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Antihypertensive and cognitive effects of grape polyphenols in estrogen-depleted, female, spontaneously hypertensive rats

Ning Peng,¹ John T. Clark,² Jeevan Prasain,³ Helen Kim,³ C. Roger White,⁴ and J. Michael Wyss^{1,4}

Departments of ¹Cell Biology, ³Pharmacology, and ⁴Medicine, University of Alabama at Birmingham, Birmingham, Alabama; and ²Department of Physiology, Meharry Medical College, Nashville, Tennessee

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Peng, Ning, John T. Clark, Jeevan Prasain, Helen Kim, C. Roger White, and J. Michael Wyss. Antihypertensive and cognitive effects of grape polyphenols in estrogen-depleted, female, spontaneously hypertensive rats. *Am J Physiol Regul Integr Comp Physiol* 289: R771–R775, 2005; doi:10.1152/ajpregu.00147.2005.—Both endogenous and dietary estrogens reduce hypertension and enhance cognitive abilities in estrogen-depleted female spontaneously hypertensive rats (SHR). Many of the beneficial effects of estrogens/phytoestrogens also appear to be provided by other polyphenols (e.g., proanthocyanidins) in grape seed, which lack appreciable estrogenic receptor binding. The present study tested the hypothesis that similar to phytoestrogens, proanthocyanidins in grape seed polyphenols reduce salt-sensitive hypertension in young, estrogen-depleted SHR. SHR were ovariectomized at 4 wk of age and placed on phytoestrogen-free diets with or without 0.5% grape seed extract added and with high (8.0%) or basal (0.6%) NaCl. After 10 wk on the diets, grape proanthocyanidin supplementation significantly reduced arterial pressure in the rats fed the basal (10 mmHg) and high (26 mmHg)-NaCl diet, compared with the nonsupplemented controls. In vitro superoxide production was significantly reduced (23%) by the grape seed polyphenols. Spatial learning (8-arm-radial maze) in the SHR on the basal NaCl diets was improved by dietary grape seed polyphenols. These results indicate that grape seed polyphenols decrease arterial pressure in SHR, probably via an antioxidant mechanism.

blood pressure; antioxidant; learning; memory

CARDIOVASCULAR DISEASE, including stroke, is the greatest cause of morbidity and mortality in the world, and it can lead to cognitive impairment. Before menopause, women have significantly lower arterial pressure and risk of cardiovascular disease than age-matched men; however, after menopause, women rapidly lose their gender-related advantage, and their rates of hypertension, cardiovascular disease, and stroke become equal or greater than those of age-matched men. This acceleration in the development of cardiovascular disease in postmenopausal women likely relates to a postmenopausal decline in sex steroids, especially estrogen. Hormone replacement therapy has often been suggested as a method to blunt these effects. However, in the wake of the findings of the Women's Health Initiative, the clinical use of hormone replacement therapy for the prevention/reduction of cardiovascular disease has been largely discontinued due to the reported increased health risks associated with it (see e.g., Refs. 12, 20, 30). As an alternative, many women have searched for a safer therapy to combat the adverse effects of menopause, and a growing number of women have turned to botanical products that contain estrogen-like compounds.

Phytoestrogens are plant-derived polyphenols that structurally and functionally mimic mammalian estrogens and appear to provide protection from cardiovascular disease (5, 9, 17, 28). Previous work in our laboratory demonstrates that soy phytoestrogens can protect estrogen-depleted spontaneously hypertensive rats (SHR) from NaCl-sensitive hypertension (11, 29). In contrast to age-matched male SHR, young female SHR are resistant to the hypertensive effects of a high-NaCl diet (8). Depletion of most of the endogenous estrogen (via ovariectomy) produces a modest hypertensive response to a high-NaCl diet (<10 mmHg), but depletion of both endogenous and exogenous estrogens (phytoestrogens) results in a much greater hypertensive response to the high-NaCl diet (11). Although these effects have typically been attributed to the estrogenic effect of the phytoestrogens, other groups have suggested that the effects may be related to nonestrogen receptor-mediated effects on superoxides or nitric oxide (NO) availability (15).

Other classes of polyphenols have antihypertensive effects, but many of these appear to act through mechanisms other than the estrogen receptor. For instance, tea catechins reduce arterial pressure in male stroke-prone SHR, apparently via antioxidant properties (26). Grape seed polyphenols also have several beneficial health effects, most notably, anticancer (1, 33) and vasorelaxant effects (3, 22), but again, grape polyphenols do not appear to bind to either α - or β -estrogen receptors.

Grapes (*Vitis vinifera*) are one of the most widely consumed fruits worldwide and are rich in polyphenols. Grape seed extract is composed of ~90% proanthocyanidins (i.e., dimers, trimers, and other oligomers of flavan-3-ols) and 7% other polyphenols (flavonoids, including catechin, epicatechin, gallocatechin, epigallocatechin, and epicatechin 3-O-gallate; unpublished from Kikkoman, Chiba, Japan). Although the mechanism of the beneficial health effects of grape seed polyphenols remains unclear, several lines of evidence strongly suggest that they do not act via estrogen receptor binding (1, 10, 33). Grape seed polyphenols are powerful antioxidants with greater potency than vitamin E and C (3), and upregulation of reactive oxygen species appears to play an important role in some forms of cardiovascular diseases, including hypertension (see e.g., Refs. 18, 23, 42).

The present study investigated whether grape seed polyphenols had antihypertensive and cognitive enhancement effects similar to other polyphenols and whether this effect was likely mediated via an antioxidant mechanism. A positive finding would suggest that nonestrogenic polyphenols may be beneficial to cardiovascular and cognitive function and that the

Address for reprint requests and other correspondence: J. M. Wyss, 1900 Univ. Blvd./THT 950, Dept. of Cell Biology, Univ. of Alabama at Birmingham, Birmingham, AL 35294-0006 (e-mail: jmwys@uab.edu).

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mechanism underlying the beneficial health effects of phytoestrogenic polyphenols may be related, at least in part, to characteristics other than their estrogenic binding properties. The results are the first demonstration that dietary grape seed polyphenols blunt hypertension and cognitive decline in an animal model.

METHODS

One-month-old female SHR ($n = 32$, Harlan Sprague-Dawley) were ovariectomized and allowed ad libitum access to one of four diets ($n = 8$ /group except in the high-NaCl diet group, in which two rats had strokes at 9–10 wk on the diets and were, therefore, eliminated from the study): a phytoestrogen-free diet (PE⁻, AIN 76A diet) containing either basal (0.6%) or high (8%) NaCl and 0.0 or 0.5% grape seed extract (Kikkoman) (40). The NaCl and polyphenols were added to the powdered diet by overnight blending in a rotating mixer and subsequent pelleting. Food intake measurements demonstrated that the rats in the appropriate control and experimental groups ate approximately the same amount of salt and polyphenols. Rats were housed three per cage at constant humidity ($65 \pm 5\%$), temperature ($24 \pm 1\%$), and light-dark cycle (0600–1800, lights on), and allowed ad libitum access to tap water and diet throughout the experimental protocols. All experimental procedures were conducted in accordance with Institutional Animal Care and Use Committee of the University of Alabama, Birmingham, and National Institutes of Health guidelines.

Measurement of the blood pressure and heart rate. At 9 wk of age, all rats were anesthetized with isoflurane, and each rat was instrumented with an implantable telemetry probe (TA11PA-C40; Data Sciences, St. Paul, MN) for arterial pressure and heart rate measurements (7). The catheter was inserted into the abdominal aorta immediately caudal to the renal arteries, and the body of transmitter was sutured to the inside of the anterior abdominal wall. The rats were allowed at least 1 wk to recover from the operation and were housed in their individual cages throughout the experiment. At 10 wk of age (6 wk on the diets), each animal's cage was placed on a receiver panel for recording data by the Dataquest IV software system (Data Sciences). Arterial pressure, heart rate, and activity for each rat were continuously recorded every 5 min for 4 wk. The blood pressure, heart rate, and activity were analyzed, and the mean arterial pressure and heart rate and activity were calculated using Pharmfit (21).

Behavior test. After 10 wk on the diets, the rats in the basal NaCl diet groups were tested in an eight-arm-radial maze cognitive task as previously reported (38). In brief, the rats were deprived to 85% body weight and fed once per day to maintain them at that weight. After the rats attained the 85% body weight (3 days), before each day's feeding, the rats were run in an eight-arm maze task. After an initial 2 days of training to acclimate the rats to the apparatus, the spatial learning task was initiated. For the task, six of the eight arms were baited each day with one-half of a Froot Loop (the same arms were always baited for an individual rat). The task for the rat was to gain the reward at the end of the six runways without reentering an already visited runway (working memory error) or entering an unbaited runway (reference memory, error). The working memory correlates with short-term memory, and the reference memory is more closely related to long-term memory. A 30-s delay was imposed between each trial to prevent the rats from using proprioceptive cues to solve the maze. All rats were run in the maze until they made correct choices on five out of the first six trials of the day for 4 consecutive days. The rats were run for either eight trials/day or until all baited arms were visited, whichever came first. In past studies, we have found that this task is very sensitive to subtle changes in spatial learning and memory capacity. Furthermore, the task differentiates long- vs. short-term memory deficits (38).

Measurement of vascular superoxide anion production. To better understand the underlying mechanism by which grape seed polyphenols

decrease arterial pressure, we treated animals on a high-NaCl diet with or without grape seed supplementation for 6 wk, as above ($n = 6$ per group). Superoxide formation was measured in vitro in the aorta of SHR with coelenterazine-dependent chemiluminescence (35). The $O_2^{\cdot-}$ -dependent oxidation of coelenterazine results in the formation of a high energy intermediate, which emits light as it relaxes to the ground state. Aortas were excised from SHR, the luminal surface of the artery was exposed, and tissue segments ($\approx 16 \text{ mm}^2$) were cut and placed in cell culture wells containing PBS. Basal $O_2^{\cdot-}$ production was monitored on a luminometer (BMG Labtechnologies). At the beginning of each experiment, coelenterazine was injected into each well to yield a final concentration of $10 \mu\text{M}$. $O_2^{\cdot-}$ -dependent chemiluminescence increased with time and stabilized after ~ 3 – 5 min. Photon emission was measured during this plateau phase. The assay was calibrated by monitoring the chemiluminescence signal of known amounts of $O_2^{\cdot-}$ generated by xanthine ($50 \mu\text{M}$) and xanthine oxidase (0.1 – 0.5 mU/ml). Rates of $O_2^{\cdot-}$ production associated with these xanthine/xanthine oxidase incubation conditions were determined spectrophotometrically by measuring the $O_2^{\cdot-}$ -dependent reduction of ferricytochrome *c*. Chemiluminescence signals measured in isolated aortic tissues were then converted to rates of $O_2^{\cdot-}$ formation and normalized to tissue weight.

Statistics. All data from experiments were evaluated by two-way analysis of variance followed by post hoc Tukey's test to determine the source of main effects and interactions (SPSS, Chicago, IL). The significance criterion for all experiments was $P < 0.05$.

RESULTS

Blood pressure control. In SHR on the basal-NaCl diet for 6 wk, the grape seed supplementation (compared with control diet) decreased 24-h mean arterial pressure from 142 ± 2 to $129 \pm 1 \text{ mmHg}$ ($P < 0.05$, Fig. 1). Exposure to the high-NaCl diet during the initial 6 wk increased mean arterial pressure in both groups, but the polyphenols-supplemented rats continued to have significantly lower arterial pressures than the non-supplemented rats (175 ± 2 vs. 189 ± 4 , $P < 0.05$, Fig. 1).

After 10 wk on the diets, in the SHR fed a basal NaCl diet, the grape polyphenols supplementation continued to lower arterial pressure by $\sim 10 \text{ mmHg}$ (139 ± 1 vs. $149 \pm 1 \text{ mmHg}$, $P < 0.05$, Fig. 2). Maintenance on the high-NaCl diets for 10 wk increased arterial pressure in both groups, but the increase was significantly greater in the nonsupplemented SHR (179 ± 2 vs. $205 \pm 3 \text{ mmHg}$, Fig. 2). Heart rates were not differentially altered by any of the diets (average heart rate was 340

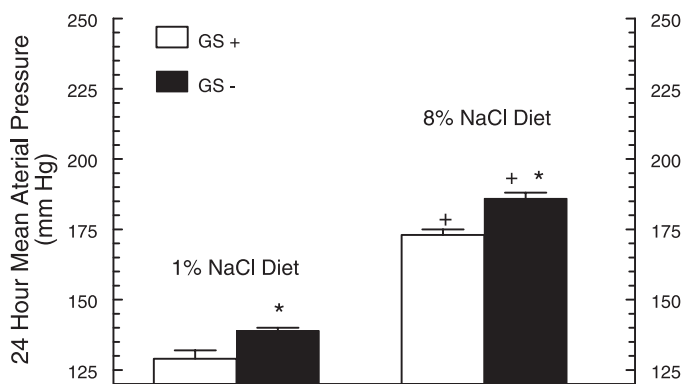


Fig. 1. Bar graph demonstrating the 24-h, average mean arterial pressure of the spontaneously hypertensive rats (SHR) after 6 wk on the 4 diets (\pm SE). * $P < 0.05$ compared with grape seed (GS)-supplemented rats on same NaCl diet; + $P < 0.05$ compared with SHR on the basal NaCl diet that received the same supplement.

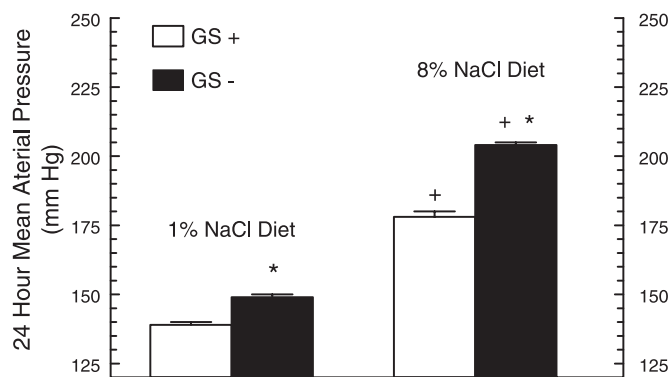


Fig. 2. Bar graph demonstrating the 24-h, average mean arterial pressure of the SHR after 10 wk on the 4 diets (\pm SE). * $P < 0.05$ compared with GS-supplemented rats on same NaCl diet; + $P < 0.05$ compared with SHR on the basal NaCl diet that received the same supplement.

beats/min). Furthermore, the diets did not differentially alter final body weights in these rats (0.6% NaCl with/without grape seed extract = 279 ± 4.7 g/ 277 ± 6.8 g; 8.0% with/without grape seed extract = 275 ± 5 g/ 273 ± 7 g).

Behavior. Over 50% of the SHR on the nonsupplemented, high-NaCl diet died or were stroke-compromised at the start of the learning component of the study. Therefore, although none of the supplemented SHR displayed signs of impaired health, we did not include either group on the high-NaCl diet in the learning arm of the study.

In the eight-arm-radial maze test, both groups learned the task, but the group receiving polyphenols supplementation reached criterion in significantly fewer days (Fig. 3) and with significantly fewer working (35 ± 2 vs. 42 ± 1) and fewer reference (30 ± 2 vs. 40 ± 3) memory errors. The learning curves were not significantly different during the initial 10 days of testing, i.e., before any rats reaching the criterion (not shown).

Superoxide production. In two additional groups of SHR fed a high-NaCl diet, a 6-wk grape seed extract supplementation significantly decreased in vitro aortic superoxide production. The maximum arterial superoxide production difference between two groups was 0.57 ± 0.017 vs. 0.44 ± 0.014 nM per minute per milligram tissue (Fig. 4)

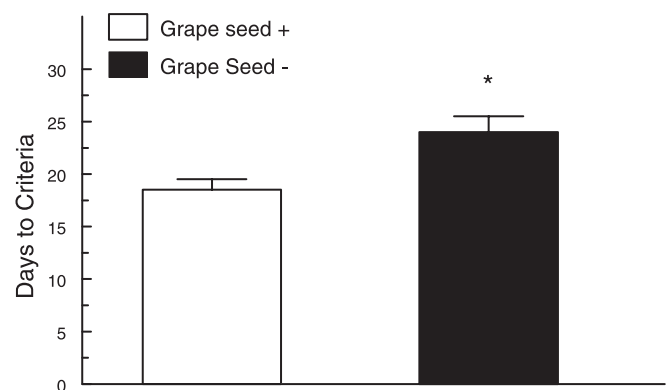


Fig. 3. Days to criterion in the 8-arm-radial-maze task. * $P < 0.05$ compared with polyphenol-supplemented group.

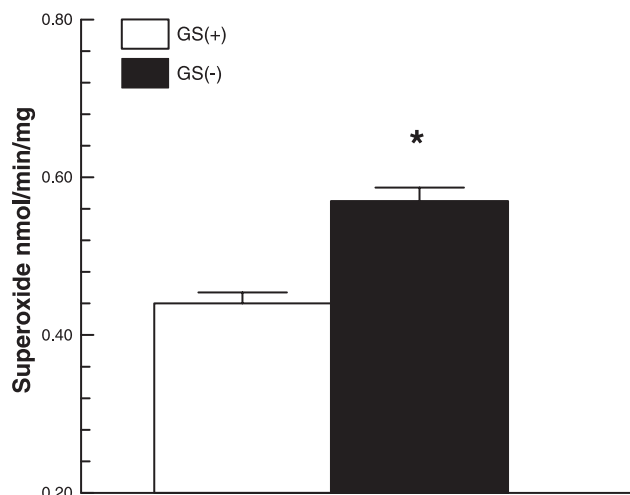


Fig. 4. In vitro measurement of superoxide anion formation in aortic ring segments of SHR rats treated with vehicle or GS extract. The assay was calibrated by monitoring the chemiluminescence signal of known amounts of O_2^- generated by xanthine + xanthine oxidase. Data are expressed as means \pm SE. * $P < 0.05$ compared with GS-supplemented group.

DISCUSSION

Polyphenols (e.g., soy phytoestrogens) are increasingly used by postmenopausal women for cardiovascular protection. Compared with women from Western societies, Asian women have significantly lower rates of cardiovascular disease and mortality, but cardiovascular disease increases in Asian women when they shift to a Western diet. For example, in contrast to the postmenopausal rise in blood pressure in Western women, there is little change in arterial pressure after menopause in Japanese women (2). The Asian women's intake of dietary polyphenols has been suggested to play a significant role in this health benefit.

Dietary intake of polyphenols is associated with a lower blood pressure and reduced incidence of cardiovascular disease and cognition dysfunction (14, 24, 27). These beneficial effects have typically been attributed to estrogenic effects of many of these substances; however, polyphenols, like the proanthocyanidins in grapes, lack appreciable estrogenic effects, and thus their beneficial effects appear to be attributable to other mechanisms, e.g., inhibition of low-density lipoproteins oxidation (e.g., see Refs. 25, 32, 34, 37).

Our previous studies indicate that polyphenols from soy protect against NaCl-sensitive hypertension in ovariectomized SHR. Ovariectomy or removal of polyphenols from the diet only modestly increases the arterial pressure of SHR on a basal-NaCl diet, (<15 mmHg), but simultaneous removal of both sources of estrogen leads to large increases in arterial pressure (>40 mmHg) that are even greater than that seen in male SHR on a high-NaCl diet (typically 30 mmHg) (6, 11, 29). We have traditionally attributed such effects to the estrogenic action of the phytoestrogens in soy. The current results demonstrate that grape proanthocyanidins have a similar antihypertensive effect; however, the response is somewhat altered. Whereas soy isoflavones have negligible effect on arterial pressure in ovariectomized SHR on a basal NaCl diet, the grape polyphenols reduce arterial pressure in such rats by about >10 mmHg. Furthermore, in ovariectomized SHR on a high-NaCl diet, soy isoflavones have a greater early effect than

grape seed polyphenols (>20 vs. 10 mmHg decrease after 6 wk on polyphenol supplement), but by 10 wk, the antihypertensive effects of the two polyphenols are similar (>20 mmHg) (11).

We believe that the grape seed polyphenols responsible for the action of the dietary supplement are largely proanthocyanidins (oligomeric catechins). Nearly all (90%) of the content of the extract is in the form of oligomeric catechins, whereas only 6% is in the form of monomeric catechins (19). Humans and rats absorb B1 and B2 procyanidins into their blood (4, 31). We have detected proanthocyanidins up to tetrameric in size in grape seed extract using electrospray ionization mass spectrometry analysis, but the metabolic fate of these larger oligomers remains to be determined. We should also note that the concentration of grape seed extract used in the present study is significantly below those that will compromise the health of rats (41), and the rats on the grape seed supplement (vs. the nonsupplemented rats) did not display lower body weight.

The precise mechanisms by which the grape seed proanthocyanidins affect arterial pressure and cognition remain to be uncovered; however, data from the current vessel superoxide production experiment indicate that grape polyphenol supplement significantly reduces vessel superoxide production in SP-SHR fed a high-NaCl diet. This indicates that the beneficial effects of grape polyphenols may result from their ability to decrease superoxide production *in vivo*. Together, this suggests that the search for the mechanism by which polyphenols are protective should reach beyond their estrogenic properties.

Several studies suggest that hypertension is associated with a decrease in learning and memory in rats (see e.g., Refs. 13, 36, 38). In our previous studies, young male SHR were equal or better than nonhypertensive controls in spatial learning; however, by 12 mo of age they showed markedly impaired spatial learning. In contrast, if these SHR received chronic antihypertensive treatment with angiotensin converting enzyme, they displayed unimpaired learning at 12 mo of age (38). Similarly, Dahl hypertensive rats display spatial learning deficits that are ameliorated by antihypertensive therapy (13, 36). Together this suggests that hypertension *per se* can decrease learning in rats. However, our recent studies show that in Sprague-Dawley rats, chronic, severe hypertension (aortic coarctation) for 12 mo has no significant effect on spatial learning (16). Treatment of aging normotensive rats with angiotensin-converting enzyme inhibitors improves memory despite negligible effects on arterial pressure (39), and chronic antihypertensive treatment of SHR with hydralazine does not improve their age-related memory impairment (39). Thus it seems unlikely that in the present study the reduction of arterial pressure by 10 mmHg is the major factor underlying the improved spatial learning performance of the supplemented rats. It also should be noted that the rats used in the present study are only young adults, and normally, at this age, SHR display spatial learning that is equal or better than that of normotensive control rats (38). Normally, the SHR learning impairment is not present until ~1 yr of age (38, 39). Thus the effect of grape seed polyphenols is likely to be an enhancement effect. Whether this is due to the polyphenol's effects on oxygen radicals or NO in the brain and/or cerebrovasculature or a more direct impact of the polyphenols on neuronal function must await further studies. However, preliminary results

indicate that the orally administered grape proanthocyanidins can access the brain in this model.

The similar effect of the grape polyphenols on working and reference memory errors indicates that both short-term (working memory) and long-term (reference memory) were beneficially and nearly equally enhanced by the grape seed polyphenols; however, a more formal test of this hypothesis, using other indexes of these forms of memory are needed before the effect can be fully interpreted.

Compared with their premenopausal counterparts, postmenopausal women are at relatively high risk for heart disease, hypertension, and cognitive impairment. The standard therapy of a decade ago promoted the pharmaceutical replacement of the lost hormones in these women; however, extensive studies have demonstrated that long-term use of hormone replacement therapy is associated with significant adverse effects in many women. Dietary polyphenols appear to provide some of the beneficial effects of hormone replacement therapy, without appreciable adverse effects. Thus in the present study, the nonestrogenic polyphenols in grape provided protection for arterial pressure and cognition, likely, at least in part, via an inhibition of superoxide production. Whereas it is unlikely that such polyphenols will provide effective monotherapy for postmenopausal women, they may provide important adjuvant therapy that complements the use of lower doses of traditional pharmaceutical compounds.

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