Dietary Calcium Reduces Blood Pressure, Parathyroid Hormone, and Platelet Cytosolic Calcium Responses in Spontaneously Hypertensive Rats

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Dietary calcium effects on blood pressure, parathyroid hormone (PTH), and platelet cytosolic calcium concentrations were investigated. The dietary calcium (low, 0.2%; medium, 0.5%; and high, 2.0% wt/wt) was supplemented in spontaneously hypertensive rats from 6 through 22 weeks of age. Mean systolic blood pressure was decreased by age 12 weeks with calcium supplementation (low, 227 ± 6 mm Hg; medium, 211 ± 6 mm Hg; and high, 182 ± 7 mm Hg; $P < .001$). By the 10th week of age, the low calcium group had significantly ($P < .05$) more elevated (44 ± 2.3 pg/ml) plasma PTH compared with the high calcium-supplemented group (15 ± 4.5 pg/mL). Regression analysis showed a significant ($P < .001$) positive correlation (r = 0.3) between systolic blood pressure and PTH.

The platelet cytosolic calcium concentration was determined using the fura-2 method. The basal calcium was 134 ± 5.5 nmol/L for the low calcium group and thrombin increased to 228 ± 8 nmol/L ($P < .0001$; +70% change). The normal calcium group had 202 ± 8 nmol/L; thrombin increased to 239 ± 10 nmol/L ($P < .0026$; +19% change). The high calcium group had basal levels 145 ± 7 nmol/L, with thrombin stimulating to 212 ± 8 nmol/L ($P < .0001$; +46% change). Although thrombin increased platelet cytosolic calcium concentration in all groups, normal and high dietary calcium groups had smaller percentage increases (51% and 24% lesser, respectively) compared with the low dietary calcium group. The blood pressure-lowering effects of dietary calcium is associated with blunting of thrombin-induced increases in platelet cytosolic calcium, suggesting the involvement of an intracellular second messenger system mediated by receptors. Am J Hypertens 1994;7:1052–1057

KEY WORDS: **Blood pressure, dietary calcium, platelet cytosolic calcium, plasma PTH spontaneously hypertensive rat.**

Dietary calcium supplementation has blood pressure-lowering effects in spontaneously hypertensive rats (SHR)[1,2] and in humans.[3] Hypertensive animals and humans do manifest several abnormalities in calcium handling at the systemic level that alters the calcium balance in the whole body. SHR had higher circulating parathyroid hormone (PTH) levels compared with normotensive strain (WKY) animals[4] and these elevated levels of PTH might be a secondary response to depressed serum-ionized calcium,[4] probably brought in by increased urinary calcium excretion.[5] McCarron et al[6] and Strazzullo et al[7] postulated a renal "calcium leak" in essential hypertension and in addition dietary calcium supplementation induced a "membrane stabilization" effect that leads to the correction of this calcium leak.[8] Substantial evidence ex-

Received December 10, 1993. Accepted July 26, 1994.

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This study was supported by National Institutes of Health grants No. GM 08140 and RR 03026.

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0895-7061/94/$7.00

ists linking calcium transport-related membrane defects to hypertension.[9,10] Previously, we reported a significant reduction in the expression of two calcium-binding proteins in aortic smooth muscle of SHR.[11]

The information on the basal levels of platelet cytosolic calcium is conflicting in the hypertensive condition. Some investigators[12,13] showed higher levels of basal platelet cytosolic calcium in SHR and in essential hypertensive patients, wheres others[14] found no change in basal platelet cytosolic calcium in SHR. Also, when measured in the Ca^{2+} free buffer,[15] basal levels platelet cytosolic was not higher in SHR. The agonist-induced responses of platelet cytosolic calcium were greater in prehypertensive rats.[16] The addition of thrombin induced a significantly greater increase in the platelet cytosolic calcium of hypertensive rats compared with the normotensive strain, but ionomycin could not, suggesting a receptor-linked second messenger pathway abnormality.[17]

To date, two studies are available pertaining to the influence of dietary calcium supplementation on platelet cytosolic calcium concentration. Wuorela et al[18] have shown that 12 weeks of dietary calcium supplementation decreased basal levels of platelet cytosolic calcium in SHR, without data on agonist-stimulated levels. The second study using stroke-prone SHR on a high sodium-low calcium diet from 3 weeks of age through 9 weeks demonstrated greater basal and ionomycin-stimulated responses in platelet cytosolic calcium.[19] But no information exists regarding the thrombin-stimulated responses in platelet cytosolic calcium with dietary calcium supplementation alone. Hence in this study three levels of dietary calcium (0.2, 0.5, and 2.0% wt/wt) were supplemented from 6 through 22 weeks of age in SHR. We measured systolic blood pressure (SBP), plasma PTH, and plasma-ionized calcium every 2 weeks throughout the supplementation period. At the end of the supplementation, basal and thrombin-stimulated responses in platelet cytosolic calcium were determined. Probably this is the first longitudinal study reporting the results of 16 weeks of dietary calcium supplementation starting from a stage before the hypertension has been fully established in SHR.

METHODS

Eighteen male SHR (Aoki-Okamato strain) at 4 weeks of age were purchased from Harlan Sprague Dawley (San Diego, CA) and were acclimatized to the vivarium conditions. Beginning at 6 weeks of age, the animals were randomly placed on three diet groups (n = 6) based on the dietary composition of calcium (wt/wt, low [0.2%], medium [0.5%], and high [2.0%] Teklad Research Diets, Madison, WI). The same dietary ingredients were maintained except for the varying levels of calcium. All animals were given unlimited and free access to diet and water and were maintained on diets for 16 weeks.

Every 2 weeks SBPs were measured three times by the indirect tail-cuff method with a PE-300 programmable sphygmomanometer (NARCO Biotechnologies, Austin, TX). After blood pressures were taken, blood was collected (0.5 to 0.8 mL) from the caudal vein into a tube with 50 μL of heparin. At the end of the calcium supplementation period, all animals were anesthetized with intraperitoneal injection of ketamine (15 mg/100 g body weight). While under anesthesia, the thoracic cavity was surgically opened and blood (6 to 8 mL) was collected with a syringe containing 0.2 mL of heparin, for the isolation of platelets and determination of cytosolic calcium concentration.

Plasma PTH for intact molecule was assayed using the radioimmunoassay method.[20] Plasma-ionized calcium was measured by COBAS-FARA computerized autoanalyzer. Each sample was run in duplicate and an average value was considered as one determination. All the samples from the study were assayed in one batch.

The centrifugation technique of Tepel et al[21] was used for isolation of platelets. Briefly, to isolate platelets, blood samples were centrifuged immediately at 300 *g* for 8 min without the usage of brake at 22°C and platelet-rich plasma was aspirated into a separate tube from the rest of the sedimented cells. Platelets were isolated from the platelet-rich plasma by immediate centrifugation at 600 *g* for 10 min at 22°C and platelets were resuspended into a buffer containing (mmol/L) sodium chloride 145, potassium chloride 5, magnesium sulfate 1, dibasic sodium phosphate (Na_2HPO_4) 0.5, *N*-2 hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES) 10, and glucose 5, at pH 7.4.

Platelet cytosolic calcium concentration was measured using the fura-2 method as published previously by Grynkiewicz et al.[22] Briefly, platelet suspension in buffer was incubated with 2 μmol fura-2 acetoxymethyl ester (fura 2-AM) (Sigma Co., St. Louis, MO) for 30 min at room temperature (22°C). At the end of the incubation period, the platelet suspension was centrifuged at 600 *g* for 8 min at 22°C to wash extraneous fura-2 AM and again resuspended in buffer. Platelets were washed again with buffer and centrifuged at 600 *g* and final preparation of platelet suspension contained 1 mmol/L CaCl. A random aliquot of platelet preparation was tested for viability by the trypan blue exclusion method and found that >95% platelets were viable. Two milliliters of this preparation was placed in a disposable cuvette and fluorescence was measured in Hitachi F2000 Dual Scanning spectrofluorometer, with excitation wavelengths of 340/380 nm and emission wavelength of

510 nm. Agonist-stimulated responses were obtained by the direct addition of 0.1 U/mL thrombin (Sigma) into the platelet suspension in the cuvette, through a syringe. Equivalent volume of vehicle was added in a separate run to determine and correct for the effects of medium in which thrombin was added. All the values were corrected for autofluorescence from unloaded platelets. Maximum fluorescence (R_{max}) was obtained by the addition of 1 mL digitonin and minimum fluorescence (R_{min}) was determined after adding 1 mmol/L alkaline Tris-EGTA (pH 8.4). Intracellular calcium concentration was calculated by the use of the following formula:

$$[Ca^{2+}]_i = K_d \times (R - R_{min}/R_{max}),$$

where $K_d = 224$.

As and when the data was collected, the values were entered into a Macintosh LCII and a second operator has rechecked the data for entry and accuracy. We used the "StatView 4.0" program on the LCII for all statistical analysis. Statistical analysis of the data was performed by one-way analysis of variance. When differences were found among groups, Fisher's *t* test was used for further determination of significant differences. Simple regression analysis was used to investigate the relationship between SBP and plasma PTH. Statistical significance was established at a *P* value of $<.05$.

RESULTS

The SBP values of SHR on low, medium, and high dietary calcium are presented in Figure 1. At 12 weeks of age the high calcium group had significantly ($P < .001$) lower (182 ± 7 mm Hg) SBP compared with the low (227 ± 6 mm Hg) and medium calcium groups (211 ± 6 mm Hg). The reduction in SBP in the high calcium group compared with the medium and normal groups was persistent throughout the study period. Simple regression analysis showed that SBP was strongly correlated ($r = 0.7$; $P < .001$) with basal and thrombin-induced platelet cytosolic calcium concentrations.

Plasma PTH concentrations of the three groups of animals are given in Figure 2. By the 10th week of age, the low calcium group had significantly ($P < .05$) higher (44 ± 2.3 pg/ml) levels compared with the high calcium group (15 ± 4.5 pg/ml). The higher plasma levels of PTH in the low calcium group and the depressed values in the high calcium groups were consistently observed. A significant ($P < .001$) positive correlation ($r = 0.3$) was observed between SBP and plasma PTH. Plasma PTH values had no correlation with platelet cytosolic calcium levels.

Plasma-ionized calcium concentrations (not presented) were modulated throughout our study. Simple regression analysis showed a weak ($r = -0.161$) and insignificant ($P > .05$) correlation between plasma-ionized calcium levels and plasma PTH. Also, plasma-ionized calcium levels were correlated ($r = -0.423$) with basal and thrombin-induced levels ($r = -0.272$) of platelet cytosolic calcium, but were not statistically significant.

Basal and thrombin-stimulated platelet cytosolic calcium concentrations are presented in a scatterplot on Figure 3. The basal levels were 134 ± 5.5 nmol/L for the low calcium group and thrombin significantly ($P < .0001$) increased to 228 ± 8 nmol/L (+70% change). The basal cytosolic calcium for the medium calcium group was 202 ± 8 nmol/L; thrombin increased to 239 ± 10 nmol/L ($P < .0026$) (+19%

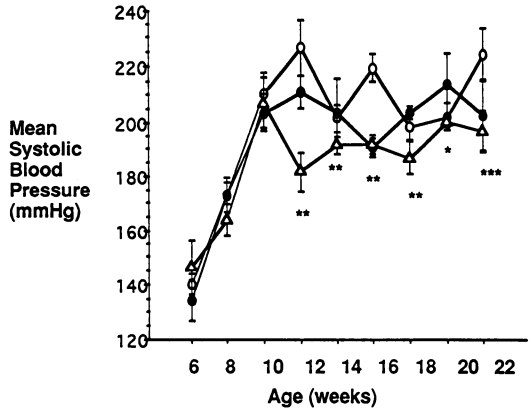

FIGURE 1. *Mean systolic blood pressure from 6 through 22 weeks of age of SHR on selected calcium diets (-○-, low; -●-, medium; -△-, high). Each value is the average of six (n = 6) observations at that time point. *Low group significantly different from medium group; **low group significantly different from high group; ***low group significantly different from medium and high groups (P <.05).*

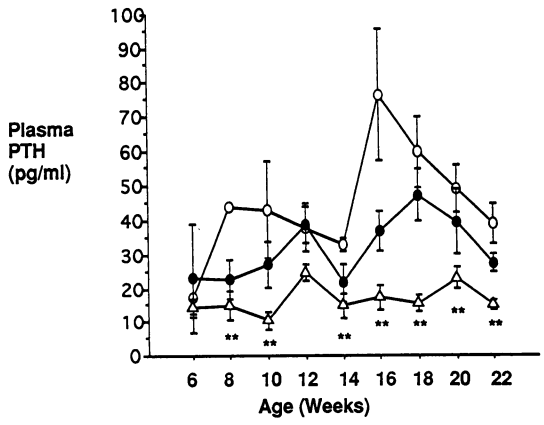

FIGURE 2. *Plasma parathyroid hormone (PTH) from 6 to 22 weeks of age of SHR on selected calcium diets (-○-, low; -●-, medium; -△-, high). Each value is the average of six (n = 6) observations at that time point. **Low group significantly different from high group (P <.05).*

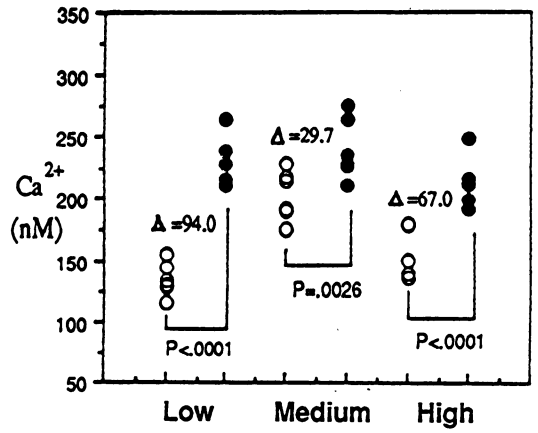

FIGURE 3. *Scatterplot showing platelet cytosolic concentration of SHR. (open circle) Basal; (closed circle) thrombin-stimulated level. P values were obtained by the Fisher's t test. Each value is the average of six (n = 6) observations. Delta values represent nanomoles per liter of calcium increase in the mean platelet cytosolic calcium concentration stimulated by 0.1 U/mL thrombin addition.*

change). The high calcium group had a basal platelet cytosolic calcium concentration of 145 ± 7 nmol/L and was significantly (P <.0001) increased by thrombin addition to 212 ± 8 nmol/L (+46% change). Although all groups had demonstrated significantly increased platelet cytosolic calcium concentration by thrombin addition, the medium and high dietary calcium groups had smaller percent increases (51% and 24% lesser, respectively) compared with the low dietary calcium group.

DISCUSSION

Dietary calcium supplementation lowered blood pressure in SHR[1,23] and in humans.[3,12] Our current study presents results of the longest dietary calcium supplementation study, starting from 6 weeks of age in SHR and determines the SBP, plasma PTH, platelet cytosolic calcium, and the thrombin-stimulated responses (Figures 1 to 3). The objective of this longitudinal study was to explore the hypertension preventive potential of dietary calcium therapy, if supplementation starts from a stage before hypertension has been fully established in SHR, with an explanation of cellular calcium basis for such an effect. As we have found in our study (Figure 1), the higher the calcium in the diet, the lower the blood pressure and vice versa. This inverse relationship between dietary calcium and blood pressure was significant (P <.0001) in our study by age 12 weeks, which was after 6 weeks on calcium diets. Similar reduction in blood pressure in SHR[24,25] have been reported, with variations.[26,27] The reduction in SBP might be mediated through changes in circulating calciotropic hormones or changes in the intracellular calcium concentration or both.

Plasma PTH levels were significantly higher in the low calcium group compared with the medium and high calcium groups, with a positive correlation between PTH and SBP (r = 0.296, P <.0001). Higher blood pressures were associated with hypocalcemia,[4] a status that in turn stimulates plasma PTH. In this study, we have observed a similar physiologic process: the lower the dietary calcium, the higher the blood pressure and PTH; the higher the dietary calcium, the lower the blood pressure and PTH, suggesting that blood pressure-reducing effects of dietary calcium might be mediated through the modulation of calciotropic hormone, PTH.

Several investigators have reported basal and agonist-stimulated platelet cytosolic calcium concentration in SHR[16] compared with WKY. Presently, much controversy exists on the issue of positive correlation between blood pressure and basal platelet cytosolic calcium concentrations. Although some observed elevated basal platelet cytosolic calcium concentration,[17] others found no changes in basal levels.[15] This discrepancy might be attributable to the variations in methodology of fura-2, or isolation or preparation of platelets before incubation or age of the animal. In this study, we preferred the centrifugation method to isolate platelets because 1) it minimizes the time lapse between the drawing of blood and the final calcium measurements; and 2) it maximizes the number of samples to be studied. The viability (trypan blue exclusion method) of platelet cells was checked just before the measurement of cytosolic calcium and found that approximately 95% of cells were viable.

Although significant information is available on platelet cytosolic calcium in SHR, few studies focused on the effects of dietary calcium supplementation. Dietary calcium supplementation for 12 weeks decreased basal platelet cytosolic calcium[18] in SHR, which used first generation fluorescent probe quin-2, but no information on agonist-stimulated levels was available. The interactive actions of dietary sodium and calcium for 6 weeks of supplementation using stroke-prone SHR found higher basal and ionomycin-stimulated platelet cytosolic calcium concentration in the high sodium-low calcium group compared with other groups. In the present study, the basal and thrombin-stimulated levels of platelet cytosolic calcium concentration are reported in SHR on three calcium diets. The basal calcium levels were higher in our study compared with the values reported by others. That might be attributable to the methodology used, either in the preparation of platelets or the removal process of extraneous fura-2 from the reaction buffer. The observed lowest basal platelet cytosolic

calcium in the low dietary calcium group could be due to differential membrane permeability for fura-2. Platelet cytosolic calcium concentrations were increased in all groups by thrombin addition, but the medium and high dietary calcium groups demonstrated lower percent rises compared with the low calcium group. Thrombin-induced increases in platelet cytosolic calcium concentration could be mediated through the receptor-linked second messenger system in the cell.[17] The present observation of blunted thrombin-induced response in the group on the higher calcium diet suggests a possibility that dietary calcium supplementation alters a receptor-linked second messenger system in the cells. Cytosolic calcium is a critical intracellular cation in triggering the contraction of smooth muscle.[28] Diminished increases by thrombin in the higher dietary calcium group might be one of the important cellular changes in calcium handling in response to dietary calcium supplementation and consequential blood pressure reduction of SHR.

In conclusion, the present study indicated that dietary calcium supplementation lowered mean SBP in SHR with simultaneous decrease in plasma PTH and a concomitant blunting of thrombin-induced rises in platelet cytosolic calcium.

ACKNOWLEDGMENTS

We thank Mary F. Quash and Nycal Anthony for their administrative and secretarial help. We appreciate Yolanda Nunn, MBA, PhD, for her skilled assistance in the preparation of manuscript.

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