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The effect of nutrient profiles of the Dietary Approaches to Stop Hypertension (DASH) diets on blood pressure and bone metabolism and composition in normotensive and hypertensive rats

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Hypertension has been associated with abnormalities of Ca and bone metabolism. Consequently, dietary strategies aimed at reducing blood pressure may also benefit bone health; however, this issue has received little attention. Therefore, the objective of the present study was to investigate the effect of two antihypertensive-type diets on blood pressure and bone metabolism and composition in normotensive (Wistar-Kyoto NHsd, WKY) and hypertensive (spontaneously hypertensive NHsd, SHR) rats. Thirty WKY and thirty SHR male rats, 14 weeks old, were separately randomized by weight into three groups of ten rats each. One group from each strain was given a control diet while the other two groups were fed two anti-hypertensive (high fruit and vegetable (F/V) and high fruit and vegetable and low-fat dairy produce (combination)) diets for 8 weeks. SHR rats were significantly ($P<0{\cdot}01$) heavier than WKY rats. Blood pressure and femoral length, width, dry weight, ash, Ca, Mg, P and bone mineral mass were significantly ($P<0{\cdot}0001$) greater in SHR than WKY rats, but were unaffected by diet, irrespective of strain. While markers of bone formation (serum osteocalcin) and bone resorption (urinary pyridinoline and deoxypyridinoline) were similar in both strains, these markers were significantly ($P<0{\cdot}05$) lower (28–31, 16–23, 31–33 % respectively) in the SHR rats fed the combination diet relative to those fed the control and F/V diets. Bone turnover in WKY rats was unaffected by diet. In conclusion, these findings suggest that the combination diet may benefit bone metabolism in hypertensive animals. However, as blood pressure was unaffected by this diet, the mechanism by which it reduced bone turnover requires further investigation.

Bone: Hypertension: Dietary intervention

Hypertension has been associated with abnormalities of Ca and bone metabolism in human subjects (McCarron *et al.* 1980; Cappuccio *et al.* 1999) and rats (McCarron *et al.* 1981, 1985; Pörsti, 1992). For example, lower circulating levels of 1,25-dihydroxycholecalciferol and reduced duodenal Ca absorption, bone density and bone Ca content have been reported in hypertensive rats as compared with normotensive rats (McCarron *et al.* 1985; Lucas *et al.* 1986; Blakeborough *et al.* 1990). In human subjects, reduced serum ionized Ca levels and increased levels of Ca and cyclic AMP in urine and increased 1,25-dihydroxycholecalciferol and parathyroid hormone in serum have been associated with hypertension (McCarron *et al.* 1980; Brickman *et al.* 1990; Jorde *et al.* 2000). MacGregor & Cappuccio (1993) have hypothesized that the increased urinary Ca excretion in hypertensive patients is compensated not by increased intestinal absorption of Ca, but by that liberated by resorption of bone, and that hypertensive patients are thus more prone to bone demineralization, bone fracture and osteoporosis.

Recent evidence suggests that dietary modification can positively influence blood pressure regulation (Svetkey *et al.* 1999; Dakshinamurti & Dakshinamurti, 2001; Fleet, 2001). For example, the findings of the original Dietary Approaches to Stop Hypertension (DASH) study and the subsequent DASH-Sodium study suggest that increasing the fruit and vegetable intake, either alone (in the case of the DASH trial) or in combination with increased consumption of low-fat dairy produce (referred to as the 'combination diet' (Comb), used in both DASH and

Abbreviations: DASH, Dietary Approaches to Stop Hypertension; Comb, high fruit and vegetable and low-fat dairy produce diet; Dpyr, deoxypyridinoline; F/V, high fruit and vegetable diet; pyr, pyridinoline; SHR, spontaneously hypertensive; WKY, Wistar-Kyoto.

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DASH-Sodium trials), can lower both systolic and diastolic blood pressure in individuals with high-normal and mildly elevated blood pressure (Appel *et al.* 1997, 2001; Moore *et al.* 1999; Conlin *et al.* 2000; Vollmer *et al.* 2001). Furthermore, a recent report arising from the original DASH study has suggested that the DASH Comb diet can also reduce serum homocysteine levels (Appel *et al.* 2000) and plasma total-, LDL- and HDL-cholesterol levels (Obarzanek *et al.* 2001) in these normotensive and mildly hypertensive subjects. The mechanism by which this type of diet lowers these independent risk factors for cardiovascular disease is still unclear (Appel *et al.* 2000). Consumption of the DASH Comb diet in place of the more typical Western-type diet will lead to substantially increased intake of several nutrients, including Ca, Mg, K and various vitamins, all of which have been investigated individually for their anti-hypertensive effects (Allender *et al.* 1996; Touyz & Milne, 1999; Dakshinamurti & Dakshinamurti, 2001), as well as increased intake of several non-nutrients, including various phytochemicals. It is likely that the anti-hypertensive effects of the DASH Comb diet are due to the increased intake of one or more of these nutrients and/or food components, by their interaction, or indeed by their displacement of other food components typical of a Western-type diet.

It is possible that in addition to having a beneficial effect on the regulation of blood pressure and circulating homocysteine and cholesterol levels, the DASH Comb diet may also have a beneficial effect on Ca and bone metabolism, and thus bone health, particularly in hypertensive subjects, who may be at increased risk of osteoporosis (Cappuccio *et al.* 2000). This is based on the fact that many of the nutrients that are rich in the DASH Comb diet (e.g. Ca, Mg, K, vitamin C) have been individually shown to have important roles in bone health (Stendig-Lindberg *et al.* 1993; Sebastian *et al.* 1994; for review, see Institute of Medicine, 1997; New *et al.* 1997, 2000; Hall & Greendale, 1998; Tucker *et al.* 1999; Morton *et al.* 2001; Sellmeyer *et al.* 2002). In support of this contention, a recent preliminary report of an ancillary study to the DASH-Sodium trial has shown that in comparison with the DASH control diet (a typical Western-type diet), consumption of the DASH Comb diet for 30 d reduced the levels of biochemical markers of bone formation (serum osteocalcin) and bone resorption (serum C-terminal telopeptide of type I collagen) at each of three dietary Na intake levels (low, intermediate and high) in adult men and women (Lin *et al.* 2001). These findings suggest that the DASH Comb diet may reduce the rate of bone turnover, which in turn, may lower risk of fracture by maximizing development of peak bone mass in young adults and slowing the rate of bone loss in later life (Cashman, 2002). Interestingly, the reduction in the rate of bone turnover arising from consumption of the DASH Comb diet was found in all participants, regardless of age, gender and hypertension status (Lin *et al.* 2001), suggesting a possible beneficial impact of this diet on bone health in normotensive and hypertensive individuals of several population subgroups.

The objective of the present study was to investigate the effect of the nutrient profiles of the three diets used in the original DASH study (namely, control, high fruit and vegetable (F/V), and Comb diets; Appel *et al.* 1997) on blood pressure and bone metabolism and composition in spontaneously hypertensive and normotensive adult rats.

Materials and methods

Preparation of rat diets

Three semipurified diets, namely control, F/V and Comb diet were used in the present study (Tables 1–3). These were based on the AIN-93M diet (that recommended for adult rats by the American Institute of Nutrition (Reeves *et al.* 1993)), but modified to allow the nutrient profile to reflect those reported for each of the three diets used in the DASH trial (Karanja *et al.* 1999). The nutrient profiles of the control, F/V and Comb diets in the present study were formulated by expressing the content of each nutrient in the three DASH diets (as described by Karanja *et al.* 1999) as a percentage of the related US recommended dietary allowances for these nutrients (see Table 4; National Research Council, 1989) and then adjusting the nutrient contents of the AIN-93M by similar percentages (see Tables 1–3). This produced similar percentage differences between nutrients in the three human diets (i.e. the DASH diets) and the three rodent diets used in the present study. The potential renal acid load of each of the three rodent diets was estimated using the nutrient composition data of the diets and the calculation model of Remer & Manz (1995), which was derived for the prediction of the effects of diets on the acidity of urine in human subjects. The estimated potential renal acid load for the control, F/V and Comb diets used in the present study were 86, −44 and −41 mEq/kg diet, respectively.

Table 1. Composition (g/kg) of the control, high fruit and vegetable (F/V) and combination (Comb) diets

Diet ... Ingredient	Control	F/V	Comb
Maizestarch*	599·717	555·808	558·779
Casein	140·000	129·750	151·351
Sucrose	120·250	112·141	95·495
Fibre	36·110	90·102	85·081
Oil mix*	49·300	45·690	32·441
Modified AIN mineral mix†	35·000	32·437	31·532
Modified AIN vitamin mix‡	10·000	9·268	9·009
Calcium carbonate	5·361	7·642	20·567
Potassium citrate	–	13·083	7·865
Potassium phosphate	–	0·500	3·995
Choline bitartrate	2·500	2·317	2·252
L-Cystine	1·800	1·668	1·946
t-Butylhydroquinone	0·008	0·007	0·007

* Representing control, F/V and Comb diets containing respectively (g/kg): coconut oil, 16·5, 15·3, 5·86, olive oil 18·8, 17·4, 12·6, soyabean oil 14·0, 13·0, 13·9. These oil mixtures were designed to provide diets that contained saturated, monounsaturated and polyunsaturated fatty acids in the same proportions as the three Dietary Approaches to Stop Hypertension diets (see Karanja *et al.* 1999).

† For the composition of mineral mixtures used in the three diets see Table 2.

‡ For the composition of vitamin mixtures used in the three diets see Table 3.

Table 2. Composition (g/kg mineral mix) of modified AIN 93-M mineral mixtures used in the control, high fruit and vegetable (F/V) and combination (Comb) diets

Diet ... Ingredient	Control	F/V	Comb
Sucrose	410·24000	418·09800	307·24500
Potassium phosphate	300·78000	427·33000	517·06000
Calcium phosphate	111·11000	–	–
Sodium chloride	74·00000	74·00000	74·00000
Potassium sulfate	46·60000	46·60000	46·60000
Magnesium oxide	14·04000	32·56000	36·68000
Potassium citrate	28·00000	–	–
Ferric citrate	11·75000	12·96000	14·48000
Zinc carbonate	1·04000	1·28700	1·33000
Sodium meta-silicate hydrate	1·08300	1·08300	1·08300
Manganous carbonate	0·63000	0·63000	0·63000
Cupric carbonate	0·18750	0·35600	0·35600
Chromium potassium sulfate	0·27500	0·27500	0·27500
Boric acid	0·08150	0·08150	0·08150
Nickel chloride	0·06370	0·06370	0·06370
Sodium fluoride	0·06350	0·06350	0·06350
Lithium chloride	0·01740	0·01740	0·01740
Potassium iodate	0·01000	0·01000	0·01000
Sodium selenate	0·01025	0·01025	0·01025
Ammonium molybdate	0·00795	0·00795	0·00795
Ammonium vanadate	0·00660	0·00660	0·00660

Table 3. Composition (g/kg vitamin mix) of the modified AIN 93-M vitamin mixtures used in the control, high fruit and vegetable (F/V) and combination (Comb) diets

Diet ... Ingredient	Control	F/V	Comb
Sucrose	916·8950	838·2770	780·1870
Ascorbic acid	54·1360	125·8000	178·3000
α-Tocopherol acetate	13·6500	18·4500	22·5000
Cyanocobalamin	4·6250	4·7500	6·1250
Nicotinic acid	4·5780	4·4200	4·1040
Retinyl palmitate	1·7550	3·5560	4·0096
Calcium pantothenate	1·6000	1·6000	1·6000
Pyridoxine hydrochloride	0·6300	1·0850	0·9450
Thiamine hydrochloride	0·9198	0·7980	0·6798
Riboflavin	0·6348	0·5290	0·7764
Folic acid	0·2070	0·3900	0·4280
Cholecalciferol	0·2500	0·2500	0·2500
D-Biotin	0·0200	0·0200	0·0200
Phylloquinone	0·0750	0·0750	0·0750

Experimental design

Sixty male rats, 14 weeks old (*n* 30, Wistar–Kyoto/NHsd (WKY) strain; *n* 30, Spontaneously Hypertensive/NHsd (SHR) strain; average weight 308 g for SHR rats and 286 g for WKY rats), were obtained from Harlan UK Ltd (Bicester, Oxon., UK). All rats were fed *ad libitum* on a semipurified control diet (see earlier) for 1 week until they were aged 15 weeks. At this stage, both normotensive (WKY) and hypertensive (SHR) rats were separately randomized by weight into three groups of ten rats each (i.e. three groups of normotensive and three groups of hypertensive rats). One group from each strain was given the control diet while the other two groups were fed the F/V and Comb diets respectively (see earlier). Food intake of all three dietary groups in both strains was monitored for 1 week to establish whether dietary preferences between the three diets existed. To assure equivalent food consumption and avoid differences in body-weight

Table 4. Nutrient profiles of the three Dietary Approaches to Stop Hypertension (DASH) diets expressed in absolute terms and as a percentage of the recommended dietary allowances provided by each diet*†

Diet ...	Control		F/V		Comb	
Nutrient	Absolute value	% RDA‡	Absolute value	% RDA‡	Absolute value	% RDA‡
Protein (g)	97·5	144	97·5	144	117	186
Fat (g)	107	123	107	123	78	90
Carbohydrate (g)	325	110	338	115	384	130
Fibre§ (g)	13	72	35	194	34	189
Vitamin C (mg)	145	181	231	289	294	367
Vitamin E (mg tocopherol units)	9·1	91	12·3	123	15·0	150
Vitamin B_{12} (μg)	3·7	185	3·8	190	4·9	245
Niacin (mg niacin equivalents)	29	153	28	147	26	137
Vitamin A	2215	219	4499	444	5062	501
Vitamin B_6 (mg)	1·8	90	1·5	155	2·2	135
Thiamin (mg)	2·3	153	2·0	133	1·7	113
Riboflavin (mg)	1·8	106	1·5	88	2·2	129
Folic acid (μg)	207	103	390	195	428	214
Calcium (mg)	527	66	528	66	1462	183
Magnesium (mg)	205	58	475	136	535	153
Phosphorus (mg)	1144	143	1177	147	1690	211
Potassium (mg)	2051	114	5067	281	5181	289
Iron (mg)	19·4	194	21·4	214	23·9	239
Copper (mg)	1·0	62	1·9	119	1·9	119
Zinc (mg)	9·5	63	11·7	78	12·1	81

F/V; fruit and vegetable; Comb, combination.
* For details of DASH diets, see Karanja *et al.* (1999).
† Based on the US recommended dietary allowance values (National Research Council, 1989).
‡ Content per 10·88 MJ (2600 kcal) diet (Karanja *et al.* 1999).
§ Fibre recommendation was based on those of the Department of Health (1991).

gain among the dietary groups during the intervention period, an equalized feeding paradigm was used. The amount of food offered to all rats within a strain was limited to the *ad libitum* food intake (adjusted for the energy content of the diets) of the group that ate the least amount of food during the initial 1 week food-intake monitoring period. All animals were given distilled water *ad libitum* for the duration of the study. Rats were housed individually and feed was provided at 10·00 hours each day. Blood pressure of each animal was measured at baseline, week 4 and week 8 of the dietary intervention study. During the last week of the study, all rats were placed in individual metabolism cages with a grid floor and a facility for separate collection of faeces and urine.

Urine samples (24 h) were collected for each animal over the last 2 d of the study in vessels covered with Al foil to prevent degradation by light of the pyridinium crosslinks. The urine samples for each animal were pooled and the volumes recorded. Portions of the pooled urine samples were acidified with 12 M-HCl (2250 μl/l urine) and stored at −20°C until required for analysis.

After 56 d on their respective diets, all animals were anaesthetized with diethyl ether and blood was drawn from the heart into vacutainer tubes, processed to serum, and immediately stored at −80°C until required. Final body weights were recorded and femora were harvested. Femora were cleaned of adhering soft tissue, and stored in sealed containers at −20°C until required for determination of physical properties and macromineral content.

Experimental techniques

Blood pressure. Systolic blood pressure measurements were recorded by a non-invasive computerized rat-tail cuff blood pressure system (RTBP1007 rat-tail blood pressure system; Kent Scientific, Litchfield, CT, USA), connected to a MacLab database acquisition system (AD Instruments Ltd., NSW, Australia). Briefly, arterial pulsation was detected with a piezoelectric sensor attached to the rat tails. Using an occlusion air cuff placed upstream of the sensor, arterial blood flow was stopped. The pressure inside the cuff was then progressively released. When the pressure inside the cuff became analogous to the systolic blood pressure, the blood was allowed to flow again in the tail artery. At this point, the cuff pressure was recorded. Prior to the experimental days, rats were accustomed to the experimental process; rats were maintained in the restrained chamber for 15 min on two consecutive days before any blood pressure measurement. This process was shown to reduce animal stress during experiments. On the experimental day, rats were first placed in a restraining chamber, maintained at 37°C, where they were allowed to rest for 5 min. The cuff and the sensor were then placed around the tail of the rat and five measurements were performed per rat. The five values were averaged, resulting in daily blood pressure values. It is important to note that the five values for a given day were only kept as a result when they showed relative constancy. For example, stressed animals demonstrated, during the same day, high variability in their measured blood pressure values; therefore, only values within the same range of ± 15 mmHg were considered for further calculations. The contribution of unreliable results due to animal stress to any final data was thus minimized.

Urinary pyridinoline and deoxypyridinoline. Pooled urine samples were analysed in duplicate using an automated analysis system (Gilson ASPEC (Automated Sample Preparation with Extraction Columns); Gilson S.A., Villiers-le-Bel, France) linked directly to a gradient HPLC system comprising a Gilson 321 pump and a Shimadzu RF-10AXL fluorescence detector (Shimadzu Scientific Instruments Inc., Colombia, MD, USA). In brief, portions of pooled urine (250 μl) were first hydrolysed with an equal volume of 12 M-HCL at 107°C for 18 h. The crosslinks from urine hydrolysates where then extracted with cellulose partition chromatography, with the use of an internal standard (acetylated pyridinoline (pyr); MetraBiosystems Ltd, Wheatley, Oxon., UK) (Pratt *et al.* 1992). The acetylated Pyr was used in accordance with the method described by Calabresi *et al.* (1994) and Robins *et al.* (1994). The crosslinks contents of urine samples were quantified by external standardization using a commercially available Pyr–deoxypyridinoline (Dpyr) HPLC calibrator (MetraBiosystems Ltd). The intra-assay CV for Pyr and Dpyr measured as the variation between ten chromatograms obtained between column regenerations as described by Colwell *et al.* (1993) were 5 and 3 % respectively. The inter-assay CV for Pyr and Dpyr were 9 and 11 % respectively.

Urinary and serum creatinine levels. Urinary and serum creatinine were measured in duplicate by a colourimetric procedure using a diagnostic kit (catalogue no. 555A; Sigma Diagnostics, St Louis, MO, USA). Intra- and inter-assay CV were 3·6 and 7·9 % respectively.

Femoral calcium, phosphorus and magnesium levels. Weighed femora (dried overnight at 110°C) were dry ashed at 600°C for 16 h as described by Hoshino *et al.* (1998), and the ash content was calculated by weight loss on a dry basis. The ash was digested with 16 M-HNO_3. Ca and Mg were analysed in duplicate in femoral digests by atomic absorption spectrophotometry (Pye-Unicam Atomic Absorption Spectrophotometer, Model SP9; Pye-Unicam, Cambridge, Cambs., UK) after appropriate dilution with $LaCl_3$ solution (5 g/l; BDH Ltd, Poole, Dorset, UK). A range of Ca and Mg standards were used to obtain Ca and Mg calibration curves. The intra- and inter-assay CV for Ca were 2·8 and 7·8 %, and for Mg were 3·2 and 8·8 % respectively. P was determined in the femoral digests by the method of Weissman & Pileggi (1974). The intra- and inter-assay CV for P were 4·2 and 6·1 % respectively. The accuracy of mineral analysis was assured in each analytical run by appropriate recovery of mineral in dry-ashed samples of National Institute of Standards and Technology-certified bone meal (Standard Reference Material 1486; Laboratory of the Government Chemist, Teddington, Middlesex, UK).

Femoral mass, length, volume and density. The length of each right femur was measured with a vernier caliper. The width of the femur (at the midpoint between epiphyses) was also measured with a vernier caliper. Bone volume and density were measured by Archimedes' principle (Kalu *et al.* 1991). Briefly, each bone was cut at

the mid-diaphysis and the marrow was washed out. Then each bone was put in an unstoppered vial filled with distilled water, and the vial was placed under a vacuum for 90 min to ensure that all the trapped air diffused out of the bone. Each bone was removed from its vial, blotted with gauze, weighed and returned to the vial containing distilled water. The bone was re-weighed in a boat suspended, but completely immersed, in water previously equilibrated to room temperature, and the density (g/cm^3 bone volume) was calculated. Bone mass was expressed in terms of the dry weight, and bone density as the dry weight per unit volume. Bone mineral mass was taken as the ash weight per bone length.

Serum osteocalcin levels. Serum osteocalcin concentrations were measured in duplicate using the Rat-MID osteocalcin ELISA (Osteometer Biotech A/S, Herlev, Denmark). The intra- and inter-assay CV were 4·0 and 6·6 % respectively.

Statistical analysis

Before the start of the experiment, the required sample size at α 0·05 and β 0·80 was calculated (Dallal, 1990) using the variability around the mean biomarker levels in rats and a selected minimum detectable percentage difference (i.e. Δ) in bone biomarker levels among groups of 15 %. A value of 15 % was chosen as a meaningful difference in the absence of reported data on the magnitude of the reduction in bone biomarker levels following the DASH Comb diet (Lin *et al.* 2001). Statistical analysis of the data was performed using DataDesk® (version 6·0; Data Description Inc., Ithaca, NY, USA). Data for all variables was normally distributed and allowed for parametric tests of significance. Results are presented as mean values with their pooled standard errors. Where appropriate, data were subjected to two-way ANOVA, with variation attributed to diet and rat strain (Snedecor & Cochran, 1967). Where significant ($P<0·05$) differences (i.e. either an effect of diet, rat strain or an interaction between the two) were found, Fisher's least significant difference test was used to perform *post hoc* comparison of all pairs of means (Snedecor & Cochran, 1967). To test the effect of either diet or strain alone, data was subject to one-way ANOVA, and where significant ($P<0·05$) differences (i.e. an independent effect of diet or rat strain) were found, Fisher's least significant difference test was used to perform *post hoc* comparison of all pairs of means (Snedecor & Cochran, 1967). In addition, the effect of rat strain on urinary pyridinium crosslink excretion was also assessed by ANOVA after the urinary crosslink values had been adjusted for creatinine clearance rates.

Results

Mean body-weight gain did not differ significantly among dietary groups in either the SHR or WKY rats (Fig. 1). SHR rats were significantly ($P<0·01$) heavier than WKY rats at all time points of the intervention. This was particularly evident at the beginning of the 8-week dietary intervention period, but there appeared to be a convergence of body weights between the two strains over time.

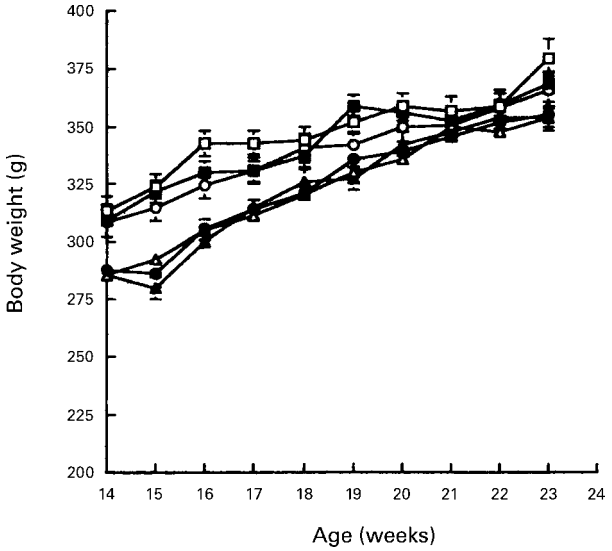

Fig. 1. Body weights over time in normotensive (WKY) and spontaneously hypertensive (SHR) rats fed on the control, high fruit and vegetable (F/V) and high fruit and vegetable and low-fat dairy produce (Comb) diets. Groups were: (□), control diet (SHR rats); (■) F/V diet (SHR rats); (○) Comb diet (SHR); (●) control diet (WKY rats); (△), F/V diet (WKY rats); (▲), Comb diet (WKY rats). For details of diets and procedures, see Tables 1–3 and p. 714, 715. Values are means with their standard errors shown by vertical bars for ten rats per group. SHR rats were significantly heavier ($P<0·01$) than WKY rats at all time points. There were no significant differences ($P>0·05$) in body weight between dietary groups within a strain.

The effect of rat strain and diet on blood pressure is shown in Table 5. As expected, blood pressure was significantly ($P<0·0001$) greater in the SHR rats than WKY rats at baseline, week 4 and week 8 of the dietary intervention period. There was no effect of dietary treatment on blood pressure at any time point, irrespective of strain.

The effect of rat strain and dietary treatment on physical properties (length, width, dry weight, ash and density) and macronutrient content (Ca, Mg, P and bone mineral mass) of femora is shown in Table 6. All of the physical variables, with the exception of bone density ($P>0·05$), were significantly ($P<0·0001$) greater in SHR rats compared with WKY rats. In addition, when femoral dry weight and ash weight were expressed relative to body weight, these variables were still significantly ($P<0·0001$) greater in SHR rats than WKY rats. There was no effect of dietary treatment on length, width, dry weight, ash weight and density, irrespective of strain.

The concentration (mg/g bone) of Ca and P in femora was unaffected by rat strain. However, when the femoral Ca and P were expressed as content (mg/bone), SHR rats had significantly ($P<0·0001$) greater Ca and P than WKY rats. Neither the content nor concentration of Ca or P was affected by diet, irrespective of strain. While femoral Mg concentration was significantly ($P<0·0001$) lower in the SHR rats compared with the WKY rats, femoral Mg content (mg/bone) was significantly ($P<0·001$) greater in SHR rats than WKY rats. Two-way ANOVA showed that femoral Mg concentration was affected ($P<0·05$) by diet. *Post hoc* analysis showed that SHR rats fed the F/V diet had significantly ($P<0·01$)

Table 5. Blood pressure of adult male normotensive and spontaneously hypertensive rats fed the control, high fruit and vegetable (F/V) and combination (Comb) diets*

(Mean values with their pooled standard errors for ten rats per group)

Rat strain.....	Hypertensive rats			Normotensive rats				Statistical significance of variance ratio (*P*)		
Dietary treatment...	Control	F/V	Comb	Control	F/V	Comb	Pooled SEM	Strain	Diet	Strain×Diet
Blood pressure (mmHg)										
Baseline	181	178	176	127	127	128	4	<0·0001	0·856	0·727
Week 4	176	180	177	130	128	130	2	<0·0001	0·887	0·317
Week 8	176	179	178	130	129	125	3	<0·0001	0·538	0·450

* For details of diets and procedures see Tables 1–3 and p. 714, 715.

higher femoral Mg concentration compared with SHR rats fed the control or Comb diets. Femoral Mg content was unaffected by diet, irrespective of strain. Bone mineral mass was significantly ($P<0·0001$) higher in SHR rats compared with that in WKY rats. Bone mineral mass was unaffected by diet, irrespective of strain.

The effects of rat strain and dietary treatment on creatinine clearance and biochemical markers of bone resorption (urinary Pyr and Dpyr) and bone formation (serum osteocalcin) are shown in Table 7. Daily creatinine output in urine was significantly ($P<0·05$) lower in SHR than WKY rats. Serum creatinine concentrations tended ($P=0·052$) to be greater in SHR rats than WKY rats. Creatinine clearance was significantly ($P<0·05$) lower in SHR rats than in WKY rats. Urinary and serum creatinine and creatinine clearance was unaffected by diet, irrespective of strain. Urinary Pyr and Dpyr excretion (expressed as daily output) was similar in SHR and WKY rats. In addition, urinary crosslink excretion (adjusted for creatinine clearance rates) was similar in SHR and WKY rats. Urinary Pyr and Dpyr excretion was significantly ($P<0·05$) lower in SHR rats fed the combination diet than that in SHR rats given the F/V and control diets. On the other hand, urinary Pyr and Dpyr excretion in WKY rats was unaffected by diet.

In the present study, serum osteocalcin was unaffected by rat strain (Table 7). Serum osteocalcin was affected ($P<0·05$) by diet. There was, however, a significant ($P<0·05$) interaction between rat strain and diet. *Post hoc* analysis showed that SHR rats fed the Comb diet had significantly ($P<0·05$) lower serum osteocalcin than SHR rats given the F/V and control diets. Serum osteocalcin in WKY was unaffected by diet.

Discussion

Effect of dietary intervention to stop hypertension-type diet on blood pressure

In the present study, SHR rats were heavier than WKY rats, in agreement with the findings of several other studies (Schedl *et al.* 1984; Hatton *et al.* 1989; Pörsti, 1992). However, other studies have reported that WKY rats were heavier than SHR rats (Lau *et al.* 1984; Izawa *et al.* 1985; Galletti *et al.* 1991; Touyz & Milne, 1999; Pezeshk & Dalhouse, 2000; Vasedv *et al.* 2000). The reasons for this discrepancy between studies are not clear. In the present study, as expected, SHR rats had significantly elevated blood pressure relative to WKY rats. Blood pressure of the SHR and WKY animals (which were aged 15 weeks at the beginning of the dietary intervention) did not change over the 8-week supplementation period. This is in agreement with the findings of some studies which have used similarly aged normotensive and hypertensive rats (Yoshioka *et al.* 1985; Sato *et al.* 1995; Vasdev *et al.* 2000); however, increased blood pressure development over time in similarly aged SHR rats has been noted in some studies (Tolavnen *et al.* 1998; Newaz *et al.* 1999; Touyz & Milne, 1999). In the present study, dietary provision of the nutrient profiles of the DASH-type diets (Karanja *et al.* 1999) over an 8-week period had no effect on blood pressure of male SHR or WKY rats. This is in contrast to the reductions in blood pressure seen in human subjects, with high-normal to mildly elevated blood pressure, that were given either the DASH F/V or Comb diet for 8 weeks (Appel *et al.* 1997; Moore *et al.* 2001). Several previous studies in hypertensive rats have shown a beneficial blood pressure-lowering effect of diets supplemented with one or two individual nutrients, such as Ca (McCarron *et al.* 1981; Blakeborough *et al.* 1990; Pörsti, 1992; Mäkynen *et al.* 1995), Mg (Touyz & Milne, 1999), K (Tolvanen *et al.* 1998), vitamin E (Pezehhk & Dalhouse, 2000; Chen *et al.* 2001) or vitamin C (Chen *et al.* 2001). For example, McCarron *et al.* (1981) reported that Ca supplementation of the diet of 10-week-old male SHR rats attenuated the development of hypertension in these animals. Pörsti (1992) also found that Ca supplementation of 8-week-old male rats attenuated the development of blood pressure in SHR rats, but had no effect in WKY rats. Mäkynen *et al.* (1995) reported that Ca supplementation, either alone or in combination with Mg, of 8-week-old rats, attenuated the development of blood pressure in SHR rats, whereas Mg supplementation alone had no effect on blood pressure in SHR rats. Blood pressure in normotensive animals was unaffected by Ca, Mg or combined Ca and Mg supplementation (Mäkynen *et al.* 1995). Tolvanen *et al.* (1998) reported that both Ca and K supplementation markedly lowered blood pressure development in 7-week-old SHR rats, with their combined supplementation reducing blood pressure even further.

There are several possible reasons for the lack of antihypertensive effect of increased Ca, Mg, K and vitamin intake (provided by the F/V and Comb diets) in SHR rats used in the present study. In the present study, the intake

Table 6. Bone physical properties and mineral analysis of right femurs of adult male normotensive and spontaneously hypertensive rats fed the control, high fruit and vegetable (F/V) and combination (Comb) diets*

(Mean values with their pooled standard errors for ten rats per group)

Rat strain...	Hypertensive rats			Normotensive rats				Statistical significance of variance ratio (*P*)		
Dietary treatment...	Control	F/V	Comb	Control	F/V	Comb	Pooled SEM	Strain	Diet	Strain×Diet
Femur										
Length (mm)	34·53	34·07	34·14	33·71	33·56	33·88	0·19	<0·001	0·059	0·439
Width (mm)	3·27	3·33	3·26	3·08	3·09	3·10	0·03	<0·0001	0·454	0·366
Dry wt (mg)	623	599	613	531	524	531	10·0	<0·0001	0·018	0·371
Dry wt (g/kg body wt)	1·64	1·66	1·68	1·46	1·47	1·49	0·02	<0·0001	0·223	0·931
Ash wt (mg)	407	393	402	345	341	346	20·0	<0·0001	0·253	0·736
Ash wt (g/kg body wt)	1·07	1·09	1·10	0·95	0·95	0·97	0·01	<0·0001	0·163	0·870
Density (g/cm^3)	1·56	1·57	1·58	1·52	1·54	1·55	0·02	0·108	0·478	0·933
Bone mineral mass (mg/mm)	11·8	11·5	11·8	10·2	10·1	10·2	0·18	<0·0001	0·613	0·654
Ca (mg/g dry wt)	236	237	239	235	235	236	2·7	0·815	0·743	0·702
Ca (mg/bone)	147	142	146	125	123	125	2·4	<0·0001	0·304	0·522
Mg (mg/g dry wt)	4·07^a	4·24^b	4·17^{ab}	4·48^a	4·55^a	4·56^a	0·04	<0·0001	0·012	0·479
Mg (mg/bone)	2·54	2·53	2·56	2·38	2·37	2·42	0·05	<0·001	0·789	0·949
P (mg/g dry wt)	111	111	110	109	111	112	1·6	0·934	0·647	0·518
P (mg/bone)	68·9	66·7	67·7	57·8	58·2	59·2	1·0	<0·0001	0·560	0·328

* For details of diets and procedures, see Tables 1–3 and pp. 714, 715.

^{a,b} Mean values within a row with unlike superscript letters were significantly difference (ANOVA following by least significant difference test; $P<0·05$).

Table 7. Biochemical markers of bone turnover in adult male normotensive and spontaneously hypertensive rats fed the control, high fruit and vegetable (F/V) and combination (Comb) diets*
(Mean values with their pooled standard errors for ten rats per group)

Rat strain	Hypertensive rats			Normotensive rats				Statistical significance of variance ratio (*P*)		
Dietary treatment	Control	F/V	Comb	Control	F/V	Comb	Pooled SEM	Strain	Diet	Strain×Diet
Urine										
Cr (mmol/24 h)	0·082	0·085	0·097	0·104	0·104	0·102	0·008	0·019	0·686	0·510
Pyr (nmol/24 h)	2·37[a]	2·18[a]	1·83[b]	2·36[a]	2·29[a]	2·39[a]	0·10	0·142	0·013	0·549
Dpyr (nmol/24 h)	2·05[a]	1·98[a]	1·37[b]	1·88[a]	1·91[a]	1·95[a]	0·17	0·908	0·006	0·048
Serum										
Osteocalcin (ng/ml)	162[a]	168[a]	116[b]	110[a]	129[a]	122[a]	17	0·104	0·042	0·048
Cr (mg/l)	5·5	5·4	5·7	4·1	4·0	3·5	1·0	0·052	0·974	0·917
Cr clearance (mg/min)	1·99	3·31	3·38	4·67	5·3	5·67	1·08	0·013	0·523	0·951

Cr, creatinine; Pyr, pyridinoline; Dpyr, deoxypyridinoline.
* For details of diets and procedures, see Tables 1–3 and pp. 714, 715.
[a,b]Mean values within a row with unlike superscript letters were significantly different (ANOVA followed by least significant difference test; $P<0·05$).

of twenty nutrients and/or dietary components were altered (which could have lead to several nutrient–nutrient interactions), whereas in the studies mentioned earlier the intake of only one or two nutrients was increased (McCarron *et al.* 1981; Blakeborough *et al.* 1990; Pörsti, 1992; Mäkynen *et al.* 1995; Tolvanen *et al.* 1998; Touyz & Milne, 1999; Pezehhk & Dalhouse, 2000; Chen *et al.* 2001). In addition, the greatest increment in dietary Ca content between the three diets used in the present study was from 3·3 to 9·1 g/kg diet, whereas the minimum level of Ca supplementation above the recommended level (5 g Ca/kg diet; that recommended for adult rats by the American Institute of Nutrition (Reeves *et al.* 1993)) that produced a blood pressure-lowering effect in previously reported studies was 20 g Ca/kg diet (Lewanczuk *et al.* 1990; Rao *et al.* 1994). In the present study, the greatest increment in dietary K content between the three diets was from 4·0 to 10·4 g/kg diet, whereas in previous studies, the level of K supplementation which led to a significant reduction in blood pressure in hypertensive rats was 35 g/kg diet (Tolvanen *et al.* 1998; Wu *et al.* 1998). Similarly, in the present study, the dietary vitamin C and E contents were increased from 541 to 1783 mg/kg diet, and from 30·9 mg/kg diet to 51·4 mg/kg diet respectively. These dietary levels are far below the amounts which have been shown in previous studies to produce a significant reduction in blood pressure in SHR rats (Pezehhk & Dalhouse, 2000; Chen *et al.* 2001).

The increments in various nutrient contents in the F/V and Comb diets used in the present study were designed to reflect those in the two equivalent DASH study diets (Karanja *et al.* 1999), and consequently were not as great as those used in previous studies of hypertensive animals (McCarron *et al.* 1981; Blakeborough *et al.* 1990; Pörsti, 1992; Mäkynen *et al.* 1995; Tolvanen *et al.* 1998; Touyz & Milne, 1999; Pezehhk & Dalhouse, 2000; Chen *et al.* 2001). Furthermore, in keeping with the DASH study design, the diets in the present study were fed to the normotensive and hypertensive animals for 8 weeks. This time-scale of dietary intervention is shorter than that used in previous rat studies (in the range 10–38 weeks) investigating the blood pressure-lowering potential of various nutrients (McCarron *et al.* 1981; Blakeborough *et al.* 1990). In addition, the age of the animals at initiation of the dietary intervention may modulate the impact of a certain nutrient(s) on blood pressure regulation in hypertensive rats. For example, McCarron *et al.* (1981) found that Ca supplementation of male SHR rats from age 10 to 34 weeks had no significant effect on blood pressure, whereas continued Ca supplementation from age 34 to 48 weeks lead to a significant ($P<0·0001$) attenuation of blood pressure elevation. Mäkynen *et al.* (1995) reported that divergence in blood pressure between Ca-supplemented and -unsupplemented SHR rats (beginning at age 8 weeks) was only significant at 22 weeks of age. On the other hand, Touyz & Milne (1999) have suggested that, in terms of blood pressure regulation, younger rats (aged 6–14 weeks) respond to Mg supplementation or deprivation, whereas adult rats (aged 16–18 weeks) do not. Therefore, the dietary interventions in the present study may either not have been of sufficient length, or were

not begun early enough in the development of hypertension, to lead to a significant reduction in blood pressure in SHR rats.

Proposed link between hypertension and osteoporosis

As mentioned previously, hypertension has been associated with abnormalities of Ca and bone metabolism in human subjects (McCarron *et al.* 1980; Cappuccio *et al.* 1999, 2000) and rats (McCarron *et al.* 1981, 1985; Pörsti, 1992). Moreover, MacGregor & Cappuccio (1993) have hypothesized that the calciuria in hypertensive patients is compensated not by increased intestinal absorption of Ca, but by that liberated by resorption of bone, and that hypertensive patients are thus more prone to bone demineralization, bone fracture and osteoporosis. In support of this contention, bone mineral density among hypertensive subjects has been reported as being lower than that of normotensive subjects (Metz *et al.* 1999; Tsuda *et al.* 2001). Furthermore, the temporal sequence whereby high blood pressure precedes and predicts the loss of bone mineral has now been established in a large prospective study of 3676 white postmenopausal women (Cappuccio *et al.* 1999).

Effect of Dietary Intervention to stop hypertension-type diet on bone metabolism and mass

In light of the proposed link between hypertension and osteopenia (MacGregor & Cappuccio, 1993; Cappuccio *et al.* 2000) and the anti-hypertensive effect of the DASH diets (Appel *et al.* 1997, 2001; Moore *et al.* 1999; Conlin *et al.* 2000; Vollmer *et al.* 2001), it seemed timely to investigate whether the DASH-type diets have an osteoprotective effect. In the present study, while femoral bone mineral density and Ca and P concentrations were similar in both strains of rat, femoral length, width, dry weight, ash weight, Ca, Mg and P content and bone mineral mass were greater in SHR than WKY rats. These findings are in agreement with the findings of some studies which show that SHR animals have a greater bone mass and/or macromineral content than WKY rats (Lau *et al.* 1984; DeMoss & Wright, 1998; Patel *et al.* 2000); however, they are in contrast with the findings of other studies which show that these variables are greater in WKY rats than SHR rats (Izawa *et al.* 1985; Lucas *et al.* 1986; Wallach & Verch, 1986; Barbagallo *et al.* 1990, 1991; Metz *et al.* 1990).

In the present study, the nutrient profiles of the DASH F/V and Comb diets had no effect on physical properties or Ca and P content of femora in either SHR or WKY rats. While femoral Mg concentration was unaffected by diet in the WKY rats, it was significantly increased in SHR rats fed the F/V diet compared with that of SHR rats fed the control or Comb diets. The reasons for the increased femoral Mg concentration in rats fed the F/V diet relative to those fed the Comb diet are unclear, especially as the Comb diet contained slightly more Mg than the F/V diet (782 and 693 mg Mg/kg diet respectively). However, the Comb diet also contained approximately 3-fold the Ca content of the F/V diet. A high-Ca diet (containing 4-fold the recommended Ca content, 5 g Ca/kg diet) has been shown to lead to reduced serum and bone Mg levels in male rats over a 12-week period, relative to rats receiving the recommended dietary Ca level (Patwardhan *et al.* 2001). Therefore, it is possible that the high Ca content of the Comb diet competed with Mg and thus impeded its uptake into femora of SHR rats.

In the present study, the rate of bone turnover in hypertensive rats, as indicated by biochemical markers of bone resorption and bone formation, was reduced by consumption of the Comb diet over an 8-week period. On the other hand, there was no effect of diet on bone turnover in the normotensive rats. These findings in hypertensive rats support the recent preliminary findings of Lin *et al.* (2001) that showed that consumption of the DASH Comb diet for 30 d reduced the levels of biochemical markers of bone formation (serum osteocalcin) and bone resorption (serum C-terminal telopeptide of type I collagen) in adult men and women (Lin *et al.* 2001). The reduced rate of bone turnover in SHR rats fed the Comb diet for 8 weeks in the present study did not appear to be translated into detectable differences in bone mass or composition in these animals. However, Sinha *et al.* (1988) have suggested that such a relatively short dietary intervention may not be of sufficient time for measurable changes in femoral mass that would follow dietary-induced alterations in the rate of bone metabolism.

Mechanism by which the dietary intervention to stop hypertension combination diet lowers bone turnover

The mechanism by which the DASH Comb diet in the study by Lin *et al.* (2001) and the Comb diet in the present study reduced bone turnover is unclear. If one accepts the proposed association between hypertension, hypercalciuria and osteopenia (Cappuccio *et al.* 1999), then by virtue of its blood pressure-lowering effect alone, consumption of the DASH Comb diet may lead to a reduction in the rate of bone resorption. However, there was no anti-hypertensive effect of the Comb diet in SHR rats in the present study. The reduced rate of bone resorption in SHR rats fed the DASH Comb diet may be due to its low estimated potential renal acid load (−41 mEq/kg), relative to that of the control diet (86 mEq/kg), as recently suggested by New (2002). However, the F/V diet, which also had a low estimated potential renal acid load (− 44 mEq/kg), had no effect on the rate of bone resorption in SHR rats in the present study. It is also possible that the mechanism by which the Comb-type diet lead to reduction in bone turnover in both human subjects and rats may be independent of an effect on blood pressure, but may be due to the fact that many of the nutrients that are rich in the DASH Comb diet (e.g. Ca, Mg, K, vitamin C) have been individually shown to have important roles in bone health (Stendig-Lindberg *et al.* 1993; Sebastian *et al.* 1994; for review, see Institute of Medicine, 1997; New *et al.* 1997, 2000; Hall & Greendale, 1998; Tucker *et al.* 1999; Morton *et al.* 2001; Sellmeyer *et al.* 2002. While this would help explain the observed reduction in bone turnover in the SHR rats fed the Comb diet in the present study, despite its lack of effect on blood pressure regulation, it would not explain the lack of effect of the same diet on bone

turnover in the normotensive rats. The SHR rats in the present study had significantly lower creatinine clearance rates relative to the WKY rats, suggesting differences in renal function between strains. Reduced creatinine clearance rates have also been noted in hypertensive patients (Catena *et al.* 2000; Kadiri & Ajayi, 2000). Differences in renal function between hypertensive and normotensive animals may have some bearing on whether the Comb diet can regulate bone turnover or not. The mechanism by which the Comb diet reduces bone turnover in the face of hypertension requires further investigation.

Conclusion

The findings of the present study show that a Comb-type diet, rich in several minerals and vitamins, can reduce the rate of bone turnover in hypertensive animals. These findings in rats would suggest that in addition to benefits for blood pressure regulation and cardiovascular health, the DASH-type Comb diet could also benefit skeletal health of hypertensive individuals, but this potential benefit would need to be confirmed in studies with human subjects.

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