Effects of Various Antihypertensive Drugs on the Function of Osteoblast

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Several studies have suggested that high blood pressure is associated with the risk of bone loss. Since various antihypertensive drugs are in wide use for the treatment of hypertension, it is important to investigate the influences of these drugs on bone metabolism. Osteoblasts play a pivotal role in the regulation of bone formation. During differentiation, they sequentially express type I collagen, alkaline phosphatase (ALP), other bone matrix proteins, and finally undergo mineral deposition. In this study, we examined the effects of various antihypertensive drugs on the function of osteoblast using clonal MC3T3-E1 cells. Drugs examined include dihydropyridine-type calcium channel blockers (benidipine, amlodipine, and nifedipine), angiotensin-converting enzyme (ACE) inhibitors (captopril, lisinopril, and enalapril), and angiotensin II receptor type1 (AT1) antagonists (TCV-116 and KW-3433). None of the ACE inhibitors or AT1 antagonists affected ALP activity or cellular DNA content significantly. In contrast, benidipine, amlodipine, and nifedipine increased ALP activity when used in amounts 1 μM, 100 nM, and 100 nM, respectively. Benidipine blocked calcium influx through the L-type voltage dependent calcium channel of MC3T3-E1 more potently than amlodipine or nifedipine. These calcium channel blockers did not change collagen accumulation. Benidipine significantly increased in vitro mineralization at a concentration of 1 μM and higher, while amlodipine did so at 1 μM and nifedipine did not. Comparison of the effective concentration of each calcium channel blocker in our study with the reported maximum serum concentration of each drug suggests that benidipine, but not amlodipine or nifedipine, promotes mineral deposition in human.

Key words antihypertension; differentiation; bone; osteoporosis; benidipine

Several studies have suggested that high blood pressure is associated with abnormalities in calcium metabolism, leading to increased calcium loss, secondary activation of the parathyroid gland, and increased removal of calcium from bone.1—7) Consistently, studies in hypertensive rats have shown that hypercalciuria and ensuing hyperparathyroidism lead to reduced growth and detectable decrease in total bone-mineral content later in life.8,9) Recently, it was also shown that higher blood pressure in elderly white women is statistically associated with increased bone loss at the femoral neck, which may contribute to bone fracture.10)

Drugs used as primary choice for the treatment of hypertension include calcium channel blockers, angiotensin-converting enzyme (ACE) inhibitors, angiotensin II receptor type1 (AT1) antagonists, diuretics, β-blockers, and α-blockers.11) The first three drugs are especially widely used in Japan. The mechanism by which each drug lowers blood pressure differs. Calcium channel blockers primarily inhibit calcium influx through the L-type voltage-dependent calcium channel at the level of vascular smooth muscle, thereby disrupting the excitation contraction process.12,13) Both ACE inhibitors and AT1 antagonists interfere with the renin-angiotensin system (RAS). The former inactivates the conversion of angiotensin I into angiotensin II (Ang II), which is a vasoconstrictive peptide, while the latter blocks the binding of Ang II to its receptor.14,15)

Osteoblasts are derived from mesenchymal stem cells and play a pivotal role in bone formation. During differentiation, they first express type I collagen, then alkaline phosphatase (ALP), and other bone matrix proteins and finally form mineralized bone. Osteoblasts express voltage-dependent calcium channels.16) Various bone regulatory factors such as vitamin D₃,17—19) parathyroid hormone,17,20,21) and prostaglandin E₂,17,22) cause a rise in intracellular calcium concentration ([Ca²⁺])ₙ, at least part of which is decreased by dihydropyridine-type calcium channel blockers. These factors also promote or inhibit osteoblast differentiation.23—26) Thus, it is suggested that signaling through the L-type calcium channel may be important for osteoblast functions. However, the fact that these factors give rise to multiple signals independent of calcium influx (i.e., transcription of specific genes,27) cAMP production,28,29) release of calcium ion from intracellular store[17]) obscures the role of L-type calcium channel. Recently, Kosaka and Uchii found that a calcium channel blocker benidipine, but not amlodipine or nifedipine, increased ALP activity of osteoblast cells isolated from neonatal mouse calvaria.29) We further investigated the effects of benidipine on osteoblastic functions using osteoblastic cell line MC3T3-E1,30) which is widely used in studies on various aspects of osteoblast differentiation since it expresses osteoblast markers and forms a mineralized extracellular matrix.31,32) There we showed that benidipine stimulated not only ALP activity but also mineral deposition. In an attempt to elucidate its target of action, we first demonstrated that blocking L-type calcium channel promotes osteoblast differentiation from the following observations: 1) The effect of benidipine was completely abolished by an excess amount of Bay K 8644, a calcium channel agonist; 2) Verapamil and diltiazem, which belong to a structurally different class of calcium channel blockers from benidipine, also enhanced ALP activity. At present, it is not clear how blockade of the calcium channel stimulates differentiation, nor is it known whether [Ca²⁺]ₙ affects osteoblast differentiation. Considering the above findings, calcium channel blockers in general are believed to promote osteoblast differentiation, but the character of each drug remains to be determined.

Several lines of evidence suggest that Ang II might play some role in bone cell metabolism. In vitro, Ang II promotes cell growth and inhibits differentiation of osteoblast, and these effects have been shown to be mediated through AT1.33—35) An AT1 antagonist, DuP 753, itself is reported to have no effect on ALP activity of rat calvarial osteoblast[36],
However, other AT1 antagonists were not examined in that referenced study. In vitro effects of ACE inhibitors on osteoblast have not been well studied.

In this study, we examined the effects of antihypertensive drugs widely used in Japan: calcium channel blockers (benidipine, amlodipine, and nifedipine), ACE inhibitors (enalapril, lisinopril, and captopril), and AT1 antagonists (candesartan cilexetil (TCV-116) and KW-3433) on proliferation and differentiation of MC3T3-E1 cells. We also studied the effects of the calcium channel blockers on collagen accumulation and mineralization in vitro. We discuss the osteogenic effect of each calcium channel blocker when used clinically by comparing the potency in osteoblast differentiation and maximum serum concentration of each drug in human.

MATERIALS AND METHODS

Reagents Benidipine hydrochloride, TCV-116, and KW-3433 were synthesized in our laboratories. Amlodipine besilate was extracted from Norvasc® tablet (Pfizer, U.K.). Nifedipine was purchased from Wako Pure Chemical Industries, Ltd. (Osaka). Captopril, lisinopril, enalapril maleate, and Bay K 8644 were obtained from Sigma Chemical Co., (St. Louis, MO, U.S.A.). All drugs were dissolved in dimethyl sulfoxide and kept at −80 °C until use. Other chemicals were of reagent grade and were from commercial sources.

Cell Culture Osteoblastic MC3T3-E1 cells were generously provided by Dr. H. Kodama (Ohu University). Stock cultures were grown in α-modified Eagle’s medium (MEM, GIBCO BRL, Life Technologies, MD, U.S.A.), containing 10% fetal bovine serum (FBS, PA Laboratories, Linz, Austria) and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin, GIBCO BRL) at 37 °C in a humidified atmosphere (5% CO2–95% air) and passaged every 3—4 d.

ALP Activity and DNA Content The method of Franzl-Zelman et al. was modified as follows. Cells were seeded in 96-multiwell microplates (Asahi Techno Glass, Tokyo) at a density of 5×104 cells/cm2 and cultured for four days. Then the cells were transferred to serum-free medium with 0.1% bovine serum albumin (Sigma), antibiotics and various drugs. The cultures were further incubated for ten days in the presence of drugs. The medium was changed every 3—4 d.

For measurement of ALP activity, the medium was removed and cells were washed with phosphate-buffered saline (PBS). Thereafter, the cells were incubated for 15 to 30 min at 23 °C with 150 μl of 100 mM NaHCO3, pH 10.0, containing 6.7 mM 2-nitrophenylphosphate and 1 mM MgCl2. The reaction was stopped with 50 μl of 1 N NaOH. The absorption was measured at 405 nm in a microplate reader.

For measurement of DNA content, the cells were washed with PBS and frozen at −20 °C. During subsequent thawing, 50 μl of 5 μg/ml Hoechst 33258 solution in 10 mM Tris HCl, pH 8.0, 150 mM NaCl, and 0.1 mM ethylene diamine tetracetic acid was added to each well. The amount of DNA was quantitated by measuring the fluorescence (excitation 355 nm/emission 460 nm) using calf thymus DNA as a standard (Worthington Biochemicals, NJ, U.S.A.).

Hydroxyproline Content and Mineralization Cells were seeded on 24-multiwell plates (Asahi Techno Glass) at a density of 2.5×104 cells/cm2 and cultured in α MEM containing 10% FBS, antibiotics, and 10 mM β-glycerophosphate. After 4 d, various concentrations of calcium channel blockers were added to the medium. The medium was changed every 3—4 d. After the indicated period of incubation, the cells were washed with physiological saline and treated with 15% trichloroacetic acid at 4 °C for 18 h. Cells were scraped off and centrifuged. Supernatants were then assayed for calcium using a Calcium-C Test Wako kit (Wako Pure Chemical) based on the cresolphthalein complexone method and for phosphates using the method of Heinonen and Lahti. Precipitates were hydrolyzed in 6 N HCl for 24 h at 110 °C and then evaporated. After resuspension in H2O, hydroxyproline was measured using the method of Woessner.

Cytosolic Calcium Measurement Cells were seeded onto coverslips at a density of 5×105 cells/cm2 and cultured overnight. The medium was removed and the cells were rinsed with Hank’s balanced salt solution (HBSS) and then loaded with 10 μM fura-2 AM in HBSS for 60 min in 5% CO2 humidified incubator at 37 °C. The loaded cells were incubated for further 30 min with HBSS alone to allow the complete deesterification of fluorescent probe, then placed into the cuvette holder of a spectrofluorimeter CAF-110 (Japan Spectroscopic, Tokyo), and stirred constantly throughout the procedure while being maintained at 37 °C. Fluorescence ratios were measured at 500 nm with excitation at 340 and 380 nm. Ratios were used as an approximation of cytosolic calcium measurement. Various concentrations of drugs were added one minute prior to the addition of the stimulant (40 mM KC1 and 10 μM Bay K 8644). The increase in [Ca2+]i was measured and represented as a percentage of the increase in the absence of drugs.

Statistical Analysis Data are expressed as mean±S.D. The significance of treatment-mediated differences was determined by Student’s t-test.

RESULTS

To assess the effects of antihypertensive drugs on bone formation, we examined the impact of calcium channel blockers, ACE inhibitors, and AT1 antagonists on the proliferation and differentiation of MC3T3-E1 cells. We chose benidipine, amlodipine, and nifedipine as the calcium channel blockers, captopril, lisinopril, and enalapril as ACE inhibitors, and TCV-116 and KW-3433 as AT1 antagonists. Although enalapril and TCV-116 are prodrugs and converted to active form after administration in vivo, we tested these compounds along with other active drugs to evaluate their possible effects on osteoblast.

As shown in Fig. 1A, none of the drugs caused a great change in DNA content after ten days of treatment (control = 1.43±0.054 μg DNA/well), while benidipine alone, but not other drugs, significantly stimulated ALP activity (Fig. 1B). The ALP activities were 384±7.6 pmol/min/μg DNA with vehicle alone, and 625±27 pmol/min/μg DNA with 10 nm of benidipine. Other drugs including amlodipine and nifedipine did not affect ALP activity at 10 nm. ACE inhibitors and AT1 antagonists had no effect on ALP activity or DNA content even at 100 nm (data not shown). Next, the effects of the cal-
Calcium channel blockers on ALP activity in a wide range of concentrations were examined. Benidipine increased ALP activity in a concentration-dependent manner from 1 pM (Fig. 2). Amlodipine and nifedipine also significantly stimulated ALP activity at 100 nM (Fig. 2). These concentrations of the drugs had no effect on DNA content (data not shown). These results tempted us to further investigate the effects of these calcium channel blockers on other osteoblastic activities.

We examined the effect of the calcium channel blockers on collagen accumulation and calcification. Collagen accumulation was assessed by measuring protein-associated hydroxyproline. None of the calcium channel blockers tested greatly altered collagen accumulation at concentrations up to 1 μM (Fig. 3). As illustrated in Fig. 4, benidipine increased mineral deposition concentration-dependently with the maximal stimulation of 165% of the vehicle-treated control at 1 nM. Amlodipine also stimulated mineralization significantly (p < 0.05) at 1 μM (Fig. 4). In contrast, nifedipine had no significant effect on mineralization (Fig. 4). A similar result was obtained when we measured phosphate content in the same samples (data not shown).

We recently reported that benidipine upregulates the transcription of ALP gene and increases ALP activity by blocking the L-type calcium channel, followed by the enhancement of in vitro mineralization. To understand the differential effects of benidipine, amlodipine, and nifedipine on osteoblast differentiation, we compared the inhibitory effects of these drugs on calcium influx through the L-type voltage-dependent calcium channel. Calcium influx was evoked by depolarization of cells with 40 mM KCl and 10 μM Bay K 8644, an L-type calcium channel agonist. This calcium influx was only observed when both KCl and Bay K 8644 were present in the depolarizing buffer, indicating that this rise in [Ca2+]i was mediated through the L-type calcium channel. As shown in Fig. 5, benidipine inhibited the calcium influx at more than 10-fold lower concentrations than amlodipine and nifedipine did.

**DISCUSSION**

To assess the possible effects of antihypertensive drugs on bone metabolism, the direct action of these drugs on bone-

Fig. 1. Effects of Various Antihypertensive Drugs on DNA Content (A) and ALP Activity (B)

Cells (1.7×10⁴ cells) were inoculated in 96-well plates and cultured for four days, then transferred to a medium containing 10 nM of various drugs. After ten days of further incubation, DNA content (A) and ALP activity (B) were determined. ALP activity was normalized to DNA content (B). Values are expressed as percentage of the vehicle-treated control (A), ALP stimulation by 10 nM of benidipine (B) and represent the mean ± S.D. (n = 3 or 4). Statistical significance vs. control was determined by Student’s t-test. *p < 0.05.

Fig. 2. Concentration-Dependent Effects of Benidipine, Amlodipine, and Nifedipine on ALP Activity

Cells (1.7×10⁴ cells) were inoculated in 96-well plates and cultured for four days, then transferred to a medium containing various concentrations of benidipine (open circles), amlodipine (open squares), and nifedipine (closed circles). After ten days of further incubation, DNA content and ALP activity were determined. ALP activity was normalized to DNA content. Values are expressed as percentage of ALP stimulation by 10 nM of benidipine, and represent the mean ± S.D. (n = 3 or 4). Statistical significance vs. control was determined by Student’s t-test. **p < 0.01 and *p < 0.05.

Fig. 3. Effects of Benidipine, Amlodipine, and Nifedipine on Hydroxyproline Content in Collagen Matrix

Cells were seeded on 24-multwell plates at a density of 2.5×10⁴ cells/cm² and cultured for four days. Then, various concentrations of the drugs were added to the media (benidipine (open bars), amlodipine (closed bars), and nifedipine (hatched bars)). After 31 d of incubation, hydroxyproline content in collagen matrix was measured as described in Materials and Methods. Values represent the mean ± S.D. (n = 3 or 4).
forming cells was investigated using osteogenic MC3T3-E1 cells. We selected calcium channel blockers, ACE inhibitors, and AT1 antagonists since these drugs are commonly used for hypertension therapy.

Ang II is the major effector peptide of the RAS and is a well-known systemic regulator of the cardiovascular system and body fluid homeostasis. It has been suggested that Ang II might affect bone cells as well. Ang II stimulates proliferation and decreases the ALP activity of rat calvarial cells. These effects were antagonized by AT1 antagonist, indicating that the effect of Ang II was mediated through AT1 receptors. Therefore, it is conceivable that agents that interfere RAS might affect bone metabolism. At present, ACE inhibitors are believed to have no major impact on bone metabolism in the post-neonatal period. In line with this, a study by Stimpel et al. with young rats did not reveal any effect of the ACE inhibitor moexipril on the skeleton when it was given alone or in combination with 17-β estradiol. Ma et al. reported that moexipril had no apparent effect on the proximal tibial metaphysis or the tibial shaft of ovariectomized spontaneously hypertensive rats. However, no skull formation was present above brain tissue in an aborted fetus exposed to ACE inhibitors, suggesting the possible involvement of Ang II in the development of calvaria. Thus, the use of ACE inhibitors during pregnancy has been considered the high risk example, 1 nM of benidipine, but not nifedipine or amloidipine, increased ALP activity of osteoblastic cells derived from mouse calvaria. In the present study, we examined these calcium channel blockers in the wide range of concentrations and found a great difference in potencies between benidipine and the others: benidipine increased ALP activity from 1 pM, while amloidipine and nifedipine did only at 100 nM. This great difference is possibly the reason why Kosaka and Uchii only detected the stimulatory effect of benidipine in their study. The difference in culture system may be another reason. We have recently found that blocking calcium channel stimulates ALP activity and mineral deposition. Here we showed that nifedipine and amloidipine weakly block calcium channel on osteoblast (Fig. 5). Therefore, it is not surprising that nifedipine and amloidipine also partially elevated ALP activity at higher concentrations.

However, concentrations effective in calcium influx assays are much higher than those in differentiation assays. For example, 1 nM of benidipine showed the maximum increase in ALP activity and mineral deposition, while the same concentration of benidipine exhibited no inhibition of calcium influx. These differences in effective concentration may be partially explained by the time period during which cells were treated with calcium channel blockers. In the calcium influx assays, calcium channel blockers were added to the solution one minute prior to depolarization stimuli. By contrast, cells were cultured for 10—34 d for the measurement of ALP activity and mineralization with periodical medium exchanges containing the calcium channel blockers. Dihydropyridine-type calcium channel blockers are lipophilic in general and are believed to exert their effects by a two-stage binding mechanism that involves partitioning of the drugs into the lipid bilayer of cell membrane, with subsequent lateral diffusion to the receptor binding site. Herbette and Katz cal-
culated that even at an nM aqueous concentration of nifedipine, which is less lipophilic than benidipine or amlodipine, the "membrane" concentration is 10^4-fold higher than in the aqueous medium. Therefore, in the longer period of incubation, the three drugs we used probably accumulated in plasma membranes, resulting in their higher concentrations in the vicinity of calcium channels.

The greater potency of benidipine in osteoblast differentiation than amlodipine or nifedipine may be partially due to the strong blockade of L-type calcium channel. As we showed in the calcium influx inhibition assay (Fig. 5), benidipine blocked L-type calcium channel at concentrations 1–2 orders lower than amlodipine or nifedipine, which is consistent with the results obtained from canine coronary artery or ventricular cells. However, greater differences in potencies between benidipine and others were observed in the ALP and mineralization assays (Figs. 2 and 4). Benidipine increased ALP activity and mineralization 4–5 and 3–4 orders of magnitude, respectively, more strongly than the others. As we mentioned above, one factor that affects the potency of each compound is lipophilicity. Benidipine and amlodipine are reported to be highly lipophilic and retained in the membranes of organs, whereas nifedipine is less retained than either of these. Benidipine has eight-fold higher partition coefficient to biological membrane than nifedipine, whereas that of amlodipine is four-fold higher, which indicates that benidipine is more lipophilic than amlodipine. Thus, the high ability to block calcium channel and high lipophilicity may both contribute to the strong potency of benidipine. It is not clear why amlodipine has almost the same level of low activity in ALP assay and mineralization assay as nifedipine, despite being more lipophilic. There might be other factors that affect drug potency, such as duration, the extent of calcium blockade in a resting membrane potential. Further investigations are required to understand the differences in the potency of each compound in our assay system.

Tanaka et al. have shown that the administration of benidipine increased bone mass and bone strength in spontaneously hypertensive rats. On the other hand, nifedipine had adverse effects on bone volume and bone morphology in growing rabbits, while it has no significant effect on bone metabolism in human males. These results seem to correlate with the results here. The adverse effect of nifedipine might be the reflection of its weak but significant negative effect in ALP activity at 100 μM and in mineral deposition at 10 nM.

Since these calcium channel blockers are clinically used, it is tempting to know whether these drugs have a bone-forming effect on human as well. One possible approach to this issue is to compare the serum concentration of each drug. In the clinical trial of each calcium channel blocker with healthy Japanese males, the maximum serum concentration (C_max) of each drug was investigated. In the single dose trial, the C_max of benidipine (Coniel®), amlodipine (Norvasc®), and nifedipine (Adalat®-CR: sustained-release formulation) was 7.18, 8.29, and 139 nM, respectively (tested highest dose of 8, 5, and 40 mg, respectively). In the multiple dose trial, the C_max values for benidipine, amlodipine, and nifedipine were 6.11 nM (6 mg × 2/d), 8.56 nM (2.5 mg/d), and 198 nM (40 mg/d), respectively. At the C_max of each drug, our results demonstrated that benidipine fully increased ALP activity, whereas nifedipine partially increased the activity and amlodipine had no effect (Fig. 2). In mineral deposition studies with these three calcium channel blockers (Fig. 4), only benidipine led to a mineral deposition at C_max. Partial elevation of ALP activity by nifedipine was not followed by the increase of mineral deposition. These findings suggest that benidipine but neither amlodipine nor nifedipine has a beneficial effect on bone formation in human if human osteoblasts respond to these calcium channel blockers similarly to mouse MC3T3-E1 cells and mouse primary calvaria. Unfortunately, most human cell lines available are derived from sarcoma and do not differentiate naturally as MC3T3-E1 cells do, which currently hampers the study of osteoblastic differentiation using human cell lines. Studies on naturally differentiatable human osteoblast, which could be primary calvarial cells, are awaited to know whether calcium channel blockers affect human bone cells as well.

Collagen is the major component of bone matrix and is synthesized by osteoblast. With respect to the differentiation of MC3T3-E1 cells, collagen matrix binds to integrins on the osteoblast cell surface and leads to the expression of various osteoblastic markers, such as ALP and osteocalcin. We showed that all the calcium channel blockers examined had only slight effects on collagen accumulation at the concentrations tested. In contrast, verapamil and diltiazem, which are also calcium channel blockers belonging to structurally different classes from those we tested here, are reported to inhibit collagen synthesis of osteoblasts by about 20% at 10 and 5 μM, respectively. Based on their results, Kim et al. argued that calcium channel blockers may have adverse effects on osteoblastic functions. From a clinical point of view, however, since the in vivo therapeutic concentration does not exceed μM concentrations (C_max of verapamil and diltiazem are 446 nM and 207 nM, respectively; see above for dihydropyridines), calcium channel blockers are unlikely to have an adverse effect on collagen accumulation in vivo.

In summary, we examined the effects of antihypertensive drugs on osteoblast function, and showed that dihydropyridine-type calcium channel blockers stimulate osteoblast differentiation, while ACE inhibitors or AT1 antagonists do not appear to affect the functions of osteoblast. We observed a great difference in the potency of effects between benidipine and the other calcium channel blockers. This is possibly due to the high ability of benidipine to block the calcium channel of osteoblast and high membrane affinity. Our findings, together with previous reports, suggest that potent calcium channel blockers such as benidipine have a beneficial effect on bone metabolism as well as an antihypertensive effect.

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