Dietary approach to attenuate oxidative stress, hypertension, and inflammation in the cardiovascular system

Lingyun Wu*, M. Hossein Noyan Ashraf†, Marina Facci*, Rui Wang†, Phyllis G. Paterson‡, Alison Ferrie§, and Bernhard H. J. Juurlink*¶

Departments of *Anatomy and Cell Biology and †Physiology, College of Medicine, and ‡College of Pharmacy and Nutrition, University of Saskatchewan, Saskatoon, SK, Canada S7N 5E5; and §Plant Biotechnology Institute, National Research Council, Saskatoon, SK, Canada SW9 OW9

Communicated by Paul Talalay, The Johns Hopkins University School of Medicine, Baltimore, MD, March 22, 2004 (received for review October 17, 2003)

Imbalance between production and scavenging of superoxide anion results in hypertension by the inactivation of nitric oxide, and the increased oxidative stress from the resultant peroxynitrite that is produced promotes inflammatory processes such as atherosclerosis. Induction of phase 2 proteins promotes oxidant scavenging. We hypothesized that intake of dietary phase 2 protein inducers would ameliorate both hypertension and atherosclerotic changes in the spontaneously hypertensive stroke-prone rat. For 5 days/week for 14 weeks, we fed rats 200 mg/day of dried broccoli sprouts that contained glucoraphanin, which is metabolized into the phase 2 protein-inducer sulforaphane (Group A), sprouts in which most of the glucoraphanin was destroyed (Group B), or no sprouts (Group C). After 14 weeks of treatment, no significant differences were seen between rats in Groups B and C. Rats in Group A had significantly decreased oxidative stress in cardiovascular and kidney tissues, as shown by increased glutathione (GSH) content and decreased oxidized GSH, decreased protein nitrosylation, as well as increased GSH reductase and GSH peroxidase activities. Decreased oxidative stress correlated with better endothelial-dependent relaxation of the aorta and significantly lower (20 mm Hg) blood pressure. Tissues from Groups B and C had considerable numbers of infiltrating activated macrophages, indicative of inflammation, whereas animals in Group A had few detectable infiltrating macrophages. There is interest in dietary phase 2 protein inducers as means of reducing cancer incidence. We conclude that a diet containing phase 2 protein inducers also reduces the risk of developing cardiovascular problems of hypertension and atherosclerosis.

Oxidative stress has been implicated as a causal factor in diseases such as hypertension (1–3) and atherosclerosis (4). Increased production, or decreased scavenging, of oxidants such as superoxide anion can give rise to hypertension by superoxide interacting with nitric oxide, forming peroxynitrite (5–7), whereby decreasing nitric oxide availability for smooth muscle relaxation function. The peroxynitrite formed is a strong oxidant that promotes inflammatory processes by activating transcriptional factor complexes such as NF-κB (8). Such inflammatory processes result in the formation of atherosclerotic lesions (9). In principle, decreasing oxidative stress and associated inflammation should provide the beneficial effect of preventing and/or inhibiting the development of these cardiovascular diseases.

Central in scavenging strong oxidants is the tripeptide glutathione (GSH) (10). Indeed, GSH depletion alone will result in development of severe hypertension (11). We have shown (12) that smooth muscle cells from spontaneously hypertensive rats (SHR), in comparison with those from normotensive rats, have increased oxidative stress that was correlated with lower GSH levels. We have also shown that increasing GSH content in SHR smooth muscle cells greatly decreased the oxidative stress in these cells (12). We decreased oxidative stress by exposing the cells to the phase 2 protein inducer sulforaphane. Sulforaphane (4-methylsulfinylbutyl isothiocyanate) is the most potent phase 2 protein inducer found in our food sources (13). A major action of phase 2 proteins, mainly enzymes, is to inactivate electrophiles and strong oxidants (14); hence, there has been a research interest in dietary phase 2 protein inducers as a means to prevent cancers (15).

**Methods**

We have argued that dietary phase 2 protein inducers should ameliorate conditions such as hypertension and atherosclerosis (16). The objective of the present research was to test this idea. The animal model chosen was the stroke-prone SHR (SHRsp). This strain develops severe hypertension (17) that is related to increased peroxynitrite formation (6) and develops inflammatory changes in blood vessels (18, 19). We used Sprague–Dawley (SD) rats as the normotensive controls. We chose broccoli sprouts as the source of phase 2 protein inducer because certain cultivars of broccoli have been shown to be high in the glucosinolate (GS) glucoraphanin (Grn) that is metabolized into the phase 2 protein-inducing isothiocyanate sulforaphane (13). GS are β-thioglucoside N-hydroxysulfates present in 16 families of higher plants with the distribution in the family Brassicaceae, particularly the genus Brassica, which is well described in ref. 20. Grn is not available in sufficient quantities to perform dietary experiments. We, therefore, chose to feed animals air-dried broccoli sprouts of the calabrese cultivar that we had determined were high in Grn and low in antinutritive GS. As a placebo control, we fed animals with broccoli sprouts that were frozen, thawed, and dried. This procedure allowed the myrosinase enzyme in the sprouts to access the GS and hydrolyze them (21). The basic difference between the two forms of dried sprouts is the presence of GS in the former and low levels of GS in the latter.

**Supporting Information.** For additional details on methods, see Supporting Methods, which is published as supporting information on the PNAS web site.

**Broccoli Sprout Preparation and GS Measurements.** Broccoli seeds, obtained from Mumm’s Seeds and Sprouting (Shellbrook, Saskatoon, SK, Canada), were sprouted according to the supplier’s instructions and screened for GS content by using HPLC, as outlined in ref. 22. For calibration purposes, a small amount of Grn was obtained from Ian McGregor (Agriculture and Agrifood Canada, Saskatoon Research Station, SK, Canada). The calabrese cultivar was selected because of its major Grn peak.
and low levels of antinutritive GS. We either air-dried 4-day broccoli sprouts at room temperature for 7 days before feeding the animals, or we froze, thawed, and dried them at 60°C.

Phase 2 enzyme-inducing activity was measured by using a bioassay, as outlined in ref. 13, for the two broccoli sprout preparations.

Animals and Tissue Preparation. We used male SHRSp (originally obtained from the National Institutes of Health, Bethesda, and maintained by the University of Saskatchewan Animal Facilities) and male SD rats (Charles River Breeding Laboratories, St. Constant, Quebec). All animals were treated in accordance with the Canadian Council on Animal Care Standards. We placed 5-week-old rats on AIN-93 defined diet (23), containing no GS and low levels of antinutritive GS. We either air-dried 4-day broccoli sprouts at room temperature for 7 days before feeding the animals, or we froze, thawed, and dried them at 60°C.

Phase 2 enzyme-inducing activity was measured by using a bioassay, as outlined in ref. 13, for the two broccoli sprout preparations.

Animals and Tissue Preparation. We used male SHRSp (originally obtained from the National Institutes of Health, Bethesda, and maintained by the University of Saskatchewan Animal Facilities) and male SD rats (Charles River Breeding Laboratories, St. Constant, Quebec). All animals were treated in accordance with the Canadian Council on Animal Care Standards. We placed 5-week-old rats on AIN-93 defined diet (23), containing no GS and low levels of antinutritive GS. We either air-dried 4-day broccoli sprouts at room temperature for 7 days before feeding the animals, or we froze, thawed, and dried them at 60°C.

Phase 2 enzyme-inducing activity was measured by using a bioassay, as outlined in ref. 13, for the two broccoli sprout preparations.

Animals and Tissue Preparation. We used male SHRSp (originally obtained from the National Institutes of Health, Bethesda, and maintained by the University of Saskatchewan Animal Facilities) and male SD rats (Charles River Breeding Laboratories, St. Constant, Quebec). All animals were treated in accordance with the Canadian Council on Animal Care Standards. We placed 5-week-old rats on AIN-93 defined diet (23), containing no GS and low levels of antinutritive GS. We either air-dried 4-day broccoli sprouts at room temperature for 7 days before feeding the animals, or we froze, thawed, and dried them at 60°C.

Phase 2 enzyme-inducing activity was measured by using a bioassay, as outlined in ref. 13, for the two broccoli sprout preparations.

Animals and Tissue Preparation. We used male SHRSp (originally obtained from the National Institutes of Health, Bethesda, and maintained by the University of Saskatchewan Animal Facilities) and male SD rats (Charles River Breeding Laboratories, St. Constant, Quebec). All animals were treated in accordance with the Canadian Council on Animal Care Standards. We placed 5-week-old rats on AIN-93 defined diet (23), containing no GS and low levels of antinutritive GS. We either air-dried 4-day broccoli sprouts at room temperature for 7 days before feeding the animals, or we froze, thawed, and dried them at 60°C.

Phase 2 enzyme-inducing activity was measured by using a bioassay, as outlined in ref. 13, for the two broccoli sprout preparations.

Animals and Tissue Preparation. We used male SHRSp (originally obtained from the National Institutes of Health, Bethesda, and maintained by the University of Saskatchewan Animal Facilities) and male SD rats (Charles River Breeding Laboratories, St. Constant, Quebec). All animals were treated in accordance with the Canadian Council on Animal Care Standards. We placed 5-week-old rats on AIN-93 defined diet (23), containing no GS and low levels of antinutritive GS. We either air-dried 4-day broccoli sprouts at room temperature for 7 days before feeding the animals, or we froze, thawed, and dried them at 60°C.

Phase 2 enzyme-inducing activity was measured by using a bioassay, as outlined in ref. 13, for the two broccoli sprout preparations.

Animals and Tissue Preparation. We used male SHRSp (originally obtained from the National Institutes of Health, Bethesda, and maintained by the University of Saskatchewan Animal Facilities) and male SD rats (Charles River Breeding Laboratories, St. Constant, Quebec). All animals were treated in accordance with the Canadian Council on Animal Care Standards. We placed 5-week-old rats on AIN-93 defined diet (23), containing no GS and low levels of antinutritive GS. We either air-dried 4-day broccoli sprouts at room temperature for 7 days before feeding the animals, or we froze, thawed, and dried them at 60°C.

Phase 2 enzyme-inducing activity was measured by using a bioassay, as outlined in ref. 13, for the two broccoli sprout preparations.
frozen, thawed, and dried at 60°C released the endogenous plant myrosinase, which hydrolyzed most of the GS to isothiocyanates, which were then inactivated probably by binding to proteins and other intracellular components. The Grn+/H11002 preparation is, therefore, an appropriate control for the Grn+/H11001 preparation.

Up-Regulation of GSH Levels and Down-Regulation of Oxidative Stress by Broccoli Sprout Consumption in SHRsp. In rats on CT, the basal levels of GSH in blood vessels, heart, and kidney were significantly lower in SHRsp than in SD (Fig. 1 A and B). Although the Grn+ diet had no effect on GSH levels in SD tissues, it resulted in significant increases (~1.5- to 2-fold) in SHRsp tissues (Fig. 1 A and B). The Grn− diet had no effect on GSH levels in the tissues of SHRsp. In contrast, GSSG levels were higher in these tissues in SHRsp than SD rats on CT (Fig. 1C). The Grn− diet caused a significant decrease in tissue GSSG in SHRsp (Fig. 1C). The increase in GSH and decrease in GSSG caused by the Grn+/H11001 diet indicates a decrease in tissue oxidative stress. Congruent with increased oxidative stress was the observation that SHRsp kidney tissues had a prominent 45-kDa nitrosylated protein band (see Fig. 8, which is published as supporting information on the PNAS web site). SHRsp on Grn+/H11001 diet had a 37% reduction in this nitrosylated protein compared with rats on control and Grn+/H11002 diets.

GSH-Related Enzyme and NQ01 NAD(P)H QR Activities. Basal activities of GPx (Fig. 2A) and GRed (Fig. 2B) were significantly lower in aorta, carotid artery, heart, and kidney from SHRsp-CT compared with SD-CT. SHRsp fed Grn+ diet had significantly increased GPx and GRed activities. *, P < 0.05, for SHRsp-CT versus SD-CT; †, P < 0.05, for SHRsp-Grn+ versus SHRsp-CT.
SHRsp, and the Grn\textsuperscript{+} diet did not affect the activity (14.6 ± 0.9 units in Grn\textsuperscript{+} and 14.5 ± 2.1 units in Grn\textsuperscript{−}). In contrast, SD values were significantly higher in animals on Grn\textsuperscript{+} diet (96.6 ± 9.2 units) than on Grn\textsuperscript{−} diet (60.8 ± 14 units).

**Decrease of Macrophage Infiltration by Broccoli Sprout Consumption in SHRsp.** In SHRsp-CT, activated macrophages could be identified in the inner intimal layers of the aorta, carotid artery, and endocardium of the heart, as well as in the kidney medullary interstitium and tubules (Fig. 3), indicating an inflammatory state in these structures. Few activated macrophages were seen in the cardiovascular system in SHRsp animals on Grn\textsuperscript{+} diet (Fig. 3 A–C). There were also significantly fewer (P = 0.026) activated macrophages in the kidney medulla of SHRsp on Grn\textsuperscript{+} diet (3.4 ± 0.9 cells per microscope field) than in SHRsp on Grn\textsuperscript{−} diet (7.8 ± 1.0 cells per microscope field). Activated macrophages were not detectable in these tissues in the SD rats.

**Decreased Activation of NF-κB by Broccoli Sprout Consumption in SHRsp.** Inflammation is associated with activation of the transcriptional factor complex NF-κB (32). In SHRsp on CT, nuclei containing NF-κB p65 were seen in the endocardium of heart (Fig. 4A) and tubules of the kidney medulla (Fig. 4B). Few cells expressed nuclearly localized NF-κB p65 in these organs in SHRsp on Grn\textsuperscript{+} diet. Western blot analysis demonstrated that nuclearly localized NF-κB p65 was significantly decreased by 40% in kidneys from SHRsp-Grn\textsuperscript{+} compared with SHRsp-CT.

**Prevention of Vascular Endothelium Dysfunction and Hypertension by Broccoli Sprout Consumption in SHRsp.** Endothelial nitric oxide function was examined by precontracting aortic rings with phenylephrine, followed by exposure to acetylcholine (Fig. 5A). There was a concentration dependent decrease in tension in SD rats. There were no differences in relaxation with SD rats on CT, compared with Grn\textsuperscript{+} diet. In both cases there was an \(\sim 0.9\)-g relaxation force. SHRsp-CT had a maximal relaxation of \(\sim 0.33\) g in response to acetylcholine, whereas rats on Grn\textsuperscript{+} diet had a significantly better mean relaxation of 0.70 g. SHRsp-Grn\textsuperscript{−} had a mean relaxation of 0.57 g, which was significantly different from SHRsp-CT but only borderline significantly different (\(P = 0.0538\)) from SHRsp-Grn\textsuperscript{+}.

The ability of the nitric oxide donor sodium nitroprusside to cause a decrease in tension in aortic rings that were precontracted with phenylephrine was examined (see Fig. 9, which is published as supporting information on the PNAS web site). There was a dose-dependent decrease in tension in SD rats with a maximal decrease in tension of \(\sim 0.98\) g of force. The aortic rings obtained from all SHRsp diet groups had a significant impairment in relaxation in response to SNP with a maximal decrease in tension of \(\sim 0.6\) g.

Systolic blood pressure was significantly lower in SD rats compared with SHRsp and did not change over the course of the experiment, regardless of diet. In SHRsp, the systolic blood pressure was \(135 \pm 4.6\)-mm Hg at the beginning of the dietary intervention (Fig. 6). This increased to \(163 \pm 1.8\)-mm Hg after 8 weeks on Grn\textsuperscript{+} and showed no further increase to the end of study. In contrast, blood pressures of SHRsp-CT and SHRsp-
Normotensive SD rats on control or Grn/H11001 on Grn had a response that was 78% of that of SD. Interestingly, SHRsp compared with SD rats did not show the same ability of SD aortae to relax. SHRsp on Grn only had a 37% relaxation in phenylephrine-constricted aortae when compared with SD-CT, which is significantly lower than SD-CT. Aorta from SHRsp-Grn had significantly lower blood pressures in SHRsp-Grn and SHRsp-CT, whereas SHRsp-Grn had an intermediate response. *P < 0.001, for SHRsp-Grn versus SD-CT and SD-Grn-1. P < 0.001, for SHRsp-Grn versus SHRsp-CT.

Grn were significantly higher (184 ± 1.8-mm and 182 ± 2.3-mm Hg, respectively) by the end of the study.

Discussion

SHRsp fed a Grn-containing diet had a major improvement in the cardiovascular system, as demonstrated by decrease in the rise in blood pressure and inflammation. Unlike the normotensive SD rats for which blood pressures remained constant during the experimental period, blood pressures in all three diet groups of SHRsp increased progressively until the animals were 13 weeks of age. Thereafter, animals on the Grn diet had significantly lower blood pressures than in the two other diet groups.

The hypothesis initiating this research was that animals on a Grn diet would have decreased oxidative stress. This hypothesis was upheld. An index of oxidative stress is the GSH/GSSG ratio. Normotensive SD rats on control or Grn diets had ratios in the different tissues ranging from 17.1 to 20.9, SHRsp-CT had ratios ranging from 4.0 to 7.2, and SHRsp-Grn had significantly higher ratios ranging from 10.6 to 14.0. A decrease in oxidative stress with Grn is supported also by the increase in GPx activity in different tissues in SHRsp-Grn. GPx is an enzyme that is susceptible to oxidative inactivation (33). Also in support of our hypothesis is the decrease in quantity of the 45-kDa nitrosylated protein band in kidney tissue.

Higher oxidative stress in blood vessels should result in poorer endothelial function. Indeed, this effect is what we see with SHRsp on CT, as determined by ability of acetylcholine to cause only a 37% relaxation in phenylephrine-constricted aortae when compared with ability of SD aortae to relax. SHRsp on Grn diet had a response that was 78% of that of SD. Interestingly, SHRsp on Grn-1 diet had an endothelial cell response that was considerably better (68% of normotensive animals) than SHRsp on CT, but this better endothelial response did not translate into lower blood pressures.

One source of oxidative stress is increased expression and activity of NAD(P)H oxidase that produces superoxide anion radicals (34). The expression of NAD(P)H oxidase proteins increases greatly after 15 weeks of age in both SHRsp and related SHR strain (35, 36). Inhibition of the activity of NAD(P)H oxidase has been shown to improve endothelial function (37). Hence, possibly what the Grn diet may be promoting is scavenging of such increased production of superoxide anion in the older SHRsp. It is known that endothelial nitric oxide synthase is also down-regulated in SHRsp and that inhibiting advanced glycation endproduct formation increases endothelial nitric oxide synthase expression (38). It is possible that the decreased vascular oxidative stress seen in our study also promoted endothelial nitric oxide synthase expression. The decrease of 11% in systolic blood pressure in SHRsp-Grn compared with SHRsp-CT is similar to the decrease in SHRsp blood pressure seen when oxidative stress was minimized by feeding rats a pharmacological inhibitor of advanced glycation endproduct formation (38).

Higher oxidative stress should also increase activation of NF-κB promoting inflammatory responses. Indeed, we have shown that there is an increase in the activation of NF-κB in numerous tissues in SHRsp compared with SD rats and that a Grn diet decreases NF-κB activation. Decreased NF-κB activation correlated with decreased tissue infiltration of activated macrophages. Increased strong oxidant production in blood vessels and NF-κB activation plus infiltration of activated macrophages into tissues such as kidney is also seen in rats made hypertensive with deoxycorticosterone acetate (DOCA) (39). As with our study, DOCA rats exhibited infiltration of kidney tubules by activated macrophages. DOCA rats treated long-term with the synthetic antioxidants pyrrolinedithiocarbamate or 4-hydroxy-2,2,6,6-tetramethyl piperidinoxy have a decreased NF-κB activation and activated macrophage infiltration in kidneys (39). In these animals, the long-term administration of antioxidants also inhibited the rise in blood pressure.
Are the effects seen in SHRsp fed Grn⁺ diet due to increased expression of phase 2 proteins? Because of the centrality of GSH- and GSH-using enzymes in the antioxidant defense (10), we focused on this system. The rate-limiting enzyme for GSH/GSSG ratio may be due to induction of phase 2 proteins in the SHR-Grn diet due to increased GSH-related antioxidant system in some other way.

In 1995, the direct costs of hypertension to the health care system in the United States of America was almost 20 billion dollars (43). Our results show that even a modest change in diet has the potential to have a major impact on health and reduce health care costs significantly.

We thank Arlene Drimmie, Anita Quon, Koleen Safiniuk, Connie Wong, and Yi Gian Wang for their excellent technical assistance. We especially thank Drs. J. W. Fahey and P. Talalay (The Johns Hopkins University School of Medicine, Baltimore) for their analyses of phase 2 enzyme-inducing activity in our sprout preparations, and Dr. Albena Dinkova-Kostova for advice on the QR assay. This work was supported by the Saskatchewan Agriculture Development Fund of pharmacological antioxidants. In the present study, two doses were examined, 0.5 and 5.5 μmol sulforaphane equivalents per rat per day. An effect on oxidative stress parameters and blood pressure was seen only with the higher dose, although some positive effects on endothelial function was seen with the lower dose. It may be true that even more profound effects might be seen with higher intake of Grn. Whether the positive effects seen are due to induction of phase 2 proteins is not yet clear, and it may be true that Grn affects the GSH-related antioxidant system in some other way.