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Influence of dietary phytosterols and phytostanols on diastolic blood pressure and the expression of blood pressure regulatory genes in SHRSP and WKY inbred rats

Qixuan Chen[1], Heidi Gruber[1], Eleonora Swist[1], Catherine Pakenham[1], Walisundera M. N. Ratnayake[1] and Kylie A. Scoggan[1,2]*

[1]*Nutrition Research Division, Food Directorate, Health Products and Food Branch, Health Canada, Banting Research Centre, 251 Sir Frederick Banting Driveway, Ottawa, Ontario, Canada K1A 0K9*
[2]*Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, Ontario, Canada K1H 8M5*

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The aim of the present study was to determine the impact of increased consumption of phytosterols or phytostanols on blood pressure and renal blood pressure regulatory gene expression in stroke-prone spontaneously hypertensive (SHRSP) and normotensive Wistar–Kyoto (WKY) inbred rats. SHRSP and WKY inbred rats (10/group) were fed a control diet or a diet supplemented with phytosterols or phytostanols (2·0 g/kg diet). After 5 weeks, SHRSP rats demonstrated higher systolic and diastolic blood pressures than WKY inbred rats. SHRSP rats that consumed the phytosterol or phytostanol supplemental diets displayed a 2- or 3-fold respective increase in the diastolic blood pressure than those that consumed the control diet. Angiotensinogen (*Agt*), angiotensin I-converting enzyme 1 (*Ace1*), nitric oxide synthase (*Nos*) 1, *Nos3*, cyclooxygenase 2 (*Cox2*) and THUMP domain containing 1 were expressed at higher levels in SHRSP compared with WKY inbred rats. *Renin* and angiotensin II receptor type 1a were expressed at lower levels in SHRSP than WKY inbred rats. Phytostanol supplementation up-regulated the expression of *Ace1* and *Nos3* in SHRSP rats. Phytosterol supplementation increased the mRNA levels of *Nos1* and spondin 1 (*Spon1*) in SHRSP and WKY inbred rats. *Cox2* mRNA levels were elevated in both phytosterol- and phytostanol-supplemented SHRSP and WKY inbred rats. Therefore, the increased blood pressure in SHRSP rats may be partly due to altered renal expression of blood pressure regulatory genes. Specifically, up-regulation of *Ace1*, *Nos1*, *Nos3*, *Cox2* and *Spon1* were associated with the increased diastolic blood pressure observed in phytosterol- or phytostanol-supplemented SHRSP rats.

Phytosterols: Phytostanols: Blood pressure: Gene expression: Rats

Consumption of phytosterols and phytostanols at the level of 1·5–3·0 g/d has been documented to reduce blood LDL cholesterol between 8 and 15 %[1,2]. The average western diet provides 150–400 mg phytosterols and 20–50 mg phytostanols daily[3]. Therefore, a variety of foods have been supplemented with phytosterols and/or phytostanols to provide high enough intakes of phytosterols and phytostanols in order to market these novel foods as an aid to lower blood cholesterol levels. Phytostanol ester-supplemented margarine was first launched in Finland in 1995. Now several commercially available phytosterol/phytostanol-enriched products are available in the European Union member states[4] and in the USA.

However, whether or not phytosterols or phytostanols elicit a net beneficial effect on vascular disease remains controversial. Various national and international bodies continue to struggle with the safety issues of phytosterols and phytostanols in foods, because there are growing concerns for their potential excessive daily intake and hazards, as outlined below. First, rare individuals with phytosterolaemia, caused by mutations in either the ATP-binding cassette G5 or the ATP-binding cassette G8 half-transporter genes[5], overabsorb phytosterols and phytostanols and develop accelerated atherosclerosis and premature coronary artery disease[6]. Second, epidemiological studies have demonstrated that even a slight elevation in the serum levels of phytosterols or phytostanols is associated with an increased risk of CHD, independent of the serum cholesterol levels[7–9]. Third, the use of phytosterol- or phytostanol-enriched food products was found to elevate phytosterol and phytostanol levels in the serum of a free-living population[10] and in healthy subjects[11], as well as in the serum and aorta of patients with aortic stenosis[12]. Fourth, phytosterol ester supplementation to wild-type mice increased their phytosterol, but not

Abbreviations: *Ace1*, *Ace2*, angiotensin I-converting enzyme 1, 2; *Agt*, angiotensinogen; *Agtr*, angiotensin II receptor; *Agtr1a*, angiotensin II receptor type 1a; *Cox2*, cyclooxygenase 2; NO, nitric oxide; *Nos1*, *Nos3*, nitric oxide synthase 1, 3; RAAS, renin–angiotensin–aldosterone system; SHR, spontaneously hypertensive rats; SHRSP, stroke-prone spontaneously hypertensive rats; *Spon1*, spondin 1; TXA_2, thromboxane; *Thumpd1*, THUMP domain containing 1; WKY inbred, Wistar–Kyoto inbred.

* **Corresponding author:** Kylie A. Scoggan, fax: +1 613 941 6182, email kylie_scoggan@hc-sc.gc.ca

cholesterol, levels in plasma; endothelial function was also impaired and experimental cerebral lesion size was increased[12]. Fifth, in stroke-prone spontaneously hypertensive rats (SHRSP) that have a mutation in the *Abcg5* gene[13], dietary phytosterols or phytostanols induced the accumulation of phytosterols or phytostanols in the serum and tissues, decreased the deformability of red blood cells, accelerated the onset of stroke and reduced the lifespan[14–16].

The hypertensive SHRSP strain, a model for essential hypertension and haemorrhagic stroke[17], was derived from the spontaneously hypertensive rat (SHR) strain that was previously developed from the normotensive Wistar–Kyoto (WKY) inbred strain[18]. We have determined that both SHRSP and its 'parental' normotensive WKY inbred rats have a mutation in the *Abcg5* gene[13]. SHRSP rats are hypertensive, whereas WKY inbred rats are normotensive. The precise cause of hypertension is unclear, but is likely to be multifactorial and to involve genetic predisposition, related primarily to the kidney[19], and responses to environmental factors, such as high salt intake and obesity. In the present study, we further investigated the molecular mechanism(s) by which phytosterols and phytostanols accelerated the onset of stroke and reduced the lifespan via measuring blood pressure, and the expression of several renal genes known to be involved in blood pressure regulation in SHRSP and WKY inbred rats fed diets containing high levels of phytosterols or phytostanols.

Materials and methods

Preparation of phytosterols and phytostanols

A mixture of phytosterols was extracted from rapeseed oil deodoriser distillate (CanAmera Foods, Altona, MB, Canada), as described previously[15]. The final phytosterol product contained 22·0 % brassicasterol, 31·9 % campesterol and 43·2 % β-sitosterol as determined by GC (6890 Network GC system; Agilent Technologies, Santa Clara, CA, USA). A portion of the phytosterol product was converted to phytostanols via catalytic hydrogenation at the pilot plant facility of the POS Pilot Plant Corporation (Saskatoon, Sask., Canada). The final phytostanol product contained 54·7 % campestanol and 44·8 % sitostanol.

Experimental diets

Three diets were tested (Table 1). Each diet contained 10 % fat and was prepared according to the AIN-93G recommendations for rodent diets[20]. The diets were made in pellet form and stored at 4°C. The final total phytosterol and phytostanol content of the diets was 0·25, 2·13 and 1·96 g/kg in the control, phytosterol and phytostanol diets, respectively (Table 1). These dietary levels of phytosterols and phytostanols were used because our previous studies demonstrated that 2·0 g/kg diet of phytosterols and phytostanols significantly accelerated the onset of stroke and reduced the lifespan of SHRSP rats[14–16]. These dietary levels of phytosterols and phytostanols are similar to the amounts suggested for cholesterol lowering in humans on a dietary fat basis[2,3]. For example, consumption of 2·0 g per day of dietary phytosterols or phytostanols in an individual who consumes 100 g fat per day (average fat intake) represents 2·0 % of that person's fat intake. Our rats were also fed 2·0 g phytosterols or phytostanols in a diet that contained 100 g fat (soyabean oil) per kg of diet, which represents 2·0 % of the rat's dietary fat intake.

Table 1. Composition of test diets (g/kg)*

	Control diet	Phytosterol diet	Phytostanol diet
Ingredient			
Casein†	222·0	222·0	222·0
Soyabean oil	100·0	100·0	100·0
Phytosterols‡	–	2·0	–
Phytostanols§	–	–	2·0
Maize starch	477·5	475·5	475·5
Granulated sugar	100·0	100·0	100·0
Cellulose (Alphacel)	50·0	50·0	50·0
Miscellaneous‖	50·514	50·514	50·514
Sterol composition			
Cholesterol	0·001	0·006	0·003
Total phytosterols	0·248	2·117	0·235
Campesterol	0·061	0·680	0·059
β-Sitosterol	0·139	0·958	0·131
Stigmasterol	0·048	0·066	0·045
Brassicasterol	0	0·413	0
Total phytostanols	0·000	0·015	1·725
Campestanol	0·000	0·005	0·938
Sitostanol	0·000	0·010	0·787
Total phytosterols and phytostanols	0·248	2·132	1·960
Total sterols¶	0·249	2·138	1·963

* Based on the AIN-93G diet[20].
† Vitamin-free test casein (Harlan Teklad, Madison, WI, USA).
‡ Phytosterols isolated from rapeseed oil deodoriser distillate.
§ Phytosterols isolated from rapeseed oil deodoriser distillate were hydrogenated to produce phytostanols.
‖ 93G-MX Mineral mix (35·0 g/kg), 93-VG Vitamin mix (10·0 g/kg), L-cystine (3·0 g/kg), choline bitartrate (2·5 g/kg) and *tert*-butylhydroquinone (0·014 g/kg).
¶ Includes phytosterols, phytostanols and cholesterol.

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Animals

SHRSP and WKY inbred rats were obtained from Charles River Laboratories (Kingston, NY, USA). Thirty-five-day-old male SHRSP and WKY inbred rats were each randomly assigned to one of the three diet groups ($n = 10$/group). They had free access to drinking-water containing NaCl (10 g/l) and one of the three diets (Table 1) for 5 weeks. NaCl loading was applied to accelerate the progress of hypertension[16] for pronouncing the difference in the effects of the treatment.

Rats were housed individually in wire-bottom cages in an environmentally controlled room maintained at 22°C and 60 % relative humidity with a 12 h day/12 h night cycle. Body weight, food intake and water consumption of the rats were recorded twice weekly. After 5 weeks on the test diets, 12-h food-deprived rats were killed via exsanguination from the abdominal aorta while under 3 % isoflurane anaesthesia. Kidneys were removed and frozen immediately in liquid nitrogen. One kidney from each rat was ground under liquid nitrogen for RNA extraction. The animal protocol was approved by the Institutional Animal Care Committee at Health Canada.

Blood pressure measurements

Blood pressure was measured at the beginning, middle and end (weeks 1, 3 and 5) of the feeding phase of the study by the tail-cuff method using a II TC Model 31 Blood Pressure Apparatus (IITC Inc./Life Science Instruments, Woodland Hills, CA, USA) as per the manufacturer's instructions. Only week 1 and 5 data are presented. Briefly, the blood pressure apparatus was stored in a separate, larger, warmer room (26–27°C). The pressure channels were calibrated every day and the chamber was kept at 29–30°C. Rats were trained on the apparatus every day for 1 week before the experiment. The rats were transferred to the blood pressure room 30 min before handling to allow them to gradually get used to the heat. Each rat was placed in the chamber for 5 min before starting the measurements, allowing the rats to acclimatise. The same investigator monitored the computer screen and the animal in order to detect artefacts such as animal distress or movement. The systolic and mean blood pressures were measured by the same investigator every time. The diastolic blood pressure was calculated by the equation, diastolic = (3 × mean − systolic)/2, as per the manufacturer's instructions. The blood pressure of each rat at each measuring point was obtained as an average of three readings.

Analyses of sterols

The sterol composition of the diets was analysed by GC, as described previously[15].

RNA isolation and real-time quantitative RT-PCR

Procedures described previously were followed[21]. Total RNA was extracted from each kidney sample using two passes of TRIzol reagent (Invitrogen Life Technologies, Burlington, ON, Canada). The isolated RNA was then purified and DNase I treated on RNeasy mini columns (Qiagen, Mississauga, ON, Canada). The quality of the purified RNA was assessed by a Bioanalyzer (Agilent 2100 Bioanalyzer; Agilent Technology, Waldbronn, Germany) and by a conventional agarose gel.

The purified RNA was quantified using RiboGreen RNA Quantitation Reagent and Kit (Molecular Probes, Eugene, OR, USA) and subsequent cDNA synthesis was performed with Retroscript Kit (Ambion, Austin, TX, USA). Real-time quantitative PCR was performed on an M × 4000 Multiplex Quantitative PCR System using the Brilliant SYBR Green QPCR Core Reagent Kit (Stratagene, La Jolla, CA, USA) or TaqMan Gene Expression Assay (Applied Biosystems, Foster city, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was selected to normalise the expression of the target genes. The primers were synthesised by Integrated DNA Technologies (Coralville, IA, USA) and described in Table 2 [22–24]. For each experiment, a non-template reaction and a no-RT reaction were included as negative controls. The specificity of the PCR was confirmed by dissociation curve analysis of the products as well as by size verification of the amplicons in a conventional agarose gel.

The relative amounts of each gene-of-interest were normalised to *Gapdh* expression levels in the kidney as an endogenous internal standard. A serial dilution of a standard was run on each plate for each gene-of-interest and *Gapdh*. The relative levels of mRNA (gene-of-interest/*Gapdh*) were then calibrated to the control group, namely control rats (WKY inbred) fed the control diet (set as 1·0).

Statistical analysis

Data are presented as the means with their standard errors. All data were evaluated for equality of variance before statistical analysis. Variables with skewed distribution were log transformed. Statistical analyses were performed using Statistica 8.0 software (StatSoft, Tulsa, OK, USA). Two-way ANOVA was used to determine the effects of strain (two levels: SHRSP and WKY inbred), diet (three levels: control, high phytosterols and high phytostanols) and their interactions. Comparisons between groups were made by Tukey's honestly significant differences (HSD) test. Differences were considered significant when $P < 0{\cdot}05$.

Results

Body weight, food intake and water consumption

Body weight of the animals increased steadily during the 5-week feeding phase of the study. There were no effects of the diet on body weight gain, food efficiency and total water consumption (Table 3). However, SHRSP rats had less initial body weight ($P = 0{\cdot}0069$), more weight gain ($P = 0{\cdot}0002$), more food intake ($P < 0{\cdot}0001$) and less food efficiency ($P < 0{\cdot}0001$) compared with WKY inbred rats. SHRSP rats also consumed less, 1 % salt water, than WKY inbred rats ($P < 0{\cdot}0001$).

Blood pressure

There were no differences in the initial systolic and diastolic blood pressures among groups (Table 4). After consuming the diets for 5 weeks, SHRSP rats demonstrated higher mean systolic ($P < 0{\cdot}0001$) and diastolic ($P < 0{\cdot}0001$) blood

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Table 2. Primer sequences for real-time quantitative PCR (rat)

Gene	Forward primers (5′ → 3′)	Reverse primers (5′ → 3′)	Amplicon size (bp)	Accession no.	Sequence source
Gapdh	TCA AGA AGG TGG TGA AGC AGC C	GCA TCA AAC GTG GAA GAA TGG G	118	AF106860	Yu *et al.* [22]
Agt	ACT TGG ATA AAG AAC CCG CCT CCT	GTG TCA CCC ATC TTG CCC AGA TTT	152	NM_134432	
Renin	TTC TCT CCC AGA GGG TGC TA	CCC TCC TCA CAC AAC AAG GT	211	NM_012642	Boustany *et al.* [23]
Ace1	GAG CCA TCC TTC CCT TTT TC	GGC TGC AGC TCC TGG TAT AG	154	NM_012544	
Ace2	TGG AGG TGG ATG GTC TTT CAG GAT	ATG GAA CAG AGA TGC AGG GTC ACA	141	NM_001012006	
Agtrt1a	CAC AGT GTG CGC GTT TCA TT	TGG TAA GGC CCA GCC CTA T	66	BC078810	Nakazawa *et al.* [24]
Nos3	TAT TTG ATG CTC GGG ACT GCA GGA	ACG AAG ATT GCC TCG GTT TGT TGC	92	NM_021838	
Nos1	TaqMan Gene Expression Assay kit (Rn 00583793)				
Cox2	TaqMan Gene Expression Assay kit (Rn 01483828)				
Spon1	TaqMan Gene Expression Assay kit (Rn 00597105)				
Thumpd1	TaqMan Gene Expression Assay kit (Rn 01510734)				

pressures as well as a greater increase in the systolic ($P<0{\cdot}0001$) and diastolic ($P<0{\cdot}0001$) blood pressures when compared with WKY inbred rats. SHRSP rats that consumed the phytosterol or phytostanol supplemental diets displayed a 2- or 3-fold respective increase in the diastolic blood pressure than those that consumed the control diet (overall diet effect $P=0{\cdot}0016$).

Renal mRNA expression of genes involved in blood pressure regulation

SHRSP rats had significantly higher mRNA expression of angiotensinogen (*Agt*; $P<0{\cdot}0001$), angiotensin I-converting enzyme 1 (*Ace1*; $P<0{\cdot}0001$), nitric oxide synthase 1 (*Nos1*; $P<0{\cdot}0001$), *Nos3* ($P<0{\cdot}0001$), cyclooxygenase 2 (*Cox2*; $P<0{\cdot}0001$) and THUMP domain containing 1 (*Thumpd1*; $P<0{\cdot}0001$) than WKY inbred rats (Table 5). Whereas WKY inbred rats had slightly higher mRNA expression of *renin* ($P=0{\cdot}0003$) and angiotensin II receptor type 1a (*Agtr1a*; $P=0{\cdot}007$) than SHRSP rats.

Phytostanol supplementation up-regulated the expression of *Ace1* and *Nos3* in SHRSP rats (overall diet effect: $P=0{\cdot}029$ and 0·005, respectively). Phytosterol supplementation significantly increased the mRNA levels of *Nos1* and spondin 1 (*Spon1*) in SHRSP and WKY inbred rats (overall diet effect: $P=0{\cdot}017$ and 0·0005, respectively). *Cox2* mRNA levels were significantly elevated in both phytosterol- and phytostanol-supplemented SHRSP and WKY inbred rats (overall diet effect: $P=0{\cdot}001$).

There were no strain and diet effects on the mRNA expression of *Ace2*. There was a strain–diet interaction on the mRNA expression of *Ace1* ($P=0{\cdot}006$) and *Agtr1a* ($P=0{\cdot}016$). These interactions indicated that the high phytostanol diet up-regulated the *Ace1* and *Agtr1a* expression in SHRSP rats, but not in WKY inbred rats.

Discussion

In the present study, we demonstrated that SHRSP rats have both higher systolic and diastolic blood pressures compared with WKY inbred rats. We also demonstrated, for the first time, that a high phytosterol or phytostanol diet (2 g/kg diet) induces a greater increase in the diastolic blood pressure in SHRSP rats (Table 4). In our previous studies, diets containing vegetable oils fortified with phytosterols (2 g/kg diet)[15] and diets containing commercial margarines fortified with either phytosterols (14 g/kg diet) or phytostanols (11 g/kg diet)[14] induced the accumulation of phytosterols or phytostanols in the serum and tissues, decreased the deformability of red blood cells, accelerated the onset of stroke and reduced the lifespan of SHRSP rats. Taken together, these data suggest that increased consumption and subsequent tissue retention of either phytosterols or phytostanols exacerbates hypertension, which may contribute to the early onset of stroke and reduced lifespan of salt-loaded SHRSP rats. Our findings are supported by Ogawa *et al.* [25], who reported that a diet containing soyabean oil fortified with phytosterols (4·5 g/kg diet) elevated systolic blood pressure and promoted the onset of stroke in salt-loaded SHRSP rats. Similarly, studies from Naito *et al.* [26,27] indicated that feeding rapeseed oil, but not soyabean oil, elevated systolic blood pressure in SHRSP, SHR

Table 3. Growth, food and water intake of the rats

(Mean values with their standard errors)

	SHRSP rats						WKY-inbred rats						*P* value (two-way ANOVA)		
	Control diet (*n* 10)		Phytosterol diet (*n* 10)		Phytostanol diet (*n* 10)		Control diet (*n* 10)		Phytosterol diet (*n* 10)		Phytostanol diet (*n* 10)				
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Strain	Diet	Strain × diet
Initial body weight (g)	112·5^{a}	4·4	115·7^{a,b}	4·2	120·8^{a,b}	3·5	125·6^{a,b}	4·1	131·6^{b}	4·1	126·9^{a,b}	1·8	0·0069 (one-way ANOVA)*		
Body weight at week 5 (g)	255·7	6·1	257·3	6·7	265·5	5·4	260·6	5·2	261·1	8·0	258·4	3·2	0·9196	0·8041	0·5389
Weight gain (g/5 weeks)	143·2^{a,b}	2·5	141·5^{a,b}	3·3	144·7^{b}	4·3	134·9^{a,b}	2·4	129·5^{a}	4·4	131·5^{a,b}	2·9	0·0002	0·5638	0·7451
Total food intake (g)	639·0^{b}	21·8	656·5^{b}	18·9	660·4^{b}	11·3	520·1^{a}	10·7	509·9^{a}	14·4	500·2^{b}	11·0	<0·0001	0·9685	0·3953
Food efficiency (g gain/g intake)	0·23^{a}	0·01	0·22^{a}	0·01	0·22^{a}	0·01	0·26^{b}	0·01	0·25^{b}	0·01	0·26^{b}	0·01	<0·0001	0·4388	0·7333
Total water consumption (g)	1628^{a}	81	1631^{a}	62	1626^{a}	63	1841^{a,b}	53	1859^{a,b}	111	2083^{b}	127	<0·0001	0·3215	0·3006

^{a,b,c} Mean values within a row with unlike superscript letters are significantly different ($P<0·05$, Tukey's HSD test).
* No diet treatment at the beginning of the experiment.

Table 4. Systolic and diastolic blood pressure of the rats

(Mean values with their standard errors)

	SHRSP rats						WKY-inbred rats						*P* value (two-way ANOVA)		
	Control diet (*n* 10)		Phytosterol diet (*n* 10)		Phytostanol diet (*n* 10)		Control diet (*n* 10)		Phytosterol diet (*n* 10)		Phytostanol diet (*n* 10)				
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Strain	Diet	Strain × diet
Initial systolic BP (mmHg)	121·1	2·7	120·8	2·4	122·7	3·9	112·0	3·3	115·0	3·7	111·4	3·4	0·0595 (one-way ANOVA)*		
Mean systolic BP at week 5 (mmHg)	160·9^{b}	5·5	158·8^{b}	4·6	163·6^{b}	6·7	134·2^{a}	3·6	132·1^{a}	2·6	136·6^{a}	4·1	<0·0001	0·6192	0·9994
Increased systolic BP (mmHg/5 weeks)	39·9^{b}	6·6	37·9^{a,b}	4·6	40·9^{b}	7·5	22·2^{a,b}	4·9	17·1^{a}	5·0	25·2^{a,b}	2·5	<0·0001	0·5905	0·8909
Initial diastolic BP (mmHg)	83·1	3·1	81·1	1·6	79·4	2·4	81·9	2·1	79·5	1·9	78·5	1·7	0·6856 (one-way ANOVA)*		
Mean diastolic BP at week 5 (mmHg)	93·1^{b}	2·0	101·8^{c}	2·4	107·2^{c}	3·6	88·4^{a,b}	2·5	84·7^{a}	2·2	88·6^{a,b}	2·1	<0·0001	0·0213^{B}	0·0139
Increased diastolic BP (mmHg/5 weeks)	9·9^{a,b}	3·9	20·8^{c}	3·1	27·8^{c}	2·7	6·5^{a}	1·8	5·2^{a}	2·7	10·1^{a,b}	2·0	<0·0001	0·0016^{A,B}	0·0302

BP, blood pressure. ^{A}control *v.* sterols; ^{B}control *v.* stanols; ^{C}sterols *v.* stanols ($P<0·05$, Tukey's HSD test).
^{a,b,c} Mean values within a row with unlike superscript letters are significantly different ($P<0·05$, Tukey's HSD test).
* No diet treatment at the beginning of the experiment.

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Table 5. Renal gene expression of the rats
(Mean values with their standard errors)

Gene	SHRSP rats						WKY-inbred rats						*P* value (two-way ANOVA)		
	Control diet (*n* 9)		Phytosterol diet (*n* 9)		Phytostanol diet (*n* 9)		Control diet (*n* 9)		Phytosterol diet (*n* 9)		Phytostanol diet (*n* 9)				
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Strain	Diet	Strain × diet
Agt	1·58[b]	0·05	1·70[b]	0·09	1·66[b]	0·08	1·00[a]	0·07	1·04[a]	0·04	0·98[a]	0·05	<0·0001	0·526	0·728
Renin	0·86[a,b,c]	0·05	0·76[a,b]	0·02	0·71[a]	0·05	1·00[b,c]	0·09	0·88[a,b,c]	0·07	1·01[c]	0·05	0·0003	0·188	0·260
Ace1	1·56[b]	0·05	1·52[b]	0·06	1·87[c]	0·11	1·00[a]	0·03	0·98[a]	0·05	0·96[a]	0·04	<0·0001	0·029[C]	0·006
Ace2	0·94	0·03	1·02	0·04	0·98	0·03	1·00	0·06	1·08	0·04	1·04	0·05	0·064	0·203	0·994
Agtr1a	0·91[a]	0·02	0·95[a]	0·03	1·02[a,b]	0·04	1·00[a,b]	0·02	1·09[b]	0·04	0·99[a,b]	0·02	0·007	0·071	0·016
Nos1	1·79[b,c]	0·12	2·06[c]	0·14	1·82[b,c]	0·16	1·00[a]	0·07	1·55[abc]	0·16	1·28[a,b]	0·16	<0·0001	0·017[A]	0·559
Nos3	1·26[b,c]	0·05	1·32[c,d]	0·05	1·51[d]	0·07	1·00[a]	0·05	1·04[a]	0·02	1·08[a,b]	0·05	<0·0001	0·005[B]	0·224
Cox2	1·17[a,b]	0·09	1·53[b]	0·09	1·57[b]	0·10	1·00[a]	0·05	1·20[a,b]	0·09	1·42[a,b]	0·16	0·014	0·001[A,B]	0·658
Spon1	0·96[a]	0·03	1·11[a,b]	0·05	1·08[a,b]	0·04	1·00[a]	0·04	1·25[b]	0·05	1·09[a,b]	0·07	0·117	0·0005[A]	0·379
Thumpd1	1·99[b]	0·07	2·06[b]	0·10	2·02[b]	0·11	1·00[a]	0·05	1·17[a]	0·05	1·06[a]	0·07	<0·0001	0·328	0·808

[A] Control *v.* sterols; [B]control *v.* stanols; [C]sterols *v.* stanols ($P<0·05$, Tukey's HSD test).
[a,b,c,d] Mean values within a row with unlike superscript letters are significantly different ($P<0·05$, Tukey's HSD test).

and WKY rats. The higher levels of phytosterols present in rapeseed oil compared with soyabean oil[15,26] were speculated to contribute to this increased blood pressure.

In order to elucidate the molecular mechanism(s) by which phytosterols and phytostanols resulted in an increase in the blood pressure in SHRSP rats, the expression of several renal genes known to be involved in blood pressure regulation were investigated (Table 5). We report for the first time that phytosterols and/or phytostanols up-regulated the mRNA expression of *Ace1*, *Nos1*, *Nos3*, *Cox2* and *Spon1* in SHRSP rats. We also found that SHRSP rats have higher mRNA levels of *Agt*, *Ace1*, *Nos1*, *Nos3*, *Cox2* and *Thumpd1* and lower mRNA levels of *renin* and *Agtr1a* compared with WKY *inbred* rats.

The renin–angiotensin–aldosterone system (RAAS) is a hormone system that helps regulate long-term blood pressure and extracellular volume in the body[28]. In response to stimuli, the kidneys release the enzyme renin, which converts *Agt* into angiotensin I, and the latter is converted to angiotensin II by angiotensin I-converting enzyme 1 (*Ace1*). Angiotensin II, the major bioactive product of the RAAS, reacts mainly with angiotensin II receptor (*Agtr*) and results in increased blood pressure by mediating a powerful vasoconstrictor effect, leading to aldosterone secretion and sodium reabsorption in the kidney, and stimulating sympathetic activity[28]. In the present study, SHRSP rats had significantly higher mRNA expression of *Agt* ($P<0·0001$) and *Ace1* ($P<0·0001$) than WKY inbred rats. In addition, phytostanol supplementation further up-regulated the *Ace1* mRNA expression in SHRSP but not in WKY inbred rats. Elevation of the *Agt* and *Ace1* mRNA levels of SHRSP rats in the present study was consistent with the study by Obata *et al.* [29], who reported that SHRSP rats had significantly higher mRNA expression of *Agt* and *Ace* than WKY rats. These results are also supported by Takemori[30], who demonstrated that SHRSP rats had higher plasma angiotensin II levels than WKY rats. Therefore, the increased expression of these genes may contribute, at least in part, to the pathogenesis of hypertension in SHRSP rats (Fig. 1).

The present study also found that SHRSP rats had slightly lower mRNA levels of *renin* (1·16-fold, $P=0·0003$) and *Agtr1a* (1·10-fold, $P=0·007$) than WKY inbred rats. These results are not consistent with those of Obata *et al.* [29], who demonstrated a higher mRNA expression of *Agtr1a* in SHRSP than WKY rats. While the reason for this difference is unclear, it may be related to the different tissue samples used to isolate total RNA in these studies (whole kidney in the present study *v.* >95 % glomeruli in the study of Obata *et al.* [29] or the different methods used for measuring mRNA expression (real-time QPCR in the present study *v.* RNase protection assay in the study of Obata *et al.* [29]). Based on the present results, it is possible that SHRSP rats down-regulate the *renin* and *Agtr1a* expression in order to compensate for the up-regulation of *Agt* and *Ace1*. However, this compensatory mechanism is not sufficient to prevent the increase in blood pressure when compared with WKY inbred rats.

A reduced bioavailability of nitric oxide (NO) has also been postulated to contribute to the development of hypertension[31]. NO is biosynthesised from arginine and oxygen by a family of Nos, including neuronal Nos (Nos1 or nNos), inducible Nos (Nos2 or iNos) and endothelial Nos (Nos3 or eNos).

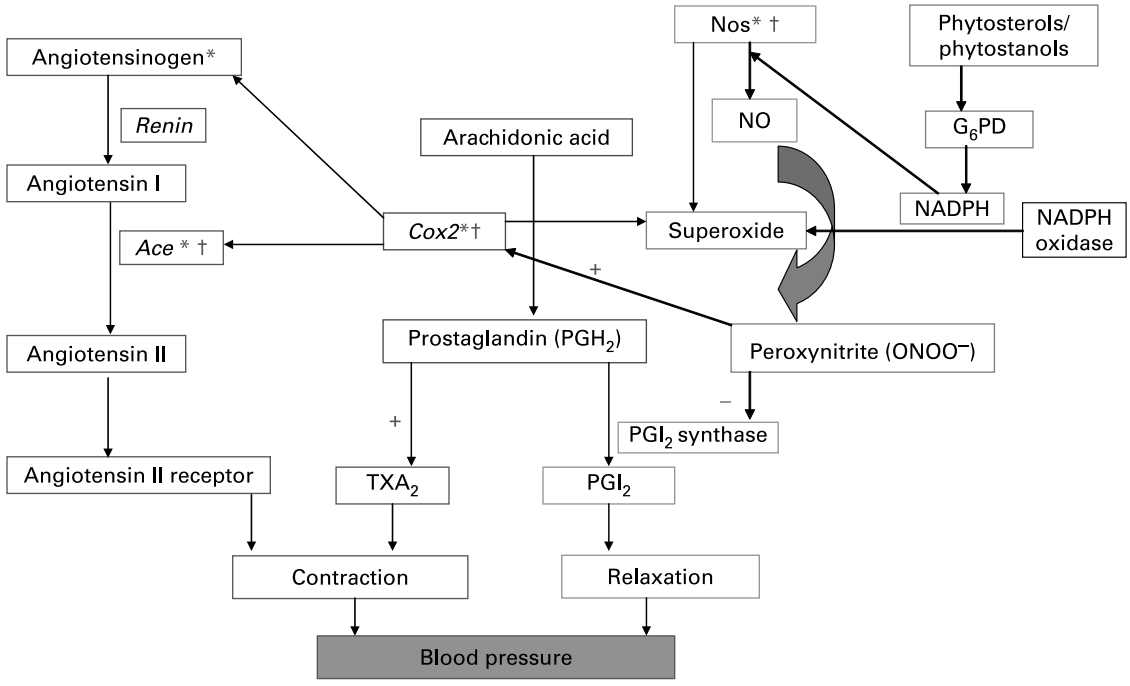

Fig. 1. Summary of the proposed mechanisms by which phytosterols and phytostanols exacerbate the development of hypertension in SHRSP rats. Overall, ingestion of high levels of phytosterols and phytostanols may exacerbate the development of hypertension in SHRSP rats by directly or indirectly activating RAAS, stimulating excessive peroxynitrite formation, increasing PGH_2/TXA_2 activity and decreasing the availability of NO and PGI_2. +/−, activated/inhibited by $ONOO^-$; *, elevated in SHRSP rats in the present study; †, up-regulated by phytosterols or phytostanols in the present study.

In the present study, SHRSP rats had higher mRNA expression of *Nos1* ($P<0{\cdot}0001$) and *Nos3* ($P<0{\cdot}0001$) than WKY inbred rats. Also, phytosterol and phytostanol supplementation up-regulated the mRNA expression of *Nos1* and *Nos3*, respectively. However, in addition to synthesising NO, Nos can also generate superoxide[32], which has been found to react with NO to produce peroxynitrite ($ONOO^-$)[33]. Thus, increased scavenging of NO by superoxide could lead to a decrease in NO availability despite increased synthesis (Fig. 1). The present results and the proposed explanation are supported by the studies from Kerr *et al.* [34] and others[35,36], who reported that *Nos3* mRNA expression and superoxide generation were significantly higher in SHRSP than in WKY counterparts.

In the present study, SHRSP rats had significantly higher *Cox2* mRNA expression than WKY inbred rats and both phytosterol and phytostanol supplementation increased *Cox2* mRNA levels. Prostanoids (including PG, prostacyclin and thromboxane (TXA_2)) have been demonstrated to play an important role in the regulation of blood pressure[37]. Cox (also known as PGH_2 synthase) converts arachidonic acid to PGH_2, the precursor of the series-2 prostanoids that cause dilation (such as PGI_2) or constriction (such as TXA_2) in vascular smooth muscle cells. Peroxynitrite, produced from a reaction of NO with superoxide (as mentioned earlier), has been shown to activate Cox[38], causing the accumulation of PGH_2, which was able to activate the TXA_2/PGH_2 receptor and mediate smooth muscle contraction[39]. Peroxynitrite has also been shown to be a potent oxidant[40], which inhibits PGI_2 synthase[39,40] leading to a decrease in PGI_2 and subsequently reducing muscle relaxation. *Cox* enzyme itself can also generate superoxide[41,42], which may further promote peroxynitrite formation. Excessive expression of *Cox2* may also stimulate RAAS, leading to increased arterial pressure and vascular lesions[43]. Therefore, excessive peroxynitrite formation from the reaction of NO with superoxide may contribute to hypertension in SHRSP rats by decreasing the availability of NO and PGI_2, and increasing vasoconstrictors, PGH_2/TXA_2 and RAAS (Fig. 1).

The observed phytosterol and phytostanol up-regulation of *Cox2* mRNA expression in the present study was supported by the results from Ohara *et al.* [44], who reported that rapeseed oil increased Cox2 protein expression in SHR rats, when compared with soyabean oil. Recent studies have also shown that ingestion of rapeseed oil significantly increased glucose-6-phosphate dehydrogenase activity in SHR[44] and WKY[27] rats, compared with soyabean oil. Glucose-6-phosphate dehydrogenase, the first enzyme in the pentose phosphate pathway, provides NADPH for the generation of NO by Nos[45,46] and for the generation of NADPH oxidase-derived superoxide[47,48]. As mentioned earlier, superoxide reacts with NO leading to a decrease in NO availability and the generation of peroxynitrite. These data suggest that NADPH production by glucose-6-phosphate dehydrogenase in response to phytosterols and phytostanols could contribute to the mechanisms by which NO availability is diminished and excessive peroxynitrite is produced, leading to an increase in blood pressure (Fig. 1). This proposed mechanism is also supported by the studies which showed that rapeseed oil lowered the activities of anti-oxidative enzymes, superoxide dismutase and catalase in SHR[44] and WKY rats[27].

Recently, Clemitson *et al.* [49] identified *Spon1* as a candidate hypertension gene. They reported that the mRNA levels of *Spon1* in the kidney, heart and aorta were significantly

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higher in SHR than in WKY rats. In the present study, strain effects on *Spon1* expression in the kidney were not observed between SHRSP and WKY inbred rats ($P=0{\cdot}117$). However, phytosterol supplementation up-regulated renal *Spon1* mRNA levels in both SHRSP and WKY inbred rats. Therefore, the role of *Spon1* in hypertension is unclear and its significance in phytosterol/phytostanol-induced blood pressure elevation warrants further study.

Thumpd1 was also recently identified as a candidate hypertension gene[49,50] and was shown to have higher mRNA expression in hypertensive SHR rats compared with normotensive Brown Norway rats. Similarly, we found that SHRSP rats have a significantly higher mRNA expression of *Thumpd1* (1·99-fold, $P<0{\cdot}0001$) than WKY inbred rats; however, diet effects were not demonstrated. Our data suggest that although *Thumpd1* may contribute to hypertension in SHRSP rats, it does not appear to be involved in the phytosterol/phytostanol-induced blood pressure increase in SHRSP rats.

In conclusion, we determined that SHRSP rats have higher systolic and diastolic blood pressures compared with WKY inbred rats. SHRSP rats fed phytosterol- or phytostanol-supplemented diets demonstrated a significant increase in the diastolic blood pressure compared with rats fed the control diet. Increased blood pressure in SHRSP rats may be due, in part, to altered renal expression of blood pressure regulatory genes. Specifically, the up-regulation of *Ace1*, *Nos1*, *Nos3*, *Cox2* and *Spon1* is associated with the increased diastolic blood pressure observed in the phytosterol- or phytostanol-supplemented SHRSP rats. Overall, we speculate that ingestion of high levels of phytosterols and phytostanols may exacerbate the development of hypertension and related conditions in SHRSP rats by directly or indirectly activating RAAS, stimulating excessive peroxynitrite formation, increasing PGH_2/TXA_2 activity and decreasing the availability of NO and PGI_2. Therefore, it would be prudent to determine whether foods fortified with high levels of phytosterols or phytostanols may have a potential health risk to individuals predisposed to hypertension.

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