through migration of VSMC into the intima and by proliferation of VSMC [3]. The renin-angiotensin system (RAS) represents a cascade of biochemical events leading to the generation of AII. Local generation and the autocrine and paracrine actions of AII have been shown in the vascular system and the heart [4]. Many previous studies have demonstrated that AII type 1 (AT1) receptor antagonists [5, 6], as well as angiotensin-converting-enzyme (ACE) inhibitors [7], significantly reduced intimal lesions in rats following balloon injury (approximately 50% to 80% reduction). It is remarkable that the administration of a single drug that blocks the growth-promoting effects of AII on VSMC caused such a strong inhibition of the intimal lesions in response to catheter-induced vascular injury. This observation is surprising because according to the response-to-injury hypothesis of Ross, platelets release several growth factors for vascular smooth muscle cells following endothelial injury, such as platelet-derived growth factor (PDGF) or EGF [3]. Thus, it is conceivable that AII may promote intimal lesions directly or in combination with other growth factors such as PDGF or EGF. Therefore, we examined the hypothesis that the prevention of intimal lesions by AT1 receptor antagonists occurs through inhibition of direct and indirect growth-promoting effects of AII in VSMC. This hypothesis was evaluated by investigating the effect of CV-11974 on the mitogenic effect of AII in the presence and absence of PDGF or EGF in VSMC.

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† *Abbreviations:* AII, angiotensin II; CV-11974, 2-ethoxy-1-[[2′-(1H-tetrazol-5-yl)biphenyl-4-yl]methyl]-1H-benzimidazole-7-carboxylic acid; TCV-116, (±)-1-cyclohexyloxycarbonyloxy)ethyl 2-ethoxy-1-[[2′-(1H-tetrazol-5-yl)biphenyl-4-yl]methyl]-1H-benzimidazole-7-carboxylate; VSMC, vascular smooth muscle cells; PDGF-BB, platelet-derived growth factor-BB; EGF, epidermal growth factor; DMEM, Dulbecco's modified Eagles medium.

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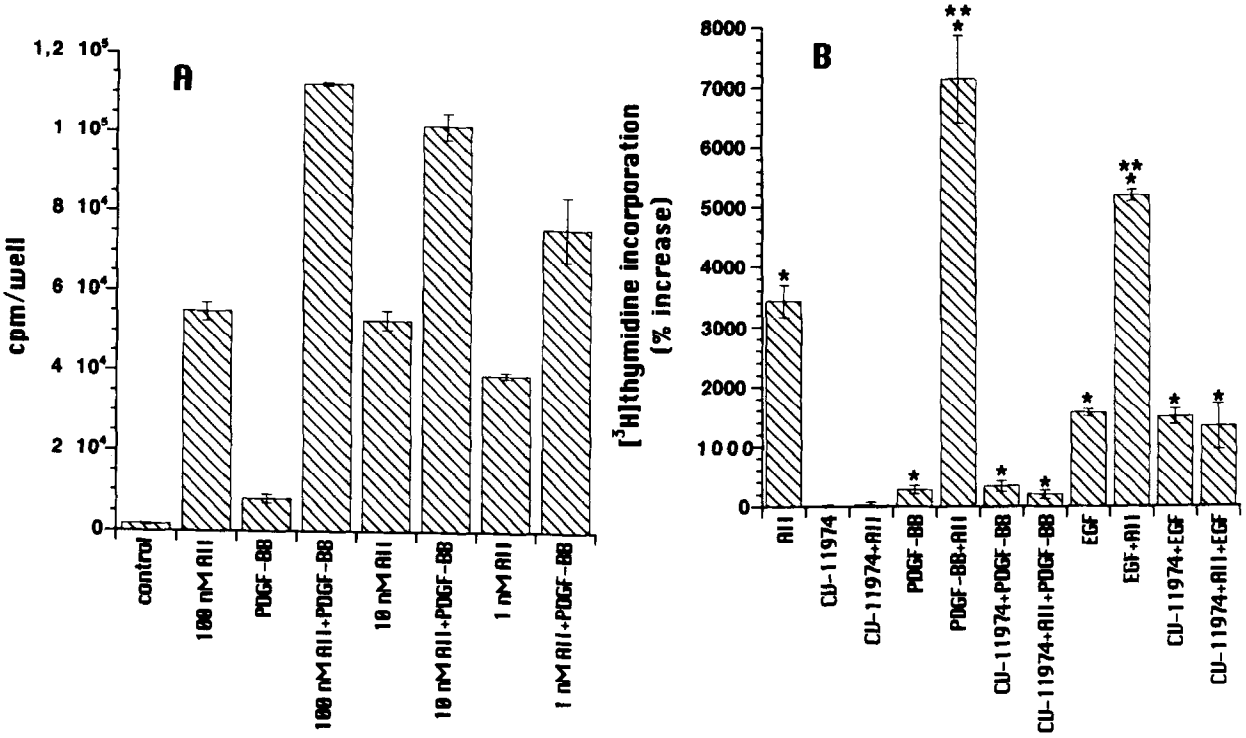

FIG. 1. Effect of AII in the presence and absence of PDGF and EGF on [^{3}H]thymidine incorporation into cell DNA. (A): Confluent cells (24-well plates) were precultured for 24 hr in the quiescent medium and, then, stimulated with AII at different concentrations and PDGF-BB (50 ng/mL). Following another 20-hr incubation, cells were exposed to 3 μCi/mL [^{3}H]thymidine. The reaction was terminated 4 hr later and [^{3}H]thymidine incorporation into cell DNA was quantified (one representative experiment performed in triplicate wells). Values are expressed as means ± SD. (B): Data from individual experiments, each performed with triplicate wells, were standardized by calculating the means ± SD of 3 individual experiments and expressed as % increase above the basal value of the unstimulated cells. CV-11974 at a concentration of 1 nM was added 5 min before cell stimulation with AII in the presence and absence of PDGF-BB or EGF. *$P < 0.05$, **$P < 0.05$ for AII + PDGF-BB vs AII effect ***$P < 0.05$ for AII + EGF vs AII effect.

MATERIALS AND METHODS

Materials

DMEM, Ham's F-10, and PBS were obtained from Gibco-BRL (Eggenstein, Germany). Other chemicals were obtained from Sigma Chemical (Deisenhofen, Germany). PDGF-BB and EGF were obtained from Boehringer Mannheim (Mannheim, Germany). CV-11974 was obtained from Takeda Chemical Industries, Ltd., Osaka, Japan, *via* Takeda Euro R&D Centre, Frankfurt/Main, Germany.

Isolation of Vascular Smooth Cells

Rat aortic smooth muscle cells were isolated using thoracic aortas from Wistar-Kyoto rats (6–8 weeks old, Charles River Wiga GmbH, Sulzfeld, Germany) by enzymatic dispersion according to the method of Chamley *et al.* [8]. Cells were cultured over several passages as previously described [9].

Determination of [^{3}H]thymidine Incorporation

The effect of AII on DNA synthesis was measured as previously described [10]. VSMC were seeded on 24-well culture plates and cultivated in culture medium until confluence. Then the medium was replaced by serum-free quiescent medium consisting of a mixture of DMEM and Ham's F-10 medium (1:1). Following another 24-hr cultivation in quiescent medium, CV-11974, and 10 min later, AII (final concentration, 10^{-7} M) were added to the cells. Following 20 hr of incubation, 3 μCi/mL [^{3}H]thymidine were added to the quiescent medium. Four hours later, the experiments were terminated as described previously [10].

Determination of Cell Number

For cell counting, VSMC were seeded in 24-well culture plates (5×10^4 cells/well, well diameter 12 mm) and cultured in DMEM, supplemented with 10% fetal calf serum nonessential amino acids, penicillin 100 IU/mL, and streptomycin 100 μg/mL at 37°C for 24 hr. Under these conditions, a cell confluence of approximately 70% was reached. The medium was, then, replaced by serum-free quiescent medium consisting of DMEM and Ham's F-10 (1:1, v/v), and VSMC were stimulated with AII in the presence and absence of PDGF or CV-11974. After 24 hr, cells were trypsinized and resuspended in DMEM plus Trypan Blue. Cell counting was performed with a Neubauer-cell-box by light microscopy.

Statistics

Values are expressed as means ± SD. Statistical analysis of the data was performed using the Mann-Whitney U-test (StatView 512^{+TM}, version 1.0, Apple Computer, Inc.). Triplicate wells were analyzed for each [^{3}H]thymidine incorporation experiment, and each experiment was performed independently a minimum of 3 times.

RESULTS

As illustrated in Fig. 1A, AII at a concentration of 100, 10, and 1 nM caused an increase in [^{3}H]thymidine incorporation into cell DNA from 1738 ± 192 to 54,966 ± 2205, 52,965 ± 729, and 39,150 ± 729 cpm/well, respectively (one representative experiment performed in triplicate, mean ± SD). PDGF-BB (50 ng/mL) induced an increase to 7902 ± 1078 cpm/well and enhanced the effect of 100, 10, and 1 nM to 112,470 ± 434, 101,950 ± 3380, and 76,143 ± 8037 cpm/well, respectively. For standardization of 3 different independent experiments (each performed in triplicate), the % increase in AII-induced [^{3}H]thymidine incorporation above basal value in the absence and presence of PDGF-BB (50 ng/mL), EGF (20 ng/m), and CV-11974 (1 nM) was evaluated. As seen in Fig. 1B, AII (100 nM), PDGF-BB, and EGF caused a 3426 ± 262%, 277 ± 69%, and 1568 ± 62% increase in [^{3}H]thymidine incorporation (mean ± SD, n = 3). PDGF-BB and EGF enhanced the effect of AII from 3426% to 7146% and 5199% above the basal value, respectively. CV-11974 completely blocked the mitogenic effect of AII *per se*, as well as abolishing the synergistic effect of PDGF-BB or the additive effect of EGF. As expected, CV-11974 had no effect on EGF and PDGF-BB mitogenic effects. Figure 2 shows the effects of AII and PDGF-BB or their combination on cell number. AII and PDGF-BB *per se* induced a 57 ± 19 and 70 ± 14% increase in cell number over the basal value, respectively (mean ± SD, n = 3). PDGF-BB in combination with AII enhanced the AII-dependent increase in cell count to 134 ± 16%. Furthermore, as demonstrated in Fig. 2, CV-11974 suppressed the effect of AII down to basal values, as well as reducing the synergistic effect of PDGF-BB down to PDGF-BB values.

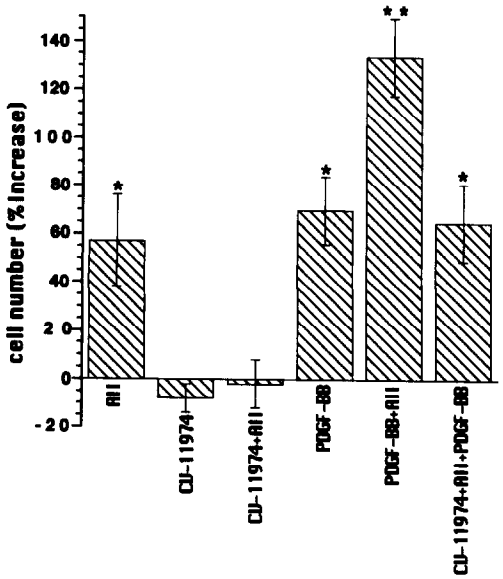

FIG. 2. Effect of CV-11974 on the Ang II-dependent increase in VSMC number in the presence and absence of PDGF-BB. Cells (5 × 10^4) were seeded per well (24-well plates) in cultured medium containing 10% FCS. After 24 hr, approximately 70% confluence was reached. Then, the medium was replaced with quiescent medium and the cells immediately stimulated with AII (100 nM) in the presence and absence of PDGF-BB (50 ng/mL). CV-11974 at a concentration of 1 nM was added 5 min before cell stimulation. After 24 hr, cells were trypsinized and counted. Data represent the means ± SD of 3 individual experiments. *P < 0.05, **P < 0.05 for AII + PDGF-BB vs AII effect.

DISCUSSION

Following binding to the AT1 receptor, AII stimulates the phosphoinositide signalling system, leading to various cellular processes, such as cell growth and constriction of vascular smooth muscle cells [11]. TCV-116 is an orally active long-lasting, and highly specific nonpeptide AT 1 receptor antagonist with antihypertensive effects in renal and genetically hypertensive rats [6].

Whether AII stimulation of VSMC induces cell hypertrophy, defined as an increase in cellular mass or size [12, 13], or cell proliferation, defined as an increase in [^{3}H]thymidine incorporation into cell DNA, is still a matter of controversy [10, 14]. Berk *et al.* and Geisterfer *et al.* reported that AII neither stimulates VSMC proliferation nor enhances PDGF-dependent cell proliferation [12, 13]. However, Daemen *et al.* demonstrated that AII induces smooth muscle cell proliferation in the normal and injured rat arterial wall [14]. Our group has recently demonstrated that the effect of angiotensin II on DNA synthesis varies considerably in cultured vascular smooth muscle cell from different Wistar-Kyoto rats [10].

In our experiments, we observed changes in [^{3}H]thymidine incorporation, as well as changes in cell number as an indicator for cell proliferation following cell stimulation with AII. In addition, the proliferative effects of AII were remarkably augmented by the classical growth factors PDGF and EGF. The proliferative effects of AII *per se* or the synergistic effect of PDGF-BB and the additive effect of EGF were all specifically blocked by preincubation of VSMC with the AT1 receptor antagonist. Although the effect of PDGF-BB on the angiotensin II-induced increase in [^{3}H]thymidine incorporation was synergistic, its effects on the angiotensin-induced elevation in cell number seem to be additive rather than synergistic. Thus, stimulation of the cells with AII in the presence of PDGF-BB may induce polyploidy of vascular smooth muscle cells.

Obviously, because the concentrations of AII used in our experiments exceeded by far those physiologically occurring *in vivo*, one has to question the physiological relevance of

our findings derived from cultured cells. Although the local concentration of such factors in vascular tissues under pathological conditions is completely unknown, one may speculate that the concentrations of such growth factors may reach values similar to those used in cultured cells for a short period of time. However, cultured VSMC cells are a useful model for explaining observations obtained *in vivo*.

It is important to note that our experiments were performed in cultured VSMC of the rat aorta, and extrapolation to *in vivo* models should be considered with caution. It is still attractive, however, to suggest that the mitogenic effects of AII *per se* or in combination with other classical growth factors may play an important role in the development of intimal lesions.

Finally, from our results, one may speculate that TCV-116 possesses a beneficial effect on the development of intimal lesions (e.g., occurring after coronary angioplasty) through inhibition of the growth-promoting effects of AII in VSMC.

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