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Angiotensin II Stimulates the Proliferation of Osteoblast-Rich Populations of Cells from Rat Calvariae

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We examined the effects of angiotensin II (Ang II) on the proliferation of osteoblast-rich populations of cells obtained from calvariae of newborn rat. Addition of Ang II to the culture medium caused dose-dependent increases in the rate of DNA synthesis. Such increases were completely inhibited by the addition of DuP753, an antagonist of AT_1 receptor. Ang II potentiated the production of inositol 1,4,5-trisphosphate (IP_3) in the culture. Ang II also stimulated the activities of mitogen-activated protein kinases (MAPKs) upon binding to the AT_1 receptor. These results suggest that Ang II might be intimately involved in the proliferation of the cells in calvariae through the AT_1 receptor.

Angiotensin II (Ang II) is the well known vasoactive peptides. The peptides are implicated in blood-pressure control and body-fluid homeostasis. In addition to its well-established role in circulatory homeostasis, Ang II has been linked to a variety of process, which include the control of nervous system activity (1, 2), cell growth (3, 4), and developmental processes (2, 5). Two different types of Ang II receptor have been cloned, namely the type 1 (AT_1) receptor (6, 7) and the type 2 (AT_2) receptor (8, 9). It has been reported that the AT_1 receptor regulates two distinct signaling pathways that involve G proteins. Ang II is converted from Ang I by angiotensin-converting enzyme (ACE). ACE inhibitors, such as captopril and enarapril, have been well used as medicine for patients with hypertension. However, ACE inhibitors cause neonatal hypotension and renal failure (10). Moreover, an aborted fetus, exposed to ACE inhibitors, in whom no skull formation was present above the brain tissue. Thus, the use of ACE inhibitors during pregnancy has been the high risk of hypocalvaria or acalvaria in the fetus (11, 12). Therefore, to see whether Ang II is indeed involved in the development of calvariae, we examined the effect of Ang II on the proliferation of calvarial cells enzymatically obtained from newborn rat.

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MATERIALS AND METHODS

Materials. Human Ang II was purchased from the Peptide Institute, Osaka, Japan. DuP 753 (a specific antagonist of AT_1 receptors) and PD-123319 (a specific antagonist of AT_2 receptors) were generous gifts from Dupont/Merck (Wilmington, DE, USA) and Dr. Hitoshi Miyazaki (Tsukuba University, Japan), respectively. α-MEM and penicillin/streptomycin antibiotic mixture were obtained from Life Technologies, Inc., Grand Island, NY, USA; and fetal bovine serum was obtained from Filtron, Victoria, Australia.

Cell culture. Cells were isolated enzymatically from calvariae of newborn Sprague-Dawley rats. The constituents of the enzyme mixture and the sequential digestion procedure for the isolation of rat calvarial cells have been described previously (13). The cells were replated in 96-well or 12-well plates at a density of 1×10^4 cells/cm^2 and grown in α-MEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 5 mM β-glycerophosphate, and 50 μg/ml ascorbic acid. During subculture, the medium was replaced every 4 days and Ang II and/or an analog was added every 2 days.

Assay of the proliferation of cells. Osteoblast-like cells were seeded in 96-well plates at a density of 3,500 cells per well and cultured for 5 days in the presence of 10% fetal bovine serum. Then cells were washed twice with serum-free α-MEM and incubated with serum-free α-MEM for 72 h. After incubation of cells in fresh serum-free α-MEM that contained Ang II and/or an analog at various concentrations for 24 h, the proliferation of cells was examined by an enzyme immunoassay (5-Bromo-2′-Deoxy-Uridine Labeling and Detection Kit III; Boehringer Mannheim, Mannheim, Germany) with 5-bromo-2′-deoxy-uridine (BrdU).

Measurement of the production of inositol 1,4,5-trisphosphate (IP_3). Cells that had been subcultured in 9-cm^2 dishes for 9 days, were incubated with serum-free α-MEM for 3 h. This culture medium was supplemented with 10^{-7} M Ang II for various brief times as indicated, and then the medium was removed by aspiration and replaced with 0.5 ml of 4% perchloric acid. After incubation of the cells in perchloric acid on ice for 20 min, the supernatant was brought to pH 7.5 by titration with ice-cold 1.5 M KOH that contained 60 mM Hepes buffer. The IP_3 generated was quantitated with a D-*myo*-inositol 1,4,5-trisphosphate (IP_3) [^{3}H] assay kit (Amersham Life Science).

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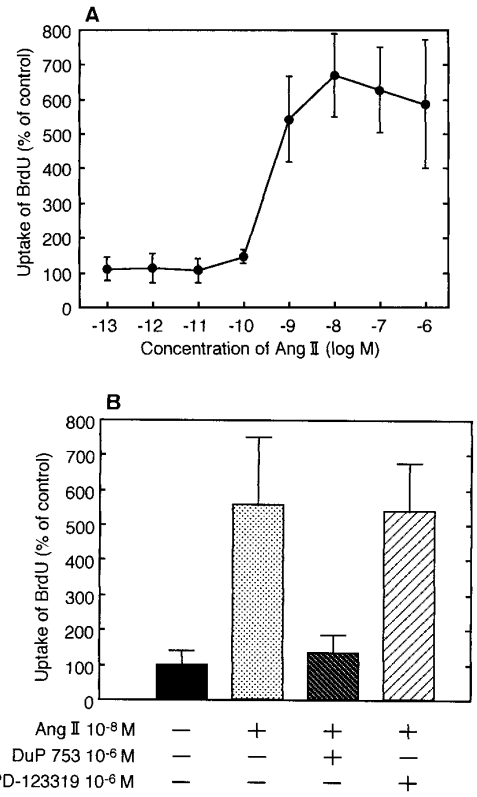

FIG. 1. Uptake of BrdU by osteoblast-like cells after exposure to Ang II. Osteoblast-like cells were grown to confluence in 96-well plates (5 days). After growth of cells had been arrested by culture in serum-free medium for 72 h, cells were exposed to Ang II at various concentrations with or without a receptor subtype-specific antagonist for 24 h. BrdU that had been incorporated into cells upon treatment with Ang II was quantitated as described in "Materials and Methods." (A) Dependence of the incorporation into DNA of BrdU on the concentration of Ang II. The control value refers to the extent of incorporation of BrdU by cells in serum-free α-MEM that contained no Ang II. Values are expressed as percentages of control values. Data are means ± SD of results from five wells. (B) The effects of receptor subtype-specific antagonists on the uptake of BrdU. Values represent the means ± SD of results from five wells.

Measurement of the activities of mitogen-activated protein kinases (MAPKs). Cells were subcultured in α-MEM that contained 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 5 mM β-glycerophosphate, and 50 μg/ml ascorbic acid in 9-cm^2 dishes for 7 days. Subsequently, cells were incubated with serum-free α-MEM for 48 h. Finally they were incubated with various concentrations of Ang II for 10 min and lysed by homogenization in 0.5 ml of 10 mM Tris-HCl buffer (pH 7.4) that contained 150 mM NaCl, 2 mM EGTA, 2 mM dithiothreitol, 1 mM orthovanadate, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. After centrifugation of the homogenate at 16,000 × g at 4 °C for 20 min, the supernatant was stored at −80 °C prior to the assay. The activities of MAPKs were determined with a p42/p44 MAP kinase enzyme assay kit (Amersham Life Science).

RESULTS AND DISCUSSION

In the present study, we examined the effects of Ang II on the incorporation of BrdU into the DNA of cells

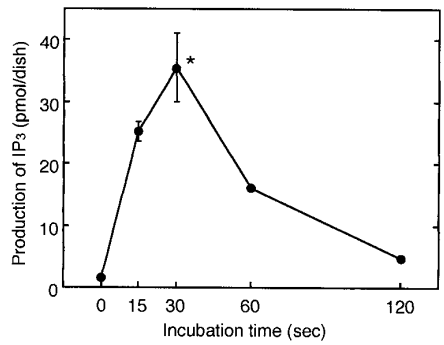

FIG. 2. The Ang II-induced accumulation of IP_3. Cells in 9-cm^2 dishes were exposed to 10^{-7} M Ang II for 0 to 120 sec at 37°C. Subsequent steps for determination of levels of IP_3 are described in "Materials and Methods." Values represent the means ± SD of results from three dishes. *, $p < 0.0005$ *versus* 0 sec.

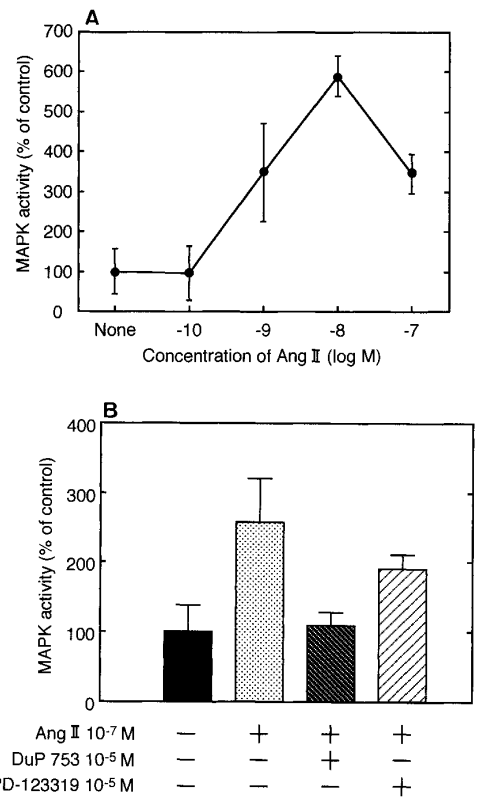

FIG. 3. Activation of MAPKs by Ang II. Cells that had been cultured for 9 days in 9-cm^2 dishes were exposed to Ang II at increasing concentrations with or without a receptor subtype-specific antagonist for 5 min at 37°C. The activity of MAPKs in the Ang II-treated cells was quantitated as described in "Materials and Methods." (A) Dose-dependent activation of MAPKs by Ang II. The control value refers to the activity of MAPKs in cells incubated in α-MEM without angiotensin II. Values are expressed as percentages of control values. Data are means ± SD of results from three dishes. (B) The effects of receptor subtype-specific antagonists on the activation of MAPKs. Values represent the means ± SD of results from three dishes.

in this culture system. Figure 1A shows the dose-dependent stimulation of mitogenic activity by Ang II. Ang II at 10^{-9} M clearly increased in the rate of uptake of BrdU. Figure 1B shows the marked enhancement (approximately 550% increase) in the mitogenic activity of cells exposed to 10^{-8} M Ang II. This acceleration of DNA synthesis by Ang II was prevented by the addition of 10^{-6} M DuP 753 to the culture medium, while 10^{-6} M PD-123319 had no effect. Next we measured the production of IP_3 and the activities of MAPKs by Ang II in osteoblast-rich populations of cells. Ang II promoted the formation of IP_3 in a time-dependent manner (Fig. 2). Incubation with 10^{-7} M Ang II for 30 sec induced the maximal formation of IP_3 (35 pmol/dish). Incubation for 5 min of the cells with Ang II at various concentrations resulted in large increases in the activities of MAPKs (Fig. 3A). As shown in Figure 3B, the MAPKs were activated *via* the action of AT_1 receptors.

From the present results, it is apparent that the response of AT_1 receptors to Ang II can stimulate the synthesis of DNA in osteoblast-rich populations of cells isolated from rat calvariae. Moreover, the Ang II-mediated activation of MAPKs occurred *via* the interaction of Ang II with its specific AT_1 receptor. As described in "Introduction", we have found some reports of hypoplastic calvariae in fetus that was associated with the use of ACE inhibitors during pregnancy (11, 12). Their reports implied that Ang II might be involved in the development of calvariae. Our observations strongly suggest that Ang II might control the growth of calvarial cells locally, alone or in concert with the other bone-regulatory factors. In further studies the types of cell in our culture system that respond to Ang II remain to be determined.

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