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Dietary Calcium Supplementation and Dopamine-β-hydroxylase in Spontaneously Hypertensive Rats

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ABSTRACT. Spontaneously hypertensive 4-week-old male rats were fed, before and after the onset of hypertension, with either commercial chow (control) or commercial chow combined with different forms of milk proteins with or without calcium supplementation. After 40 weeks, rats were still hypertensive, and dopamine-β-hydroxylase enzyme activity measured simultaneously in serum and adrenal was found to be higher than in the controls. The enzyme activity in rats fed diets with milk proteins was increased significantly in both serum and adrenal compared with the control, and such enhancement was significantly higher than that observed in animals fed the commercial diet supplemented with calcium (1.2%), suggesting that dietary calcium intake associated with dietary protein of high digestibility, such as casein, potentiates the endogenous mechanisms regulating the homeostasis of calcium more than calcium supplementation itself. Moreover, the selective and additive effect of diets supplemented with milk proteins and calcium on adrenal enzyme activity clearly suggests a relationship between cardiovascular diseases involving the genesis of hypertension and stress mechanisms through the hypothalamo-pituitary adreno-sympathetic axis. BIOCHEM PHARMACOL **53**;12: 1867–1871, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. dopamine-β-hydroxylase; dietary intake; SHR rats; calcium supplementation

DBH‡ [3,4 dihydroxyphenylethylamine ascorbate:O_2 oxidoreductase (β-hydroxylating), EC 1.14.17.1] catalyses the conversion of 3,4-dihydroxyphenylethylamine into noradrenaline and, as a constituent of the chromaffin granules of the adrenal medulla and of the nerve granules of the sympathetic nervous system [1, 2], is released with noradrenaline into the circulation during sympathetic transmission processes [3–5]. Several studies have demonstrated a sharp elevation in human serum DBH enzyme activity following various stressful situations [6–8]. It was found that serum enzyme activity in animals was influenced by nutrient deficiencies including those of copper, zinc, and calcium [9, 10] as well as by dietary proteins with or without stress [11–14]. Moreover, Fujita *et al.* [15] clearly demonstrated that stress induces a pronounced elevation of DBH activity in adrenal, serum, and brain, but not in heart of SHR compared with normotensive rats, thus suggesting a close relationship between hypertension and catecholamine release.

The purpose of this study was to compare the effects of calcium supplementation on serum and adrenal DBH enzyme activity in SHR fed prior to the onset of hypertension and for the 40 weeks that followed with either commercial chow (control) or commercial chow combined with different forms of milk proteins.

MATERIALS AND METHODS

Chemicals

Bovine serum albumin, fumaric acid, pargyline HCl, ascorbic acid, catalase, tyramine, octopamine, sodium *m*-periodate, and Dowex 50W-4X were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Trichloroacetic acid, sodium bisulphite, and monobasic and dibasic sodium phosphate were obtained from Fisher Scientific (Ste-Foy, Québec, Canada). Sodium acetate and *N*-ethylmaleimide were purchased from BDH (Ville St-Laurent, Québec, Canada). Whole milk and skim milk were furnished by "la Coopérative de la Côte-sud," Bellechasse, Québec, Canada.

Animals

Thirty-six male SHR weighing 50–55 g, obtained from Charles River (St-Constant, Québec), were adapted to the environmental conditions and fed with Purina Chow for 1 week. Rats were housed six per group in a room controlled at constant room temperature (22°) and humidity (55%), with fluorescent lights and background music from 7:00 a.m. to 7:00 p.m.

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‡ *Abbreviations:* DBH, dopamine-β-hydroxylase; SHR, spontaneously hypertensive rats; and HPAS, hypothalamo-pituitary adreno-sympathetic.

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Experimental Design

Animals were fed with commercial chow or with a mixture of commercial chow and milk proteins with or without 1.2% calcium supplementation. The Purina Chow was ground and combined with whole milk or skim milk in a 75:25 ratio, and the mixture was formed into biscuits. Dietary treatments were started in young rats (3- to 4-weeks-old) before the onset of hypertension and were carried on for 40 weeks. SHR were distributed randomly into six groups as follows: the first group (P) was fed with commercial diet (Purina Chow); the second group (PCa) received commercial chow supplemented with 1.2% calcium; the third group (W) received a mixture of 75% commercial chow complemented with 25% whole milk; the fourth group (WCa) was similar to the W group but supplemented with 1.2% calcium; the fifth group (S) was fed with a mixture of 75% commercial chow complemented with 25% skim milk; and the sixth group (SCa) was similar to the S group but supplemented with 1.2% calcium. Rats were provided diets and tap water *ad lib.* At the end of the experiment, the average body weight of the animals was 400 g without any significant weight changes among the groups. The average daily protein and calcium intakes were calculated according to Héroux and Roberge [11] and expressed in g/100 g of diet (see Table 1).

Rats were killed by decapitation, and blood samples were collected in 15-mL tubes and kept for 2 hr at room temperature, then centrifuged at 1100 *g* for 15 min in a refrigerated (4°) Sorvall RC-5B. Adrenals were dissected out, fat was removed, and both sera and adrenals were stored at −80° for subsequent biochemical analysis.

DBH

DBH activity was measured by the method of Kato *et al.* [16] as modified by Charbonneau and Roberge [14] and by Fortin *et al.* [17] for serum and adrenal, respectively. The serum (75 μL) containing the enzyme was diluted to 400 μL with buffer (sodium phosphate 0.1 M, pH 7.4). Serum DBH enzyme activity was expressed in nanomoles of octopamine formed per milliliter of serum per hour. Adrenal DBH enzyme activity was measured using 100 mg of tissue homogenized in 2 mL sodium phosphate buffer, 0.1 M, pH 7.4, containing 0.5% Cutscum. The homogenate was centrifuged at 40,000 *g* for 60 min (Sorvall RC-58, Dupont), and 400 μL of the supernatant was used for the enzymatic assay estimated as previously described [17]. Protein was assayed by the method of Bradford [18]. Adrenal enzyme activity was expressed in nanomoles of octopamine formed per milligram of protein per hour.

Statistical Analysis

Mean, standard deviation, standard error of the mean (SEM), one-way ANOVA, and paired *t*-test followed by a Duncan or a Fisher test were calculated [19].

TABLE 1. Daily dietary protein and calcium intakes after 40 weeks in SHR*

Groups†	Protein intake (g/100 g diet)	Calcium intake (g/100 g diet)
P (Purina)	22.9	0.9
PCa (1.2%)	22.6	2.1
W (whole milk)	18.1	0.7
WCa (1.2%)	18.1	1.9
S (skim milk)	18.1	0.7
SCa (1.2%)	18.1	1.9

* Dietary intake was calculated as described by Héroux and Roberge [11] for a daily dietary intake of 26 ± 0.8 g for Purina Chow and of 21 ± 0.8 g for casein diet in 300 g rats.

† A mixture of Purina Chow and milk products in a ratio of 75:25 with or without 1.2% calcium supplementation was used for groups W, WCa, S, and SCa.

RESULTS

In Table 1, the average daily calcium and protein intakes, expressed in g/100 g of diet, are described in SHR fed with the respective diets for 40 weeks. Whatever the diet used, the calcium intake was between 700 and 900 mg/100 g of diet in groups P (Purina), W (whole milk), and S (skim milk). In diets supplemented with 1.2% calcium, the average intake was at least two times higher in PCa, WCa, and SCa groups, being around 2000 mg/100 g of diet. The dietary protein intake was similar in P and PCa groups, and was higher than in all groups fed with milk proteins.

In Table 2, the effects in SHR of dietary calcium supplementation on serum DBH enzyme activity are compared with those of the P, W, and S groups. In rats fed the commercial chow (the P group), the serum enzyme activity was significantly lower ($P < 0.001$) than the activity observed in rats receiving either commercial chow supplemented with 1.2% calcium (PCa; 67%) or diets containing milk proteins with or without calcium, respectively (W and S: 118%; WCa and SCa: 140%). In rats fed a calcium-supplemented commercial chow (the PCa group), the enzyme activity was increased significantly (up to 67%; $P < 0.001$), whereas in the WCa and SCa groups, calcium supplementation did not provide any additive effect when data were compared with the W and S groups, respectively.

Table 3 shows the adrenal DBH enzyme activity measured in SHR fed with the same diets as described in Table

TABLE 2. Effects of calcium supplementation on serum dopamine-β-hydroxylase in SHR fed diets with milk proteins

Groups	Serum DBH enzyme activity* (nmol octopamine · $mL^{-1} \cdot hr^{-1}$)
P (Purina)	33.4 ± 0.21 (6)
PCa (1.2%)	55.6 ± 0.27† (6)
W (whole milk)	70.0 ± 0.12†‡ (6)
WCa (1.2%)	75.0 ± 0.26†‡ (6)
S (skim milk)	73.0 ± 0.44†‡ (6)
SCa (1.2%)	77.3 ± 0.51†‡ (6)

* Results are means ± SEM; the number of rats used is given in parentheses.

† $P < 0.001$, compared with the P group.

‡ $P < 0.001$, compared with the PCa group.

TABLE 3. Effects of calcium supplementation on adrenal dopamine-β-hydroxylase in SHR fed diets with milk proteins

Groups	Adrenal DBH enzyme activity* (nmol octopamine · mg protein^{-1} · hr^{-1})	
P (Purina)	2700 ± 8.9	(6)
PCa (1.2%)	3478 ± 14.8†	(6)
W (whole milk)	4122 ± 8.1†‡	(6)
WCa (1.2%)	4835 ± 43.6†‡§	(6)
S (skim milk)	4168 ± 8.8†‡	(6)
SCa (1.2%)	5388 ± 8.3†‡§	(6)

* Results are means ± SEM; the number of rats used is given in parentheses.
† $P < 0.001$, compared with the P group.
‡ $P < 0.001$, compared with the PCa group.
§ $P < 0.05$, compared with the W and S group, respectively.

2. In rats fed commercial chow supplemented with calcium (the PCa group), the enzyme activity was significantly higher (29%) than the activity observed in the P group ($P < 0.001$). Moreover, the enzyme activity was significantly higher in rats fed with dietary milk proteins (W and S) than in the P group, by at least 54% ($P < 0.001$). In adrenal, calcium supplementation (1.2%) significantly increased the enzyme activity in WCa and SCa groups by 79 and 100%, respectively ($P < 0.001$), when data were compared with the P group and by at least 20% when compared with the W and S groups. A significant additive effect of calcium supplementation was observed only in the adrenal (24%; $P < 0.01$).

DISCUSSION

The present study shows for the first time a link between dietary milk intake with or without additional calcium and both serum and adrenal DBH enzyme activity, the noradrenaline synthesizing enzyme. Moreover, the present findings demonstrate an inverse relationship between blood pressure and DBH enzyme activity, contribute to dissociating the mechanisms by which calcium supplementation might act on the respective enzyme activity, and point out the involvement of the quality of dietary proteins, such as casein, in endogenous mechanisms regulating the homeostasis of calcium through the HPAS axis. In fact, with a similar dietary calcium intake, a significantly higher enhancement of the enzyme activity was observed in response to dietary protein of high digestibility rather than to calcium supplementation itself; such enhancement was selectively and significantly potentiated in adrenal following calcium supplementation in SHR fed dietary milk proteins, thus supporting the capacity of adrenal to react to such a treatment.

DBH, Dietary Protein and Stress

Serum DBH enzyme activity, a clinical index for the sympathetic nervous system [20], increases in stressful conditions [6, 7, 12–15] and in human essential hypertension [21, 22], as well as in experimental hypertensive or atherosclerotic rats [15, 22, 23]. Moreover, the relationship between stress and cardiovascular diseases has been reviewed extensively [24], describing the involvement of the HPAS axis and of various biochemical and physiological parameters. The present work is in good correlation with the literature demonstrating that (1) serum and adrenal DBH enzyme activity was significantly higher (10-fold) in SHR than in normotensive Wistar rats [15–17, 22, 23], and (2) dietary protein composition selectively influences serum DBH enzyme activity in cats [12–14]. Following intake of casein as a dietary protein source, the increased serum DBH activity previously was ascribed to greater bioavailability of the serum neutral amino acids [11] that compete with tryptophan for entry into the brain to maintain the balance between serotonin and noradrenaline synthesis, mainly in the hypothalamus; this, in turn, provides the organism with a greater resistance to stressful situations as observed in rats [11], cats [12–14], and fish [25] through the activity of the HPAS axis, as also presently supported by the enhanced adrenal DBH enzyme activity. In human subjects fed with dietary milk proteins, a normalization of systolic pressure induced by swimming workload (manuscript in preparation) corresponds to increased DBH activity in the serum, thus suggesting that stress mechanisms and cardiovascular functions could share the same neuroanatomical and biochemical pathways [24–27].

Many divalent cations stimulate the activity of DBH enzyme, and calcium triggers its release from synaptic vesicles with noradrenaline [3, 5, 17, 24]. Moreover, endogenous thiol derivatives inhibit both serum and adrenal enzyme activity whereas copper promotes its activation [17, 26]. The addition of exogenous calcium concentrations or calcium blockers to the incubation mixture did not change serum and adrenal enzyme activity; the decreased activity induced by the chelating agent EDTA, however, was restored only by adding exogenous copper in a saturated ascorbate medium, thus reinforcing the discrepancy between the *in vitro* and *in vivo* studies related to calcium mechanisms (manuscript in preparation).

Dietary Calcium and Hypertension

Over the past few years, the hypothesis that dietary calcium was responsible for essential hypertension was based on the fact that reduced dietary calcium exposure depletes calcium from its membrane store sites and enhances calcium fluxes [28–31]. In both experimental and human hypertension, disturbed calcium metabolism was observed in various organ functions, biochemical parameters, and cellular fractions [28–31], thus associating the essential cardiovascular activities to the central and peripheral nervous system and adrenal glands [24, 30]. Moreover, based on experimental designs, reduced calcium intake from either food or water, as well as experimental dietary calcium deprivation, has been associated with an abnormally high blood pressure [16, 28–31]. In adult SHR, the average value of systolic blood pressure, around 200 mm Hg, was not restored to the

expected normal range following dietary calcium supplementation, since SHR were still considered as hypertensive with a blood pressure above 140 mm Hg [28], as also observed in the present study (over 180 mm Hg whatever the groups observed), even though the calcium intake was at least two times higher in supplemented calcium diets than in normal and milk protein diets. In humans, dietary calcium intake has been either inversely or directly correlated to diastolic blood pressure in women and men, respectively [32], and inversely related to systolic blood pressure among non-white men [33]. Even though several physiological factors are now recognized as influencing blood pressure [34], these conflicting results yielded the fact that serum calcium levels were not an obvious predictor of blood pressure. They led investigators [29, 35] to establish a negative correlation between calcium deficiency and elevated blood pressure and to suggest a close relationship between vascular hyperactivity and hypertension through the control of calcium ATPase enzyme activity, which modulates the intra- and extracellular calcium pools [27–29, 36, 37]. In this respect, noradrenaline, which is dependent upon DBH enzyme activity for its synthesis, activates calcium influxes in both voltage-dependent and -independent manners in vascular smooth muscles, thus supporting its involvement in the relationship between hypertension and cellular calcium homeostasis [38, 39]. In fact, whereas hypertension is alleviated after the administration of a DBH inhibitor [40], hypotension is associated with genetic DBH deficiency [41–43]. Moreover, as it was reported that the hypothalamic noradrenaline turnover rate was higher in casein-fed rats than in commercial diet-fed rats [11], the enhanced adrenal and serum DBH enzyme activity could be related to greater HPAS axis activity. In this respect, the present results contribute to elucidating how noradrenaline could modulate voltage-dependent calcium channels through nitric oxide synthase [3, 4, 44], a calmodulin calcium-dependent enzyme responsible for nitric oxide synthesis, ultimately to adjust the vascular tone [45, 46].

In summary, the increased serum and adrenal DBH enzyme activity was influenced directly by the quality of dietary protein rather than by calcium supplementation itself. In this respect, DBH enzyme activity, as the noradrenaline synthesizing enzyme, could trigger the homeostasis of calcium through the enhancement of HPAS axis activity involving the stress mechanisms without restoring the blood pressure to a normal level, at least in SHR.

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