Deceleration by angiotensin II of the differentiation and bone formation of rat calvarial osteoblastic cells

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Abstract

We examined the effects of angiotensin II (Ang II) on the differentiation of rat calvarial osteoblastic cells and on the formation of bone by these cells. Northern blotting analysis revealed that Ang II inhibited the expression of mRNA for osteocalcin, which is a protein that is specifically expressed during maturation of osteoblastic cells. Ang II decreased the activity of alkaline phosphatase, a marker of osteoblastic differentiation, in the cells, acting via the type 1 (AT1) receptor. We used von Kossa staining to examine the formation of mineralized nodules by osteoblastic cells. Both the number and the total area of mineralized nodules were quantified and shown to be decreased by $10^{-7}$ M Ang II. The accumulation of calcium in cells and the matrix layer was also decreased by Ang II. Binding analysis with subtype-specific antagonists revealed the presence of AT1 receptors for Ang II in this culture system. Ang II caused a marked increase in the rate of production of intracellular cAMP in this system. Our data suggest that Ang II might be intimately involved in osteoblastic metabolism through its interaction with the AT1 receptor.


Introduction

Bone morphogenetic protein (Yamaguchi et al. 1991), glucocorticoid (Bellows et al. 1990, Wong et al. 1990), and growth factors, such as transforming growth factor $\beta$ (Antosz et al. 1989, Harris et al. 1994), basic fibroblast growth factor (Deng et al. 1996), and insulin–like growth factor–I (Baker et al. 1993, Liu et al. 1993), have been shown to modulate differentiation-related events in cultures of osteoblastic cells. In previous studies, we demonstrated that 8-bromo cyclic AMP (8-Br-cAMP) and 8-bromo cyclic GMP (8-Br-cGMP) act reciprocally to regulate the differentiation of osteoblastic cells and the mineralization process (Inoue et al. 1995). We showed that 8-Br-cAMP inhibited the differentiation of osteoblastic cells and 8-Br-cGMP stimulated this process. Natriuretic peptides are physiological agonists that activate receptor guanylate cyclases with the resultant synthesis of the second messenger cGMP. Therefore, we postulated that natriuretic peptides might be involved in osteoblastic metabolism. We found that, indeed, natriuretic peptides did promote bone formation via the action of cGMP in a signal–transduction pathway that was mediated by receptor guanylate cyclases in osteoblastic cells (Hagiwara et al. 1996) and clonal in osteoblastic MC3T3-E1 cells (Inoue et al. 1996). In the present study, we investigated the effects of angiotensin II (Ang II), which induces the synthesis of intracellular cAMP, on osteoblastic metabolism using osteoblastic cells derived from rat calvariae.

Ang II, a bioactive octapeptide, plays a major role in the maintenance of extracellular fluid volume and blood pressure. In addition to its well-established role in circulatory homeostasis, it has been implicated in a variety of processes, which include the control of nervous system activity (Phillips 1987, Steckelings et al. 1992), cell growth (Aceto & Baker 1990), and several developmental processes (Millan et al. 1991, Steckelings et al. 1992). Pharmacological studies have shown that there are two different types of Ang II receptor, the type 1 (AT1) receptor and the type 2 (AT2) receptor. It has been reported that the AT1 receptor regulates two distinct signaling pathways that involve G protein(s). One pathway involves activation of phospholipase C, with subsequent stimulation of the production of inositol 1,4,5-trisphosphate, and the other involves the regulation of the level of cAMP via the activation or inhibition of adenylyl cyclase. The signaling pathway of AT2 receptors remains to be elucidated. Ang II is generated from Ang I by angiotensin–converting enzyme (ACE). Inhibitors of ACE, such as captopril and enalapril, have been successfully used in the treatment of patients with hypertension. However, exposure to ACE inhibitors was reported to

have resulted in an aborted fetus in which no skull tissue had formed above the brain tissue (Mehta & Modt 1989, Barr & Cohen 1991). Thus, the use of ACE inhibitors during pregnancy has been associated with high risk of hypocalvaria or acalvaria in the fetus.

The purpose of our present study was to elucidate the contribution of Ang II to osteoblastic metabolism using cultured cells from calvariae of newborn rats. In this culture system, the process of bone formation mimics that observed in vivo and mineralized nodules are formed that have the characteristics of woven bone (Antosz et al. 1989, Wong et al. 1990, Harris et al. 1994, Hagiwara et al. 1996). Also, this culture system does not require the specific reagents (e.g. dexamethasone and bone morphogenetic proteins) which bone marrow stromal and most osteoblastic lineages need for mineralization. We found evidence in the present study to suggest that Ang II might decelerate osteoblastic differentiation and the formation of bone by osteoblastic cells.

**Materials and Methods**

**Materials**

Rat 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. $^{32}$P-Labeled nucleotides and $^{125}$I-Ang II (74 TBq/mmol) were obtained from Amersham Life Science (Bucks, UK). α-Modified minimum essential medium (α-MEM), penicillin/streptomycin antibiotic mixture, and fetal bovine serum were obtained from Life Technologies, Inc. (Grand Island, NY, USA).

**Isolation and culture of cells**

Cells were isolated enzymatically from calvariae of newborn Sprague–Dawley rats as described previously (Hagiwara et al. 1996). Fourteen calvariae were dissected out and all adhering soft tissue was removed. The calvariae were cut into pieces and subjected to six sequential 20-min digestions (yielding digest 1 through 6) with 3 ml of an enzyme mixture that contained 1 mg/ml collagenase (150–250 units/mg; Wako Pure Chemical Industries, Osaka, Japan) and 0.5 mg/ml trypsin (Sigma, St Louis, MO, USA). Cells from a pool of digest 4, 5, and 6 were plated in 75 cm$^2$ dishes and grown in α-MEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere of 5% CO$_2$ in air at 37 °C. Cell populations enriched for osteoblastic cells (digests 4 through 6) were obtained by the sequential collagenase digestions. The medium was replaced by fresh medium after 24 h. After reaching confluence, the cells were detached by treatment with 0.05% trypsin and cells from three dishes (digests 4, 5, and 6) were combined. The cells were replated in 12-well plates (3.8 cm$^2$/well) at a density of $1 \times 10^4$ 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 its analog were added every 2 days.

We reported previously that in our culture system the expression of alkaline phosphatase (ALPase) activity and of mRNA for osteocalcin is upregulated at day 9 and day 14 respectively, and the formation of mineralized nodules was also observed at day 14 (Hagiwara et al. 1996). Therefore, we used these time points for assay in these experiments.

**Northern blotting analysis**

RNA was extracted from cultured osteoblastic cells derived from rat calvariae by the acid guanidinium–phenol–chloroform method (Chomczynski & Sacchi 1987). Total RNA (20 µg) was subjected to electrophoresis on a 1% agarose gel that contained 2.2 M formaldehyde and was then transferred to a MagnaGraph nylon membrane (Micron Separations Inc., Westborough, MA, USA). After baking of the membrane, the RNA on the membrane was allowed to hybridize overnight with cDNA for osteocalcin (Yamaguchi et al. 1991) or for β-tubulin at 42 °C in 50% formamide that contained 5 × SSPE (1 × SSPE is 0.15 M NaCl, 15 mM Na$_2$PO$_4$, pH 7.0, 1 mM EDTA), 2 × Denhardt’s solution (0.1% each of BSA, polyvinylpyrrolidone, and Ficoll), 1% SDS and 100 µg/ml herring sperm DNA. Each cDNA probe was radiolabeled with a Random Primer DNA Labeling Kit (Takara, Shiga, Japan). The membrane was washed twice in 1 × SSC (0.15 M NaCl, 15 mM sodium citrate, pH 7.0) that contained 0.1% SDS at room temperature for 5 min each and twice in 1 × SSC that contained 0.1% SDS at 55 °C for 1 h each and then it was exposed to an imaging plate for 4 h. The plate was analyzed with a Bioimage Analyzer (BAS 2000; Fuji Film, Tokyo, Japan).

**Assay of ALPase activity**

Cells were subcultured in α-MEM that contained 10% fetal bovine serum, 5 mM β-glycerophosphate, 50 µg/ml ascorbic acid, and various test compounds. The cells were washed twice with 50 mM Tris–HCl, pH 7.2, and sonicated in 1 ml 50 mM Tris–HCl, pH 7.2, that contained 0.1% Triton X–100 and 2 mM MgCl$_2$ for 15 s with a sonicator (Ultrasonic Disruptor UD–201; Tomy Co., Tokyo, Japan). The ALPase activity of the sonicate was determined by an established technique with p-nitrophenyl phosphate as the substrate (Lowry et al.)
von Kossa staining

Osteoblastic cells in 12-well plates were fixed with 10% formaldehyde for 30 min and washed three times with 10 mM Tris–HCl, pH 7·2. The fixed cells were incubated with 5% silver nitrate for 5 min in sunlight, washed twice with water, and then treated with 5% sodium thiosulfate (Hagiwara et al. 1996). Mineralized nodules were assessed with respect to their number and the total area of nodules using an automated imaging system, which consisted of a BH microscope (Olympus), a camera (CCD/ICD-740, Olympus) and Mac SCOPE program (Mitani Corp., Fukui, Japan).

Quantification of calcium

Mineralized nodules from 1 well of a 12-well plate were washed twice with PBS, incubated overnight in 1 ml 0·5 M HCl with gentle shaking and sonicated for 15 s with the sonicator (Ultrasonic Disruptor UD-201). The calcium in the sonicate was quantified by the o-cresolphthalein complexone method with a Calcium C-Test Wako (Wako Pure Chemical Industries) (Hagiwara et al. 1996).

Assay of binding of 125I-Ang II

Cells, grown in 12-well plates (3·8 cm²/well), were washed twice with PBS (pH 7·3; 20 mM potassium phosphate, 130 mM NaCl, and 1 mM EDTA) and incubated in 0·5 ml of PBS that contained 0·2% (w/v) BSA (fraction V; Sigma), 125I-labeled Ang II (920 Bq/well), and an unlabeled analog of Ang II at various concentrations for 1 h at 4 °C. After incubation, cells were washed twice with ice-cold PBS and solubilized with 0·5 ml 0·1 M NaOH. The radioactivity was measured with a gamma counter (ARC-300; Aloka, Tokyo, Japan).

Measurement of the accumulation of intracellular cAMP

Cells, grown in 12-well plates, were incubated at 37 °C for 15 min with serum-free α-MEM that had been supplemented with 0·5 mM 3-isobutyl-1-methylxanthine after two washes with serum-free α-MEM. Osteoblastic cells were subsequently incubated at 37 °C for 15 min with Ang II and/or its analog. After incubation, the cells were lysed by addition of 200 µl of a 0·1 M solution of HCl that contained 5 mM EDTA. The amount of cAMP in each cell lysate was measured with an RIA kit from Yamasa (Chiba, Japan).

Results

Northern blotting analysis of mRNA for osteocalcin

To determine whether Ang II is involved in osteoblastic maturation, we performed Northern blotting analysis using cDNA for osteocalcin, a protein that is a marker of osteoblastic maturation, as the probe. Ang II decreased the steady-state level of expression of the mRNA for osteocalcin in cells that had been treated with Ang II for 14 days (Fig. 1). There were no appreciable changes in the level of mRNA for β-tubulin in cells assayed similarly on the same respective days. These results suggest that Ang II might regulate the maturation of osteoblastic cells in our culture system.

Reduction of ALPase activity by Ang II

We measured the activity of ALPase on day 9 after the start of the continuous culture of cells in the presence and in the absence (control) of Ang II alone, of Ang II plus DuP 753 and of Ang II plus PD-123319, and the results are shown in Fig. 2. Ang II (ALPase activity 3·70 ± 0·19 µmol/mg protein per 30 min) and Ang II plus PD-123319 (4·12 ± 0·42 µmol/mg protein per 30 min) reduced the activity of ALPase to approximately 60% of the basal level (6·70 ± 0·18 µmol/mg protein per 30 min). DuP 753, a specific antagonist of AT1 receptors, overcame the inhibitory effects of Ang II (5·51 ± 0·51 µmol/mg protein per 30 min). Addition of DuP 753 or PD-123319 alone to the culture medium had no effects on ALPase activity (data not shown). These results indicate that Ang II reduces the activity of ALPase in osteoblastic cells via the AT1 receptors.

Effects of Ang II on the formation of mineralized nodules

Figure 3A shows the results of von Kossa staining of mineralized nodules that had been formed by osteoblastic cells during incubation for 14 days in medium supplemented with ascorbic acid and β-glycerophosphate in either the continuous presence or in the absence of 10⁻⁷ M Ang II. Ang II inhibited the formation of mineralized nodules by osteoblastic cells. The nodules were quantified with an image analyzer and the number and total area of mineralized nodules were recorded. In the presence of Ang II at 10⁻⁷ M, about 50% of the control number of mineralized nodules were formed (Fig. 3B), and the total area of mineralized nodules was about 80% smaller than that in control (Fig. 3C). Parathyroid hormone (PTH) is known to activate adenylate cyclase and to induce increases in the concentration of intracellular cAMP in osteoblastic cells. In a recent study (Yamauchi et al. 1997) it was demonstrated that culture of osteoblastic cells with PTH resulted in the inhibition of differentiation and mineralization via production of cAMP. Therefore, we
used PTH as a control in our culture system. As anticipated, PTH at $10^{-7}$ M strongly inhibited the formation of mineralized nodules (Fig. 3).

We next measured the accumulation of calcium in cells and the matrix layer in 12-well plates at day 14 (Fig. 4). Ang II reduced the accumulation of calcium ($24.8 \pm 3.9 \mu g$ calcium/well) compared with the control level ($38.4 \pm 7.8 \mu g$ calcium/well). PTH also inhibited the formation of mineralized nodules in this culture system ($18.2 \pm 1.3 \mu g$ calcium/well). These results suggest that Ang II reduced the rate of formation of mineralized nodules by osteoblastic cells.

The experiments described above indicated that Ang II might be involved in the differentiation of osteoblastic cells and in the formation of bone by these cells. 8-Br-cAMP inhibits both the differentiation of osteoblastic cells and the mineralization process (Inoue et al. 1995). Therefore, we attempted to identify the relevant subtype of angiotensin receptor and to quantitate the production of intracellular cAMP in our culture system.

**Identification of the relevant receptors for Ang II**

We examined the competitive displacement of $^{125}$I-Ang II by unlabeled Ang II, by DuP 753 (an antagonist specific for AT$_1$ receptors) and by PD-123319 (an antagonist specific for AT$_2$ receptors) in an attempt to identify the specific binding sites for Ang II in the cells. As shown in Fig. 5A, unlabeled Ang II and DuP 753 completely prevented the binding of $^{125}$I-Ang II, while PD-123319 did not displace Ang II. The level of the receptors for Ang II reached a maximum on day 9 during an 18-day culture. To confirm the nature of the receptor subtype, we examined the capacity of angiotensin antagonists to compete with $^{125}$I-Ang II for binding sites (Fig. 5B). DuP 753

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**Figure 1** Northern blotting analysis of mRNA for osteocalcin in cultured osteoblastic cells. Total RNA was isolated from osteoblastic cells after treatment with $10^{-7}$ M Ang II for 3, 9, and 14 days. Twenty micrograms of total RNA were subjected to electrophoresis in an agarose gel and were allowed to hybridize with $^{32}$P-labeled cDNA for rat osteocalcin or for β-tubulin. There were no appreciable changes in the level of mRNA for β-tubulin in cells assayed similarly on the same respective days.

**Figure 2** Activities of ALPase in osteoblastic cells treated with Ang II analog. Cells in 12-well plates were cultured for 9 days with α-MEM that contained 10% fetal bovine serum, 5 mM β-glycerophosphate, 50 μg/ml ascorbic acid, and $10^{-7}$ M Ang II, $10^{-7}$ M DuP 753, and $10^{-7}$ M PD-123319, as indicated. Data are means ± S.D. of results from four wells. Results are representative of four experiments. *P<0.0001 vs control; **P<0.005 vs Ang II; ***P<0.001 vs control.
was less active than Ang II, and PD-123319 displaced the radiolabeled ligand only very weakly. These results indicated that most of the binding sites for 125I-Ang II corresponded to AT1 receptors. Scatchard plot analysis displayed a single component of binding sites with 3·2 fmol/10⁶ cells. We have also obtained preliminary results that cloned rat osteoblastic cells, ROB-C8a, and cloned mouse pluripotent cells, C3H10T1/2, express angiotensin II receptors (data not shown).

Production of cAMP in response to Ang II

Our final experiments were designed to determine whether Ang II could induce the activity of adenylate cyclase in this culture system. Figure 6A shows that Ang II caused an increase in the level of intracellular cAMP in a dose-dependent manner. The maximal level (70 pmol/10⁶ cells), corresponding to the maximum activity of adenylate cyclase, was found on day 9. As shown in Fig. 6B, the stimulation of the formation of intracellular cAMP by 10⁻⁷ M Ang II was abolished by preincubation of cells for 15 min with 10 µM DuP 753, while preincubation with 10 µM PD-123319 barely affected the stimulatory effect of Ang II. These results indicate that the production of intracellular cAMP is promoted through the AT₁ receptor in response to Ang II.

Discussion

In this study, we showed that Ang II is a potent suppressor of the differentiation of osteoblastic cells from newborn rat
Angiotensin II was found to decrease the level of mRNA for osteocalcin, which is a well-known marker of osteoblastic differentiation. The activity of ALPase was also reduced by the addition of Ang II to the culture medium. Moreover, Ang II decreased the rate of formation of mineralized nodules (a model of bone formation in vitro) and the precipitation of calcium by osteoblastic cells. The present results provide evidence that Ang II decelerates the differentiation and mineralization of osteoblastic cells in this culture system.

As described above, Ang II acted to influence the differentiation and mineralization of osteoblastic cells from rat calvariae. We postulated that cAMP might be a candidate factor for a second messenger of Ang II, since we had demonstrated previously that 8-Br-cAMP (Inoue et al. 1995) and endothelins (Hagiwara et al. 1996), which induce production of intracellular cAMP, reduced the rate of mineralization by osteoblastic cells. We confirmed that continuous culture of cells with PTH decreased the rate of formation of mineralized nodules in our culture system.
must identify the cells that express the AT1 receptor or calvariae are heterogenous. In future, therefore, we the generation of prostaglandin (Rainey et al. 1989), rat adrenal glomerular cells (Missale et al. 1989), rat fetal skin (Johnson & Aguilera 1991), and bovine adrenocortical cells (Rainey et al. 1991). At present there is little evidence to support any direct coupling of the AT1 receptor to a G protein(s) that activates adenylate cyclase. Stimulation of the production of cAMP by Ang II might be expected to involve activation of type I adenylate cyclase, one of the isoforms of adenylate cyclase, by protein kinase C and/or Ca2+/calmodulin or via an indirect mechanism involving the generation of prostaglandin (Rainey et al. 1991). Cell populations enzymatically isolated from newborn rat calvariae are heterogenous. In future, therefore, we must identify the cells that express the AT1 receptor or adenylate cyclase by in situ hybridization. We also showed that Ang II stimulates the production of inositol 1,4,5-trisphosphate in this culture system (Hiruma et al. 1997). Accumulation of intracellular calcium may be involved in the regulation of osteoblastic metabolism.

Stein et al. (1990) provided a model for the reciprocal relationship between cell proliferation and the expression of differentiation-related genes. They proposed that cell proliferation is inhibited before events that are associated with the differentiation of osteoblastic cells can occur. We reported previously that cGMP, produced in response to natriuretic peptides, inhibited the proliferation and promoted the differentiation of osteoblastic cells (Hagiwara et al. 1996). A recent study in our laboratory demonstrated that Ang II might stimulate the synthesis of DNA via the activation of mitogen-activated protein kinases in our culture system (Hiruma et al. 1997). We showed here that Ang II seems to decelerate the differentiation and mineralization of osteoblastic cells. Thus, the effects of Ang II might support the model proposed for the process of bone formation.

A single heterozygous nucleotide substitution (a mutation encoding a change from His to Arg at position 223) in an exon of the gene for the PTH–PTH-related peptide receptor was identified recently in a patient with Jansen-type metaphyseal chondrodysplasia, a rare form of short-limbed dwarfism (Schipani et al. 1995). This mutation was associated with an increase in the basal level of cAMP and a decrease in the rate of production of cAMP in response to PTH. These observations suggest that the accumulation of cAMP might be involved in the abnormal proliferation and differentiation of growth-plate chondrocytes and bone cells. Moreover, there are some reports that ACE inhibitors, such as captopril and enalapril, might have caused severely underdeveloped calvarial bone in human fetuses (Mehta & Modt 1989, Barr & Cohen 1991). To date, little information is available on the relationship between Ang II and osteoblastic metabolism. However, our present observations support the hypothesis that Ang II might be involved in osteogenesis via the AT1 receptor.

In cultures of mouse bone marrow, C-type natriuretic peptide (CNP) increased the rate of bone resorption (Holliday et al. 1995). Those authors proposed that CNP might be a potent activator of osteoclast activity. Endothelin also interacts specifically with osteoclasts to inhibit osteoblastic bone resorption (Alam et al. 1992). Endothelins have been demonstrated to cause a reduction in ALPase activity in calvarial osteoblastic cells (Takuwa et al. 1989, 1990). We also reported that natriuretic peptides (atrial natriuretic peptide and CNP) promoted the differentiation of and formation of bone by calvarial osteoblastic cells (Hagiwara et al. 1996) and osteoblastic MC3T3-E1 cells (Inoue et al. 1996) and that endothelin suppressed these processes (Hagiwara et al. 1996). In the present study, we obtained evidence that Ang II might decelerate these processes. Bone is rich in blood vessels and bone formation requires vascularization. These observations suggest that certain relationships are likely to exist between bone cells and vasoactive peptides in the local environment. It does not seem inappropriate to propose that vasoactive peptides, including Ang II, natriuretic peptides, and endothelins, might be novel local regulators of bone remodeling.

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Angiotensin II regulates osteoblastic metabolism

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