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The Relationship of Blood Pressure with Intra- Or extracellular and Bone Calcium Metabolisms in the Elderly

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Summary We investigated the correlations of blood pressure with intra- or extracellular and bone calcium metabolisms in the elderly. We measured serum calcium (Ca), inorganic phosphorus (Pi), C-terminal parathyroid hormone fragment (PTH-C), and calcitonin. Intracellular free calcium ($[Ca^{2+}]_i$) in platelet and erythrocyte was measured by fura- 2/AM from dual excitation wavelength using a fluorescence spectrophotometer. We calculated bone mineral content (BMC) of lumbar vertebral body using a calibration phantom by computed tomography. Systolic blood pressure had significant positive correlation with platelet $[Ca^{2+}]_i$ ($r=0.691$; $p<0.01$), but had no significant correlations with other parameters of calcium metabolism. Diastolic blood pressure had no significant correlations with any of these parameters. Serum Ca, Pi, $[Ca^{2+}]_i$ of platelet and erythrocyte had no significant correlations with PTH-C, calcitonin and BMC. BMC had a significant negative correlation with age ($r=-0.684$; $p<0.01$), but had no correlations with the other examination parameters. These results suggest that systolic blood pressure in the elderly may be affected by calcium metabolism.

Key Words : Calcium metabolism, Intracellular free calcium, Hypertension, Calcium regulating hormone, Bone mineral content.

Introduction

Several abnormalities of calcium metabolism have been described in patients with essential hypertension[1)2)], as well as in spontaneous hypertensive rats (SHR)[3)4)] and ethanol-induced hypertensive rats[5)]. These abnormalities include reduced serum ionized calcium (Ca), increased intracellular free calcium ($[Ca^{2+}]_i$), increased serum levels of parathyroid hormone, and decreased levels of serum vitamin D[6-9)]. Other reports have demonstrated that SHR displays decreased bone calcium density due to reduced intestinal calcium absorption and increased urinary calcium excretion, and that this negative calcium imbalance combined with hypocalcemia ultimately results in hypertension[10)11)].

In the present study, we examined the intra- or extracellular and bone calcium metabolisms, and investigated these relevances to blood pressure in the elderly patients.

Subjects and Methods

Subjective Patients: The subjects consisted

of 18 hospitalized patients in 70s and 80s years old (6 men and 12 women, mean age 80.2±4.9 years old [mean±SD]) with the following basal diseases: 5 patients with essential hypertension; 6 with valvular and ischemic heart disease without acute myocardial infarction or heart failure; 3 with transient ischemic cerebrovascular disease; 4 with bronchopneumonia in the stage of convalescence; 1 with benign gastric ulcer without hemorrhage; and 7 with spondylosis deformans and osteoarthrosis as complications of postmenopausal or senile osteoporosis. Some patients had two or more concomitant diseases. All patients received no drug therapy at least for one week before examination.

Blood pressure measurement: Blood pressure was measured three times in each day between 9 and 12 A.M. in a supine position after resting for at least 15 minutes with standard mercury sphygmomanometer. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were defined as Korotkoff phases I and V, respectively. The mean of two or more measurements was adopted as a blood pressure. Hypertension was defined above 160 (SBP)/ 95 (DBP) mmHg.

Blood serum examinations: The serum examinations were performed during non-inflammatory periods in each disease. The serum levels of calcium (Ca) and inorganic phosphorus (Pi) were measured by orthocresolphthalein (OCPC) and p-methylaminophenol deoxydization method with modification, respectively[12]. The levels carboxyl-terminal parathyroid hormone fragment (PTH-C)[13] and calcitonin[14] were measured by immunoactive radioimmunoassay. Normal ranges in our laboratory were as follows: Ca 3.4-5.1 mEq/l; Pi 2.8-4.5 mg/dl; PTH-C under 1.3 ng/ml; Calcitonin under 100 pg/ml, respectively. Minimum sensitivity levels were 0.12 ng/ml for PTH-C[13] and 25 pg/ml for calcitonin[14].

Measurement of intracellular free calcium ($[Ca^{2+}]_i$) in platelet and erythrocyte: $[Ca^{2+}]_i$ in platelet was measured by modification of the method according to Tsien et al.[15] Blood was gently withdrawn from antecubital vein into a plastic syringe containing 3.8% acid citrate dextrose, and platelet rich plasma was prepared in a silicon-coated glass tube by centrifugation at 150×g for 10 minutes at room temperature. Platelet rich plasma was incubated for 20 minutes with 5μM fura-2-tetrakis acetoxymethyl-ester (fura-2/AM) (Dojin Laboratories, Kumamoto, Japan) in a shaking water bath at 37℃. After incubation, extracellular fura-2/AM was removed by centrifugation at 350×g for 10 minutes. Platelets were suspended to approximately 1.0×10^8 cells/ml after counting platelets by a microcell calculator (Sysmex AD-260 and F-800, Tokyo, Japan) in a calcium-free buffer solution containing 10mM N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid (HEPES), 145mM NaCl, 5 mM KCl, 1mM $MgSO_4$, 0.5mM Na_2HPO_4, and 5mM glucose at pH 7.4 and 37℃. And then, platelets were incubated for minutes at 37℃ in the buffer containing 1mM $CaCl_2$ before fluorescence measurement.

The level of $[Ca^{2+}]_i$ in erythrocyte was examined by modification of the method according to David-Dufilho et al.[16] Citrated blood was centrifugated at 150×g for 10 minutes at room temperature. Twenty microliter of the sedimentated erythrocyte were suspended in a Ca-containing buffer solution with 25mM HEPES, 123mM NaCl, 5mM KCl, 1mM $MgCl_2$, 1.3mM $CaCl_2$, and 10mM glucose at pH 7.4 and 37℃. Erythrocytes were incubated for 10 minutes in the buffer after adding 2mM N,N,N',N'-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN), which is zinc- and iron-chelator, and then incubated for 20 minutes at 37℃ after adding 5μM fura-2/AM. Subsequently, erythrocytes were centrifugated at 350×g for 10 minutes to remove the extracellular fura-2/AM and resuspended to 0.1×10^8 cells/ml in the same buffer containing 1mM $MnCl_2$, and then incubated for 3 minutes at 37℃ before measurement.

As shown in Figure 1, $[Ca^{2+}]_i$ of platelets and erythrocyte were measured using fluorescence spectrophotometer (Hitachi F-2000, Tokyo, Japan) in a thermostated quartz cuvette at 37℃. The level of $[Ca^{2+}]_i$ (nM/l) was calculated from the following equation described by Grynkiewicz et al.[17]: $[Ca^{2+}]_i = Kd\times[(R-Rmin)/(Rmax-R)]\times\beta$, where R is the ratio of fluorescence of the sample at dual

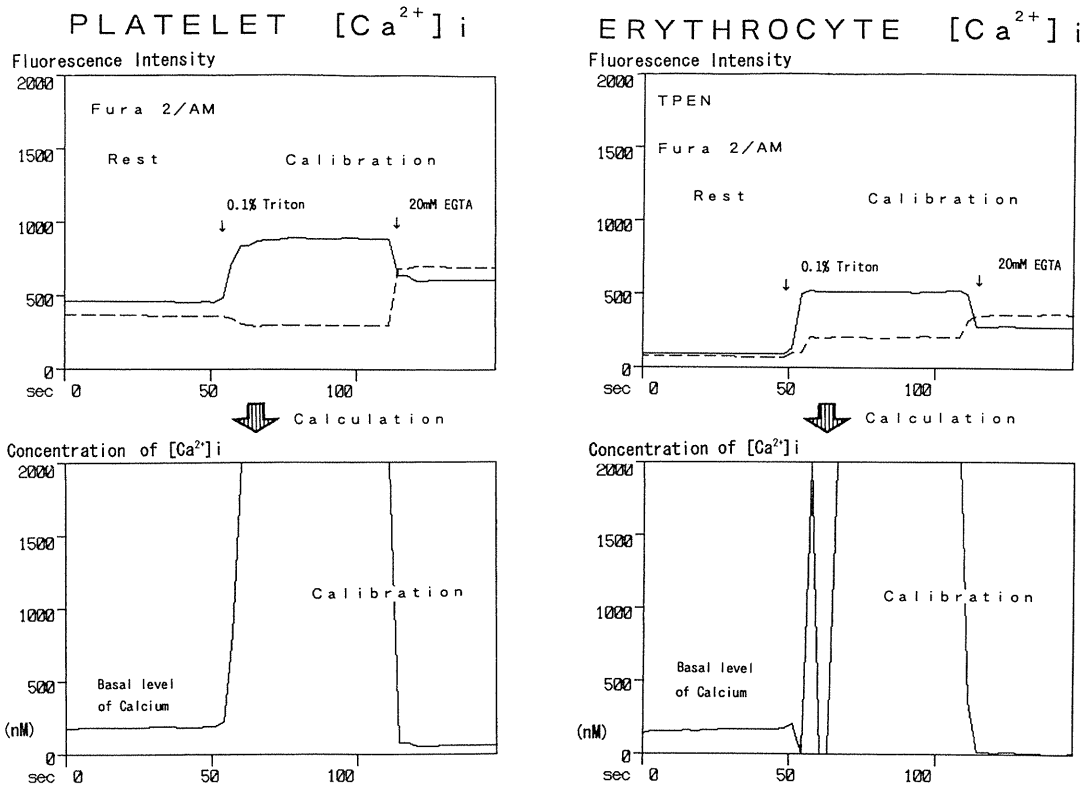

Fig. 1. The measurement method of intracellular free calcium ($[Ca^{2+}]_i$) in platelet and erythrocyte after incubation with fura-2/AM by using fluorescence spectrophotometer.

Upper pannels: Emission fluorescence (510 nm) by dual wavelength excitation (solid line is by 340 nm, and broke line is by 380 nm).

Lower pannels: Results of calculation by dual wavelength measurement according to the equation described by Grynkiewicz et al.[17]

excitation wavelengths of 340 and 380 nm, and at emission wavelength of 510 nm with 10 nm bandwidth; Rmax and Rmin are the ratios of fluorescence intensities in fura-2-Ca^{2+} complex at the same wavelengths in the presence of saturating amount of Ca^{2+} and in the normally zero Ca^{2+}, respectively; Rmax is determined by rupturing the cells with 0.1% Triton X-100 and adding a saturating amount of Ca; Rmin is determined by adding 0.1% Triton X-100 and 20mM EGTA with increasing the pH to 8.3; β is the ratio of fluorescence of fura-2 at 380nm in zero and saturating amount of Ca^{2+}; Kd is the dissociation constant of fura-2 for Ca^{2+}, which is assumed to be 224nM at 37℃. The data of fluorescence intensities were transferred from the spectrophotometer to a personal computer (NEC, PC-9801) via a RS-232C communication pathway for calculation, and stored in floppy disks. All measurements were done in duplicate and the mean values were used for statistical calculation. Intra-assay variance was 9.4% (n=5), and day-to-day individual variability was 12.5% (n=5).

Measurement of bone mineral content (BMC) in the lumbar vertebral body: As shown in Figure 2, we measured the BMC of vertebral bodies by the lumbar computed tomography (CT) using a single-energy CT equipment (Hitachi Medico CT-W400-20, Tokyo, Japan) and a calibration phantom (Chugai Pharmaceutical B-MAS, Tokyo, Japan) according to the method of Fujii et al.[18] The phantom was attached closely to the lumbar back of the patient in a supine

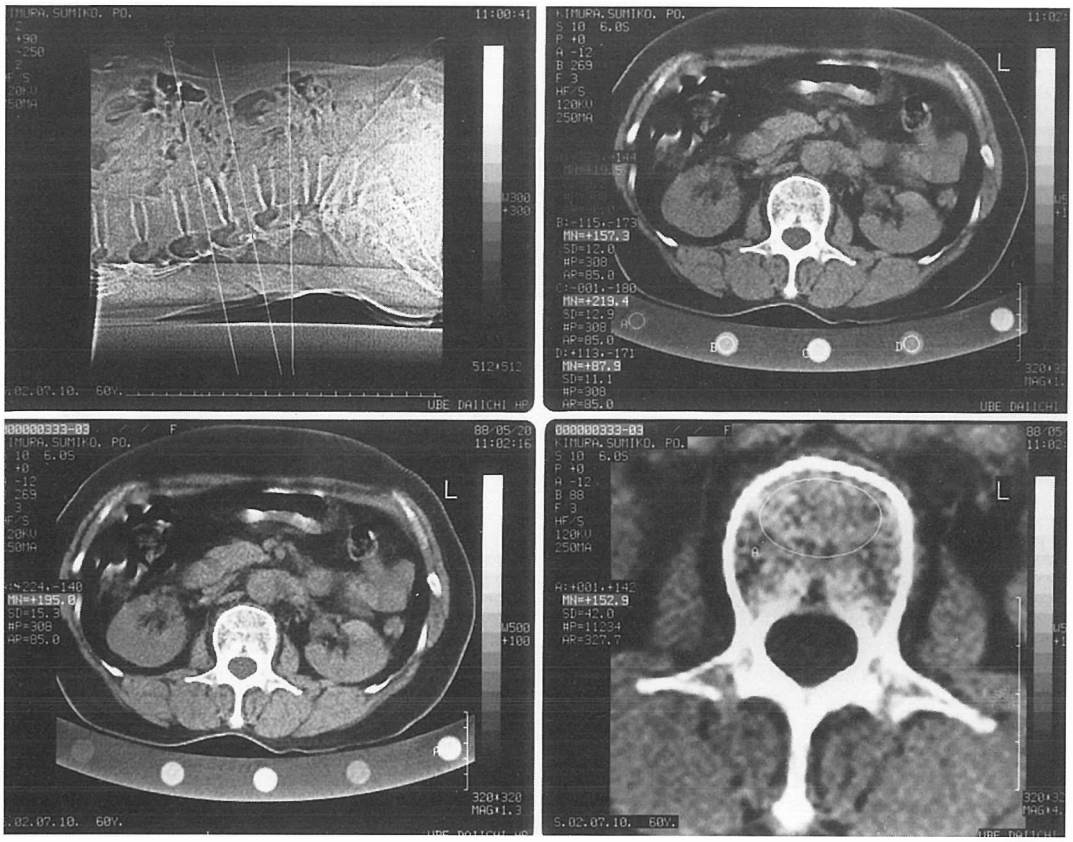

Fig. 2. The measurement method for bone mineral content (BMC) of the lumbar vertebral body.
Upper left: Scanogram and scanning lines of lumbar vertebral bodies in a scout-view image.
Upper right and lower left: The simultaneous scanning image of both the vertebral body and the calibration phantom. Five regions of interest (ROIs) were set on 5 standard substances of phantom.
Left right: ROI was set on the spongious bone of vertebral body by the largest ellipsoidal circle, excluding a cortical bone and nutritional foramen.

position at scanning slice angle perpendicular to the midplane of the vertebral body, and scanned simultaneously with the vertebral body (Fig. 2, Upper left). Subsequently, we set the region of interest (ROI) in the spongious bone of vertebral body using the largest ellipsoidal circle, excluding a cortical bone and a nutritional foramen. We calculated the mean CT value (Hounsfield unit) in the ROI of the vertebral body (Fig. 2, Lower right). Similarly, we set the 5 ROIs on 5 standard substances in the calibration phantom by using round circles, and calculated the mean CT values within the 5 ROIs (Fig. 2, Upper right and lower left). Five standard substances were composed of the following concentrations of $CaCO_3$: 32.31, 80.17, 133.47, 177.03, and 223.66 mg/cm^3, in this order[18]. The first regression line was calculated by the least squares method using the mean CT value of each standard substance. By applying the CT value for the vertebral body to the first regression line, BMC (mg/cm^3) was obtained. In all patients, we scanned the 2nd, 3rd and 4th vertebral bodies (Fig. 2, Upper left). Results of the 3rd body were used as the representative BMC for most patients[19]. In patients with compression fracture or marked callus formation in the 3rd vertebral body, results of the 2nd or 4th body were used

Table 1. Mean values of examination results, and correlation coefficients between these parameters and age, systolic or diastolic blood pressure.

Examinations	Unit	n=	Mean±SD	Correlation coefficients vs Age (year) 80.2±4.9	vs SBP (mmHg) 142.3±30.9	vs DBP(mmHg) 73.7±11.6
Serum Ca	mEq/l	18	4.5±0.3	-0.475 NS	-0.455 NS	0.133 NS
Serum Pi	mg/dl	18	2.9±1.2	-0.232 NS	-0.189 NS	0.013 NS
Serum PTH-C	ng/ml	14	0.52±0.23	0.130 NS	0.105 NS	-0.385 NS
Serum Calcitonin	pg/ml	14	40.6±25.9	-0.156 NS	-0.118 NS	-0.012 NS
BMC	mg/cm^3	13	54.4±48.8	-0.684 $p<0.01$	-0.058 NS	0.508 NS
Platelet $[Ca^{2+}]_i$	nM/l	18	119.3±40.8	0.186 NS	0.691 $p<0.01$	0.381 NS
Erythrocyte $[Ca^{2+}]_i$	nM/l	18	166.2±35.4	0.043 NS	0.167 NS	0.195 NS

Abbreviations: SBP=Systolic blood pressure; DBP=Diastolic blood pressure; Ca=Calcium; Pi=Inorganic phosphorus; PTH-C=Carboxyl-terminal parathyroid hormone fragment; BMC=Bone mineral content of lumbar vertebral body; $[Ca^{2+}]_i$=Intracellular free calcium; NS=Not significant; n=The number of patients; SD=Standard deviation.

as the representative BMC. Patients with severe compression fracture or marked scoliosis throughout the three vertebral bodies were excluded[19].

Statistics analysis: Correlation coeffiencies between variables were calculated by the first line regression of Pearson's correlation. P value lower than 0.05 was significant. Data represent mean ± standard deviation (SD).

Results

As shown in Table 1, the mean value of age was 80.2±4.9 years old; SBP 142.3±30.9 mmHg; DBP 73.7±11.6 mmHg; Ca 4.5±0.3 mEq/l; Pi 2.9±1.2 mg/dl; PTH-C 0.52±0.23 ng/ml; calcitonin 40.6±25.9 pg/ml; BMC 54.4±48.8 mg/cm^3; $[Ca^{2+}]_i$ in platelet 119.3±40.8 nM/l; $[Ca^{2+}]_i$ in erythrocyte 166.2±35.4 nM/l, respectively. BMCs were used as the representative results in 2 patients from the 2nd lumbar vertebral body, in 8 from the 3rd, and in 3 from the 4th, respectively.

As shown in Figure 3 and Table 1, SBP had significant positive correlation with $[Ca^{2+}]_i$ in platelet ($r=0.691$; $p<0.01$) (Fig. 3, Left lower), and had a tendency to negatively correlate with Ca ($r=-0.455$) (Fig. 3, Left upper). However, there were no correlations between SBP and Pi, PTH-C, calcitonin, BMC or erythrocyte $[Ca^{2+}]_i$. On the other hand, DBP had no significant correlations with all of the parameters (Fig. 4, and Table 1).

Ca was correlated negatively with Pi ($r=-0.807$; $p<0.001$). $[Ca^{2+}]_i$ in platelet had significant negative correlation with Ca ($r=-0.503$; $p<0.05$) (Fig. 5, Left upper). However, there were no significant correlations between platelet $[Ca^{2+}]_i$ and age, DBP, Pi, PTH-C, calcitonin or BMC (Fig. 5 and 6). On the other hand, erythrocyte $[Ca^{2+}]_i$ had significant negative correlations with Ca ($r=-0.550$; $p<0.05$) (Fig. 5, Right upper) and Pi ($r=-0.553$; $p<0.05$) (Fig.5, Right lower). Erythrocyte $[Ca^{2+}]_i$ had no correlations with age, PTH-C, calcitonin or BMC (Fig. 6 and Table 1). The correlation between $[Ca^{2+}]_i$ in platelet and erythrocyte was not significant ($r=0.398$). As shown in Table 1 and Figure 7, BMC had a significant negative correlation with age ($r=-0.684$; $p<0.01$), but had no correlations with other results.

Discussion

Blood pressure and calcium metabolism: Cytosolic free Ca is a fundamental regulator in intracellular response to external stimuli such as contraction of muscle cells, stimulation of secretion, and many other cellular mechanisms. Recent studies in patients with hypertension showed the increased basal levels of intracellular Ca in platelet as compared to a normotensive group[1)2)]. The contractile activity of vascular smooth muscle is regulated by the level of intracellular free Ca ions[20)]. The increased platelet intracellular Ca in patients with hypertension

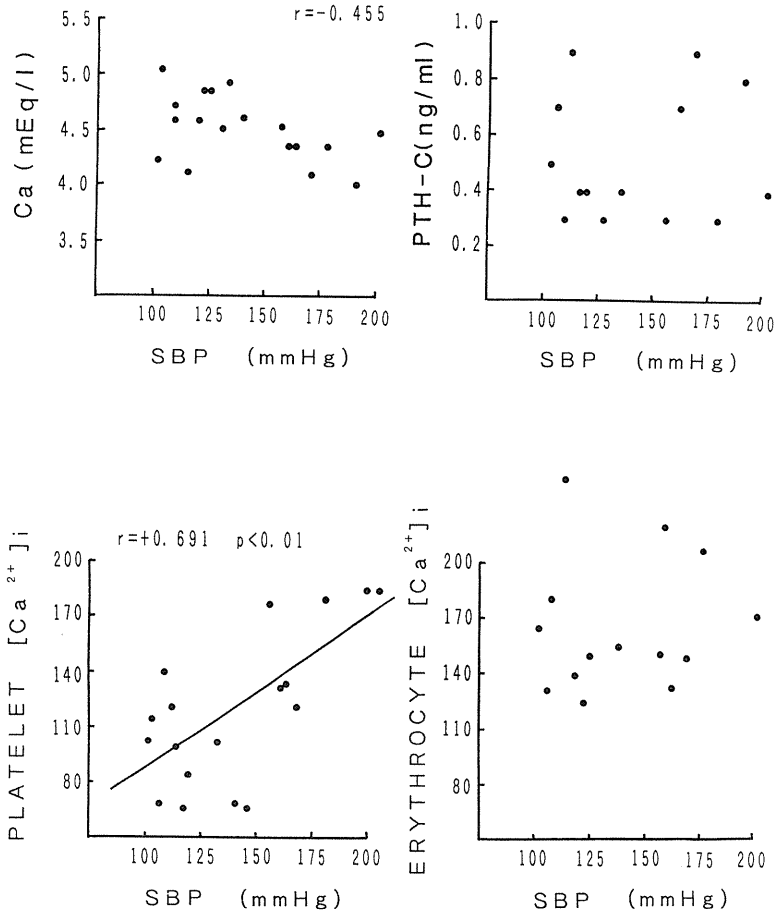

Fig. 3. The between systolic blood pressure (SBP) and serum calcium (Ca), C-terminal parathyroid hormone fragment (PTH-C), platelet intracellular free calcium ($[Ca^{2+}]_i$), or erythrocyte $[Ca^{2+}]_i$.

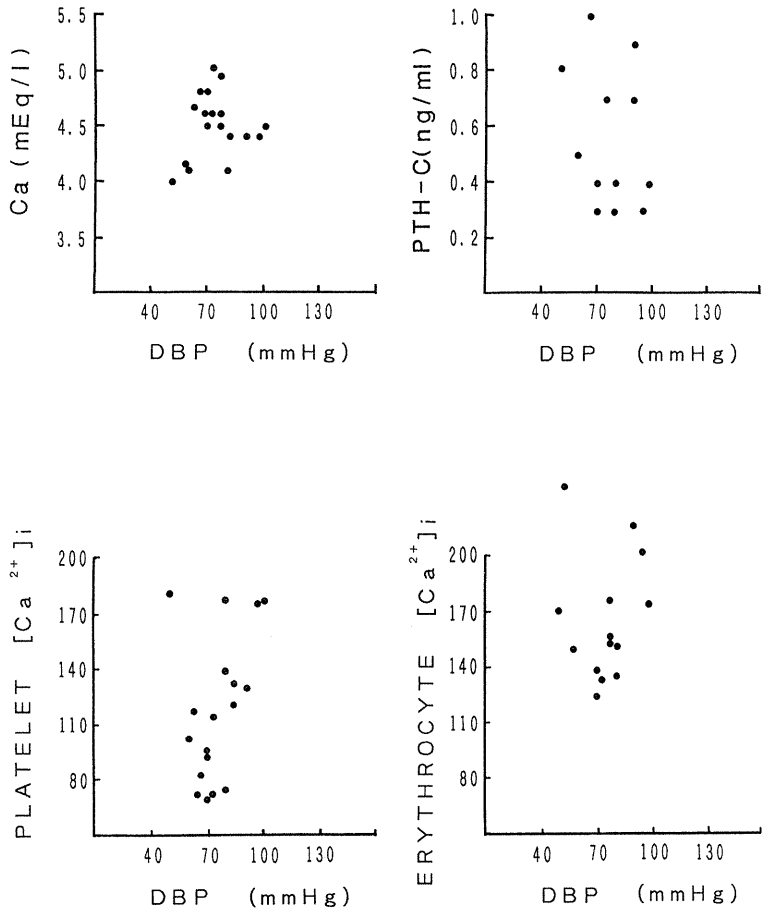

Fig. 4. The correlations between diastolic blood pressure (DBP) and serum calcium (Ca), C-terminal parathyroid hormone fragment (PTH-C), platelet intracellular free calcium ($[Ca^{2+}]_i$), or erythrocyte $[Ca^{2+}]_i$.

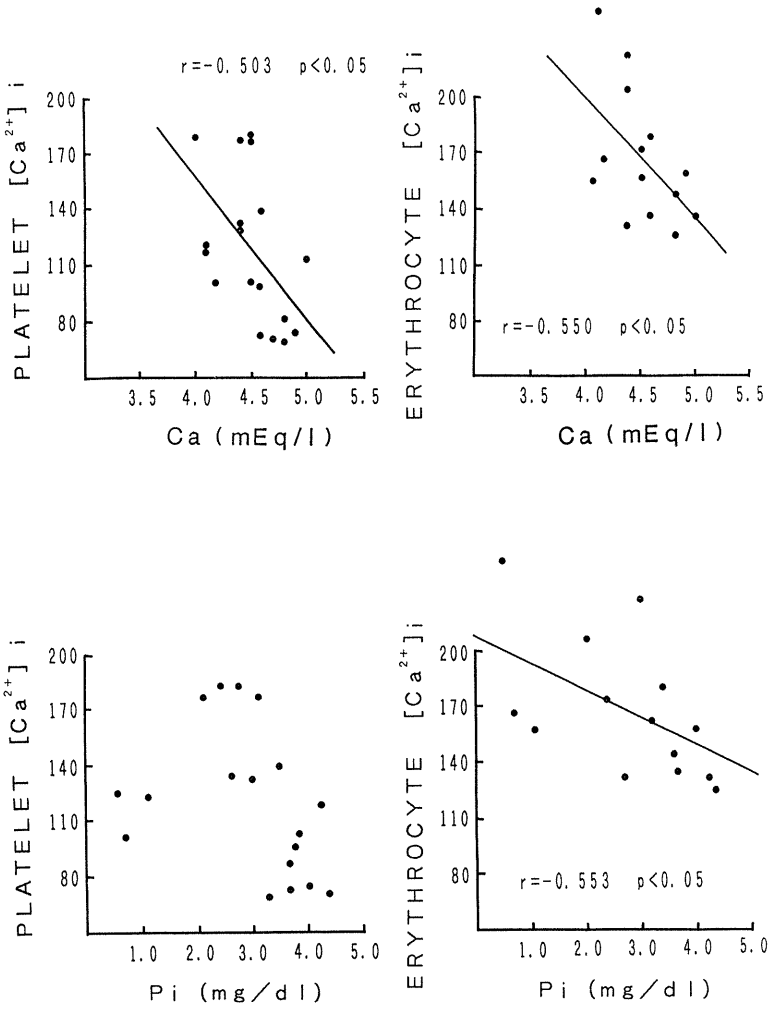

Fig. 5.

Upper figures: The correlations between serum calcium (Ca) and platelet intracellular free calcium ($[Ca^{2+}]_i$), or erythrocyte $[Ca^{2+}]_i$.

Lower figures: The correlations between serum inorganic phosphorus (Pi) and platelet $[Ca^{2+}]_i$, or erytrocyte $[Ca^{2+}]_i$.

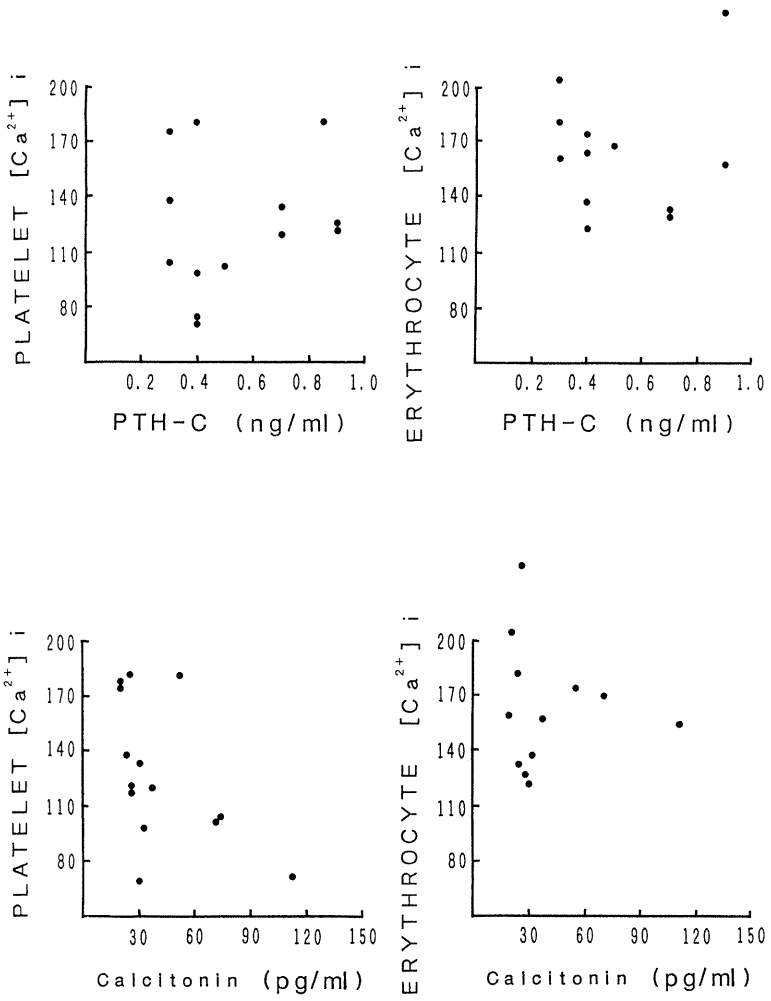

Fig. 6.

Upper figures: The correlations between C-terminal parathyroid hormone fragment (PTH -C) and platelet intracellular free calcium ($[Ca^{2+}]_i$), or erythrocyte $[Ca^{2+}]_i$.

Lower figures: The correlations between calcitonin and platelet $[Ca^{2+}]_i$, or erythrocyte $[Ca^{2+}]_i$.

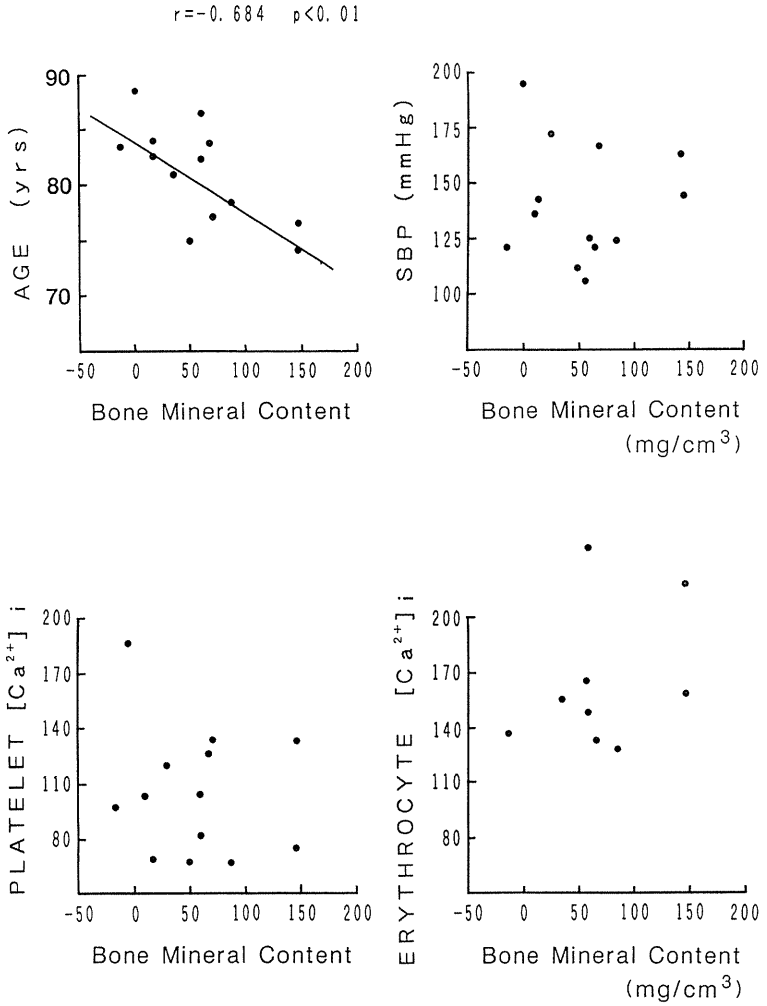

Fig. 7. The correlations between bone mineral content (BMC) and age, systolic blood pressure (SBP), platelet intracellular free calcium ($[Ca^{2+}]_i$), or erythrocyte $[Ca^{2+}]_i$.

is considered to reflect an abnormal contractile activity of vascular smooth muscle. Studies in patients with essential hypertension also showed a picture of increased renal calcium excretion, decreased serum ionized Ca and raised serum parathyroid hormone concentration[7)8)21)]. Raised parathyroid hormone increases the intracellular Ca in vascular smooth muscle and elevates blood pressure[22)]. Other studies reported that the low-renin hypertensives had an elevated serum vitamin D and parathyroid hormone concentrations, but high-renin hypertensives had a raised serum calcitonin concentration[9)]. Parathyroid hormone also reduces cellular calcium uptake and tone of vascular smooth muscle, resulting in a reduction of blood pressure[23)24)]. However, the pathophysiological roles of vitamin D, parathyroid hormone and calcitonin on essential hypertension remain to be explained satisfactorily. Previous experimental reports showed that increased activity of platelet membrane Ca^{2+}-ATPase caused the reduction of Ca efflux pump function of vascular smooth muscle cell in essential hypertension[25)26)]. Similar mecha-

nism of calcium handling in erythrocyte membrane is considered to cause the raised blood pressure in SHR[27)28)]. With respect to putative mechanism of calcium metabolism affecting on essential hypertension, some authors presumed that the genetic defect of cell membrane transport of Ca cause the decreased intestinal calcium absorption, the decreased modification of vitamin D in impaired kidney, and the increased urinary calcium excretion[8)29)]. These phenomena result in the raised parathyroid hormone concentration and the elevated intracellular Ca of vascular smooth muscle in essential hypertension[8)29)].

The present study showed that systolic blood pressure had significant correlation with concentration of platelet intracellular Ca, and that diastolic blood pressure had no statistically significant correlation with any parameters of calcium metabolism. The relationship between systolic blood pressure and platelet intracellular Ca was compatible with the report of Erne et al[1)]. In the elderly, parathyroid hormone increases and calcitonin decreases in parallel with aging[8)29)]. Experimental reports documented that SHRs reduced levels of circulating vitamin D, decreased intestinal calcium absorption, and reduced bone density or bone calcium content[10)11)21)30)]. However, the present study showed that both systolic and diastolic pressure were independent from concentration of serum calcium-regulating hormones and bone calcium metabolism. We considered that systolic blood pressure in the elderly human is influenced by calcium metabolism in some extent, but not by serum PTH-C and calcitonin levels or bone calcium metabolism.

Problems in measurement of intracellular free Ca: 1) Age is an important determinant of platelet intracellular free Ca. Duggan reported that platelet intracellular Ca has significant positive correlations with both age and mean blood pressure in normotensives[31)]. 2) A gender factor may affects intracellular Ca concentration. Young et al. reported that concentration of parathyroid hormone was increased in hypertensives in men, but not in women, as compared to normotensives[32)]. In the elderly subjects, age and sex differences of calcium metabolism should be studied. However, we could not compare these differences in the small number of subjects selected from the elderly with multiple concomitant diseases in the present study. 3) Leakage of intracellular fura-2/AM increases with incubation time[33)]. Thus, the leaked fura-2/AM combined with extracellular Ca gives gradually increasing falsely high intracellular free Ca values[33)]. In the present study, resuspension and incubation in the buffer solution with added extracellular free Ca were performed as quickly as practical. 4) The measurement of erythrocyte intracellular Ca is subjected to interference by fluorescence of hemoglobin. This should have been minimal because of the high dilution of erythrocyte suspension in this study. However, there was a possibility of such interfering emission fluorescence after rupturing the cell by Triton X -100. Also, fluorescence of fura-2 complexes with zinc, iron, manganese, and other trace in erythrocyte might interfere the measurement.

Clinical significance of calcium metabolism in essential hypertension: Essential hypertension and osteoporosis become frequent with age. Ca^{2+} antagonists which suppress of platelet intracellular Ca concentration are more effective than other types of antihypertensive drugs[1)34)35)]. Ca^{2+} antagonists are considered to be the clinically best drugs for ameliorating the abnormal contractile activity of vascular smooth muscle in essential hypertension. The study of calcium metabolism will lead to establish the future treatment for essential hypertension.

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