Dietary Calcium Alters Blood Pressure Reactivity in Spontaneously Hypertensive Rats

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Plasma catecholamines and blood pressure reactivity were investigated in spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats maintained on three levels of dietary calcium: low (0.1%), intermediate (1.0%), or high (2.0%). In the SHR, restricted dietary calcium resulted in elevations of mean arterial pressure that were most pronounced during handling and restraint stress \((p<0.05)\). There was no difference between SHR on intermediate and high calcium diets and no dietary effects in the WKY rats. Resting and stressed levels of circulating catecholamines did not differ across diet conditions in either strain. The SHR on low calcium diets had significantly larger pressor responses to infused norepinephrine \((p<0.05)\). There were no differences between the rats on intermediate and high calcium diets. The results indicate that differences observed in blood pressure reactivity across diets in this study may have been a consequence of altered postsynaptic sensitivity rather than an increase in norepinephrine release. \(\text{(Hypertension 1989;13:622-629)}\)

Epidemiological studies have consistently found restricted dietary calcium to be associated with elevations in blood pressure.\(^1\) Studies with animal models of hypertension have verified this association.\(^2,3\) They have further suggested that increases in dietary calcium lower blood pressure.\(^4-8\) Clinical trials of calcium supplementation have confirmed that dietary calcium can lower blood pressure in some hypertensive individuals.\(^9-11\) Despite numerous demonstrations of a relation between dietary calcium and blood pressure, the mechanisms underlying the alterations in blood pressure remain uncertain.

One theoretical explanation for the blood pressure-lowering effect of calcium is the induction of changes in sympathetic nervous system activity. Peuler et al\(^5\) have shown that calcium supplementation in Dahl sodium chloride-sensitive rats prevents the increase in blood pressure normally seen on high sodium chloride diets. This antihypertensive action is associated with an augmentation of baroreceptor reflex inhibition of renal sympathetic nerve activity. These authors concluded that calcium may modify blood pressure through neural mechanisms in this particular model of hypertension. Wyss et al\(^12\) have also reported that calcium supplementation prevents sodium chloride-induced hypertension through neural mechanisms. They found that calcium-enriched diets restored a sodium chloride-induced deficit in norepinephrine (NE) turnover in the anterior hypothalamus of sodium chloride-sensitive spontaneously hypertensive rats (SHR) concurrent with decreases in both arterial blood pressure and plasma NE concentrations.

In contrast, however, neither Stern et al\(^6\) nor Kageyama et al\(^7\) found evidence of altered sympathetic nervous system activity in SHR on supplemental calcium diets, even though blood pressure was lowered. Kageyama suggested that the diet-induced change in blood pressure may have been due to attenuation of vascular reactivity since calcium loading blunted the pressor response to NE in the intact animal as well as vascular reactivity in the isolated hind limb. Stern et al\(^6\) reported that NE induced a significantly greater in vivo pressor response in rats on higher calcium diets and had no effect on vascular reactivity in vitro.

An alteration in sympathetic nervous system activity, a change in vascular reactivity, or a combination of the two would be consistent with reports of calcium-induced alterations in blood pressure responses to behavioral stress. Hatton et al\(^2\) and Huie et al\(^3\) found that restricted dietary calcium potentiated both stress-induced pressor responses in SHR and chronic increases in arterial pressure
seen with prolonged exposure to crowding. Based on these observations, the present study was designed to determine the involvement of sympathetic nervous system activity and vascular responsiveness in diet-induced alterations in blood pressure.

**Materials and Methods**

Male SHR (n=36) and Wistar-Kyoto (WKY) rats (n=36) were obtained from Charles River Breeding Labs (Wilmington, Massachusetts) at 4 weeks of age. On arrival the rats were randomly assigned to one of three calcium diets (Teklad, Madison, Wisconsin): low (0.1%), medium (1.0%), or high (2.0% dry wt) calcium. The synthetic diets conformed to the AIN-76A recommendations and, with the exception of calcium, were identical in all respects. The rats were housed in groups of two or three in standard laboratory gang cages with food and water available ad libitum. The animal quarters were maintained at a temperature of 21°C with a 12-hour light/dark cycle.

After 7 weeks on the diets, a 3-ml blood sample was withdrawn via a subclavian venipuncture for determination of serum electrolytes. An equivalent volume of saline was injected subcutaneously to compensate for fluid loss. The blood samples were allowed to clot at room temperature for 40 minutes before being centrifuged. The serum was frozen (−20°C) until assayed. Ionized calcium was assayed by means of a calcium-specific electrode (Radiometer America, Westlake, Ohio). Total calcium, magnesium, and phosphorous were analyzed by spectrophotometry (CoBas-Bio centrifugal analyzer, Roche Analytical, Nutley, New Jersey); sodium and potassium were determined by flame photometry (Instrumentation Laboratories, Tustin, California).

At 12 weeks of age the animals were anesthetized with halothane and jugular and femoral catheters were implanted. The femoral catheter consisted of a length of PE-50 tubing (0.58 mm i.d. x 0.96 mm o.d.) inserted into the femoral artery and advanced into the abdominal aorta. The free end of the catheter was tunneled subcutaneously to exit at the nape of the neck. The tubing was filled with heparinized saline and plugged with stainless steel wire.

The jugular vein was catheterized with a cannula modeled after that described by Weeks. The cannula consisted of silastic tubing (0.51 mm i.d. x 0.94 mm o.d.) welded to PE-20 tubing (0.38 mm i.d. x 1.09 mm o.d.). The silastic tubing was inserted into the right jugular vein a distance of 3 cm. The free end of the catheter was tunneled subcutaneously to exit with the femoral catheter at the nape of the neck. The animals were given 72 hours to recover from surgery before testing.

Mean arterial blood pressure (MAP) was recorded with a Statham P23ID pressure transducer (Gould Inc., Oxnard, California) in line with a Grass Model 7 polygraph (Grass Instruments, Quincy, Massachusetts). MAP was calculated from the polygraph tracings as diastolic pressure plus one third of pulse pressure.

To obtain a measure of resting blood pressure, recordings were made from unrestrained rats for 30 minutes in the home cage. A 0.5-ml blood sample was then taken from the arterial catheter with a 1.0-cc syringe for the measurement of plasma epinephrine and NE. After the resting measurement, the rats were stressed by tightly restraining them in inverted, U-shaped plastic restrainers with adjustable guillotine inserts at each end. Blood pressure was recorded for 15 minutes and then a second 0.5-ml blood sample was taken for catecholamine assay. After the last blood sample, 1 ml saline was injected to replace volume loss. Blood for catecholamine assay was collected into iced, heparinized tubes that were spun in a refrigerated centrifuge within 5 minutes of collection. Samples were stored at −80°C until assayed.

On the following day, blood pressure responses to bolus infusions of NE and epinephrine were assessed. The animals were restrained and allowed to habituate for 30 minutes. Baseline blood pressure was determined as the average blood pressure over the last 15 minutes of habituation. Thereafter, blood pressure responses to infusions of 0.1, 1.0, and 3.0 µg/kg of NE (arterenol, Sigma Chemical Co., St. Louis, Missouri) and epinephrine (Sigma Chemical Co.) were determined. The sequence of doses was randomized for each drug and the order of drug presentation was counterbalanced. After each dose, blood pressure was allowed to return to baseline before the next dose was given. A minimum of 15 minutes was allowed between the two drug types.

The plasma was assayed for catecholamine content by means of high-performance liquid chromatography (HPLC) with electrochemical detection (Bioanalytical Systems 400, West Lafayette, Indiana). Two hundred microliters freshly thawed plasma and 800 µl distilled, deionized H₂O were added to 15 mg acid-washed alumina and 50 µl 5 mM sodium metabisulfite in a 1.5-cc plastic tube; 50 µl 10 mg/ml dihydroxybenzylamine (DHBA), the internal standard, and 200 µl 3 M Tris-4 g/l disodium ethylenediaminetetraacetate (EDTA), adjusted with hydrochloric acid to a pH of 8.6, was then added. The sample was shaken for 15 minutes and spun in a microcentrifuge to settle the alumina. After three washings with distilled, deionized water, 100 µl of 0.1 normal perchloric acid was added to desorb catecholamines from the alumina. Finally, 50 µl supernatant was injected into the HPLC for assay. The detection limit with this system is less than 10 pg/ml for NE and epinephrine with a test–retest reliability greater than 90%.

Analysis of variance with repeated measures was used to analyze the data. Newman–Keuls tests were used for follow-up comparisons. Data from all of the rats were not available for all of the tests due to either faulty catheters, surgical losses, or equip-
Results

Body weights and serum electrolytes obtained after 7 weeks of dietary exposure are presented in Table 1. In both strains, rats on the medium calcium diets gained the most weight. Among SHR, those rats on the high calcium diets weighed the least, whereas in WKY rats, those on the low calcium diets weighed the least. Analysis of the two strains separately showed a significant diet effect on weight in WKY rats, \( F(2,30)=5.28, p<0.01 \), but not SHR \( (p>0.05) \). The pattern of serum electrolytes was consistent with the level of calcium in the diets. In both strains, rats in the low calcium diet groups had significantly lower values \( (p<0.05) \) for serum ionized and total calcium, and significantly higher values \( (p<0.05) \) for serum magnesium and phosphorus than rats on medium calcium diets. Rats in the high calcium groups showed the opposite pattern. There was no effect of the diets on either serum sodium or potassium concentrations.

MAP for each of the unrestrained and restrained 15-minute periods are presented in Figures 1 and 2. As shown, MAP was higher in SHR than WKY rats. In both strains, MAP was elevated during the first 15 minutes after handling and declined substantially during the second 15-minute period. Blood pressure was again elevated by restraint. Across diets, SHR in the low calcium condition had higher blood pressures than rats in the other two diet conditions at each recording interval. This effect was not apparent in WKY rats.

An overall analysis of variance (ANOVA) of the data \( (strain \times diet \times measurement \ interval) \) resulted in a significant strain effect, \( F(1,54)=165, p<0.0001 \), reflecting the greater level of blood pressure in SHR. There was a significant effect for measurement intervals, \( F(2,108)=34.48, p<0.0001 \), indicative of the fall in pressure that occurs with habituation, and a significant strain by measurement interval interaction, \( F(2,108)=3.96, p<0.05 \). Subsequent analysis showed that the interaction effect was due to a greater relative increase in MAP in
WKY rats to restraint than in SHR. There was no main effect for diet, $F(2,54)=2.56$, $p=0.087$, nor was there a strain by diet interaction, $F(2,54)=1.94$, $p>0.05$.

The lack of a strain by diet interaction was surprising since visual inspection of Figure 1 suggests that low calcium SHR consistently had higher blood pressure than the other two groups of SHR. Therefore, an analysis of data only from SHR was made, which indicated that there was a significant diet effect in this strain, $F(2,24)=3.42$, $p<0.05$. Follow-up analysis confirmed that the significant effect was attributable to the higher MAP in the low diet calcium group. There was no effect of diet in WKY rats.

Further analysis of the data from SHR for each measurement interval showed that there was a significant diet effect for the first unrestrained period; after the animals had been handled $F(2,24)=3.528$, $p<0.05$; and for restraint, $F(2,24)=4.179$, $p<0.05$. This effect was not seen in the second unrestrained period when the rats were relatively less stressed. Follow-up tests indicated that the low calcium group had significantly higher MAP than the high calcium group after handling ($p<0.05$), and was higher than both the high and medium groups during restraint ($p<0.05$). No diet by measurement interval effects were found in WKY rats.

Stress-induced plasma catecholamine responses are presented in Figure 3. Absolute values obtained during stress and baseline conditions are shown in Table 2. There were no significant differences across diets or strains in either resting or stressed levels of circulating NE. Likewise, there were no differences across strains or diets in resting levels of epinephrine. There were, however, significant differences between the strains in their epinephrine responses to restraint. A strain$x$diet$x$restraint ANOVA of the epinephrine data showed that there was a significant main effect for restraint, $F(1,50)=27.12$, $p<0.0001$, and a significant strain by restraint interaction, $F(1,50)=15.43$, $p<0.0001$. Subsequent analyses showed that the interaction was due to a greater restraint-induced increase in plasma epinephrine in SHR relative to WKY rats. There were no dietary effects in the epinephrine response to restraint in either strain.

Pressor responses to infusions of NE and epinephrine are depicted in Figures 4 and 5. An overall ANOVA of the NE infusion data indicated that there was a significant main effect for dose, $F(2,135)=51.55$, $p<0.0001$, and a significant diet by

### Table 2. Plasma Catecholamines

<table>
<thead>
<tr>
<th>Catecholamines</th>
<th>SHR on calcium diet</th>
<th>WKY on calcium diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High</td>
<td>Medium</td>
</tr>
<tr>
<td>Plasma norepinephrine (pg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>409±78</td>
<td>363±63</td>
</tr>
<tr>
<td>Stress</td>
<td>455±76</td>
<td>463±49</td>
</tr>
<tr>
<td>Plasma epinephrine (pg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>174±17</td>
<td>123±22</td>
</tr>
<tr>
<td>Stress</td>
<td>266±47</td>
<td>273±36</td>
</tr>
</tbody>
</table>

Values are mean±SEM. See text for significant outcomes. SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto rats.
Dose Response to NE in the SHR

![Graph showing Dose Response to NE in the SHR](image)

**FIGURE 4.** Line graph of blood pressure (BP) response to norepinephrine (NE) (0.1, 1.0, and 3.0 μg/kg) in spontaneously hypertensive rats (SHR) maintained on different calcium diets. SHR maintained on a low calcium diet (n=9) had significantly larger pressor responses to NE infusion compared with rats on medium (n=8), or high (n=7) diets (p<0.05, df=2, F=3.84).

strain interaction, F(2,135)=6.632, p<0.002. The diet by strain interaction reflected the divergent responses of SHR and WKY rats on the high and low calcium diets. SHR on the low calcium diets tended to have the largest pressor responses, and those on the high calcium diets showed the smallest responses to infusion of NE. WKY rats exhibited a tendency for the opposite arterial pressure response to high and low calcium diets, although this did not achieve statistical significance.

Analyses of the data for SHR alone indicated that there was a significant diet effect, F(2,21)=3.84, p<0.05, in the blood pressure response to infusions of NE in this strain. Subsequent analyses showed that this was due to the consistently greater pressor response of rats in the low calcium–diet condition. There were no significant differences across diets in the responses of WKY rats to the NE infusion.

Analyses of the epinephrine infusion data demonstrated that epinephrine did not interact with diet in either strain. There was a significant strain effect, F(1,46)=4.61, p<0.05, because of the greater increase in blood pressure to the infusion in WKY rats, and significant dose response, F(2,92)=73.3, p<0.0001, but no other significant effects.

**Discussion**

The results indicate that blood pressure alterations induced by dietary calcium are most apparent in SHR during stress. Restricted dietary calcium enhanced stress-induced pressor responses in SHR, and enriched calcium diets tended to attenuate the blood pressure response to stress in this strain. These outcomes were not observed in WKY rats. Based on the enhanced pressor responses to infused NE in the low calcium diet group, it appears that the effect of dietary calcium on blood pressure may be mediated, in part, by alterations in vascular responsiveness in SHR.

If alterations in vascular responsiveness to NE are a consequence of dietary manipulations of calcium in SHR, it remains to be determined whether this effect is due to a change in the intrinsic contractile ability of vascular smooth muscle. Data from Bukoski and McCarron15 and Hatton et al16 argue against a diet-induced alteration in contractile function based on in vitro measurements of contractile responses of aortic and mesenteric arteries isolated from SHR exposed to varying calcium diets. Stern et al16 found that increased levels of dietary calcium had only a modest effect on the contractile response of isolated caudal arteries to NE and no effect on responses to transmural nerve stimulation.

The observed changes in reactivity to NE may be due to alterations in vascular smooth muscle structure. Chronic increases in blood pressure have been shown to stimulate secondary structural changes within the vasculature.17 It is possible that the vascular structure of animals maintained on low calcium diets was altered as a consequence of elevated blood pressure. Such changes may have mediated the increased reactivity to infused NE.

Although it is not possible to determine the role of structural changes from the present study, Bukoski and McCarron15 found that passive elastic properties of aortic vessels from rats on lower calcium diets were reduced. This type of alteration would be consistent with a structural alteration in the vessel. On the other hand, previous studies have shown that rats on low calcium diets may have an increased reactivity to NE but not angiotensin II.18 The lack of increased responsiveness to angiotensin II suggests that perhaps calcium's effect was not due to structural change. However, Juul et al19 found that...
some vascular beds are relatively insensitive to angiotensin II. Therefore, an enhanced pressor response to angiotensin II might not be observed despite the presence of structural changes.

Alternatively, vascular responsiveness may be influenced by circulating humoral factors that would be modified by variations in dietary calcium. For example, Resnick et al. suggested that 1,25(OH)2 vitamin D3 may act as a calcium ionophore in vascular smooth muscle. Bukoski et al. subsequently reported that chronic exposure of cultured aortic myocytes to 1,25(OH)2 vitamin D3 enhances calcium-45 uptake in both SHR and WKY rats. Acute pretreatment of isolated mesenteric resistance arteries from SHR with 1,25(OH)2 vitamin D3 potentiated the NE-induced calcium transient without altering basal calcium levels (R.D. Bukoski, unpublished observations). Thus it is possible that 1,25(OH)2 vitamin D3 may potentiate responses to pressor agents in vivo in SHR. Since dietary calcium influences circulating levels of 1,25(OH)2 vitamin D3, differential pressor responses from rats on different diets may be secondary to changes in the level of this hormone.

Kageyama et al. reported an effect of dietary calcium on renin activity in renovascular hypertensive rats. Supplemental dietary calcium attenuated the development of hypertension and suppressed plasma renin activity in two-kidney, one clip renovascular hypertensive rats. Furthermore, supplemental calcium administered after the onset of hypertension suppressed pressor responses to NE. The calcium-induced suppression of plasma renin activity is in agreement with the observations of Kotchen et al. who reported that dietary calcium suppressed renin release in sodium chloride-deprived rats. It is possible that calcium-dependent alterations in the renin-angiotensin axis influences pressor responses in the present study.

Parathyroid hormone (PTH) has also been implicated as a possible mediator of calcium-induced alterations in blood pressure. Although PTH is an acute vasodilator, parathyroidectomy consistently attenuates age-related increases in blood pressure in a number of rat strains, including SHR, and blunts the expected rise in blood pressure in the DOCA-saline hypertensive rat. These effects persist even when calcium is maintained within normal limits, and they are associated with a diminished reactivity to NE infusion. Conversely, hyperparathyroidism is associated with an increased incidence of hypertension in humans, whereas elevated levels of PTH have been observed in SHR. Therefore, the chronic effects of PTH suggest the possibility that diet-induced alterations in PTH levels may alter vascular reactivity to infused pressor agents.

Dietary calcium manipulations produce changes in serum phosphorus levels that may, in part, be responsible for altered vascular reactivity. In the present study both SHR and WKY rats fed a high calcium diet showed significantly reduced serum phosphate levels. The notion that decreased phosphate mediates calcium's hypotensive effects has been supported by Lau et al. who have shown that calcium-induced hypotension in SHR can be reversed by further supplementing the diet with phosphorus. Similarly, Saglik et al. demonstrated a reduced vascular reactivity to bolus infusions of NE and angiotensin II in Sprague-Dawley rats fed a diet low in phosphorus suggesting that calcium-induced phosphate depletion may compromise vascular responses to constrictor stimuli. In contrast, Bindels et al. found reduced blood pressure among hypertensive rats fed a diet high in phosphorus. Furthermore, McCarron et al. showed a calcium-induced reduction in blood pressure without a concomitant change in serum phosphorus levels suggesting that calcium's effects are independent of phosphorus. In view of these apparently conflicting findings, additional research will be needed to delineate the role of phosphorus in calcium-induced alterations in blood pressure.

The lack of change in plasma NE and epinephrine in the current study suggests that dietary calcium had little effect on sympathetic neural activity. This is in contrast to the effect seen by Wyss et al. in sodium chloride-sensitive SHR in which there was an inverse relation between dietary calcium and plasma NE. It is also at variance with a report by Peuler et al. that increased dietary calcium altered neural activity in the Dahl salt-sensitive rat.

A critical difference between our experiments and those of Peuler et al. and Wyss and colleagues involves the simultaneous use of elevated dietary sodium along with increases in calcium. Both the Dahl salt-sensitive rats used by Peuler and coworkers and the sodium chloride-sensitive SHR used by Wyss et al. that increased dietary calcium altered neural activity in the Dahl salt-sensitive rat. The outcome of these two studies suggests that calcium may have antagonized a sodium chloride-induced elevation in sympathetic nervous system activity. In the absence of elevated sodium chloride, calcium may have little effect on sympathetic activity. On the other hand, it is possible that, had other indexes of sympathetic nervous system activity been measured, an effect of the higher calcium diet on autonomic nervous system activity might have been observed. The principal findings of the current study are consistent with those of Kageyama et al. who showed that increased dietary calcium attenuated pressor responses to infusions of NE. They are, however, contrary to those of Stern et al. who reported increased pressor responses to NE in rats on high calcium diets. One possible reason for the disparity of results between the current study and that of Stern et al. may be the overall composition of the diets. Although the two diets were similar with respect to sodium (0.4% vs. 0.45%) and potassium (0.6%), they differed markedly in fiber (0% vs. 3%) and phosphorus content (0.4% vs. 0.9%). How-
ever, it should be noted that there were also differences between the diet used in the present study and that used by Kageyama et al., thus diminishing the possible explanatory power of dietary differences.

There were no strain differences in plasma noradrenaline during baseline or stress. This is consistent with findings from several laboratories. There was also no difference in blood pressure reactivity to NE between SHR and WKY rats. Similar results were found by Yamaguchi and Kopin when infusing comparable doses of NE. Kageyama, Davy et al., and Filep and Fejes-Toth also failed to find strain differences in pressor responses to NE infusions in the intact animal. This is in contrast to consistent findings of elevated NE sensitivity in isolated vascular preparations of SHR. This suggests that the enhanced vascular sensitivity of SHR to NE is buffered in the intact rat resulting in comparable blood pressure reactivity across strains.

In summary, the results of this study further validate the association between the restriction of dietary calcium and increasing arterial pressure in SHR. The blood pressure increase was most pronounced during behavioral stress and may be a consequence of an increase in vascular responsiveness to NE rather than an increase in sympathetic nervous system outflow. Based on the accumulated evidence to date, the observed increase in vascular responsiveness associated with restricted calcium diets in SHR is not likely a consequence of an alteration in intrinsic vascular contractility or an attenuation of sympathetic nervous system activity. It seems more likely that the change in vascular responsiveness is related to diet-induced changes in humoral factors, such as 1,25(OH)₂ vitamin D₃, PTH, or renin that may modulate the vascular response to NE.

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