Background. We investigated the effects of sulforaphane (SF), the main active isothiocyanate in cruciferous vegetables, on arachidonic acid (AA) metabolism in the kidney and its effect on arterial blood pressure, using spontaneously hypertensive rats (SHR) as models.

Methods. Rats were treated for 8 weeks with either drinking water alone (control) or SF (20 or 40 mg/kg) added to drinking water. Mean arterial pressure (MAP) was measured at 7-day intervals throughout the study. At the end of treatment rats were euthanized, and kidneys were harvested to prepare microsomes and measure enzymes involved in regulation of vasoactive metabolites: CYP4A, the key enzyme in the formation of 20-hydroxyeicosatetraenoic acid, and the soluble epoxide hydrolase, which is responsible for the degradation of the vasodilator metabolites such as epoxyeicosatetraenoic acids. Effect of SF on kidney expression of CYP4A was investigated by immunoblotting. Results. We found that treatment with SF leads to significant reductions in both the expression and activity of renal CYP4A isozymes, as well as the activity of soluble epoxide hydrolase (sEH). Consistent with these data, we have found that treatment with SF resisted the progressive rise in MAP in the developing SHR in a dose-dependent manner. Conclusion. This is the first demonstration that SF modulates the metabolism of AA by both P450 enzymes and sEH in SHR rats. This may represent a novel mechanism by which SF protects SHR rats against the progressive rise in blood pressure.
effects, to increase vascular tone in the afferent arterioles and augmenting tubuloglomerular feedback responses [2, 3]. In the human kidney, two enzymes (CYP4A1 and CYP4F2) were found to be responsible for the production of 20-HETE [2]. In the rat, CYP4A1 was found to exhibit the highest catalytic activity for the formation of 20-HETE, followed by CYP4A2 and then CYP4A3 [11]. While CYP4A1 and CYP4A3 are preferentially expressed in the proximal tubules, CYP4A2 is highly expressed in the outer-medullary and thick ascending limb of the loop of Henle [12]. This site-specific expression may explain the preferential regulation and diverse actions of 20-HETE in the kidney.

The phase of rapid elevation of blood pressure in the spontaneously hypertensive rat (SHR) is associated with increased production of 20-HETE by CYP4A isoforms expressed in the renal tubules and blood vessels [13]. Conversely, reduction in the synthesis of 20-HETE prevents the development of hypertension in SHR [14]. Consequently, modulation of AA hydroxylase activity (i.e., 20-HETE-forming activity) could potentially have a significant impact on the development and progression of hypertension. Such modulation can occur in response to administration of drugs, herbal remedies, or even dietary supplements.

On the other hand, EETs, produced by the endothelium, are potent vasodilators [15]. Several studies have led to the proposal that EETs serve as the endothelium-derived hyperpolarizing factor (EDHF) through increasing the activity of a large-conductance, calcium-activated K⁺ channel (KCa) [15]. Deficiency in EETs, especially 5,6-EET, renders the rat liable to salt-induced elevations of blood pressure [16]. The human CYP2C is the main CYP epoxygenase gene family expressed in the human kidney, with CYP2C8, CYP2C9, CYP2C18, and CYP2C19 being the predominant isoforms responsible for the metabolism of arachidonic acid to biologically active EETs [17]. The three major CYP2C isoforms expressed in the rat kidney and responsible for most of the renal epoxygenase activity are CYP2C11, CYP2C23, and CYP2C24 [3]. The CYP2J family was also found to contribute to the formation of EETs in both human and rat kidneys [18]. High-salt diet increases the expression of CYP2C23 and formation of EETs [19]. Significantly, an increased conversion of EETs to inactive metabolites, dihydroxyeicosatrienoic acids (DiHETEs), by soluble epoxide hydrolase (sEH) has been reported in the SHR [3] (Figure 1). Consistent with the latter findings, treatment of SHRs with inhibitors of sEH lowers blood pressure [3, 20].

The idea of modulation of drug-metabolizing enzymes as a principal means by which dietary constituents affect the risk of disease was first outlined by Wattenberg [21] and was further elaborated by Prochaska and colleagues [22]. This idea was particularly applied to the natural phytochemical sulforaphane glucosinolate (SGS), which is present in the cabbage family of plants (kale and cabbage). SGS is metabolized to sulforaphane (SF), a potent inducer for phase II detoxification enzymes [23]. Young sprouts of cruciferous plants (e.g., broccoli) are extremely rich dietary sources of SGS [23]. Multiple human epidemiological studies, as well as animal experimentation, indicate that SGS consumption decreases the probability of developing hypertension, atherosclerosis, and cancers [23–26]. More recent research indicates that the cardioprotective and cancer chemopreventive effects of broccoli sprout consumption are primarily attributed to SGS being metabolized to SF [24, 27, 28]. The current study was undertaken to examine the antihypertensive effect of SF in a hypertension rat model and to investigate its effect on AA-metabolizing enzymes in the kidney.
2. Materials and Methods

2.1. Materials. Purified sulforaphane was obtained from LKT laboratories (St. Paul, MN). Arachidonic acid and 20-HETE were purchased from Cayman Chemical Company (Ann Arbor, MI). Sprague-Dawley rat kidney microsomes for in vitro studies were purchased from BD Biosciences (Woburn, MA). NADPH and all reagents used for microsomal preparation, determination of protein content, and enzyme assays were purchased from Sigma-Aldrich (St. Louis, MO). CYP4A1/2/3 polyclonal antibody was purchased from LifeSpan Biosciences, Inc. (Seattle, WA). Acetonitrile and methanol were high pressure liquid chromatography (HPLC) grade and obtained from Fisher Scientific (Pittsburg, PA). All other chemicals used were analytical grade.

2.2. Animals. Male 14-week-old spontaneously hypertensive rats (SHR) were obtained from Charles River Laboratories (Wilmington, MA). All animals were maintained under controlled housing conditions of light (6 AM–6 PM) and temperature (22∘C) and received standard laboratory chow and water ad libitum. All rats were allowed at least 2 weeks to become acclimated to the housing conditions and ensure steady and reliable blood pressure readings before use in experiments. All procedures were approved by the Institutional Animal Care and Use Committee of Pacific University and Oregon Health & Science University.

2.3. Sulforaphane (SF) Treatment. Animals were randomly divided into 3 groups of 5 animals each. Group one served as the control group, whereas groups two and three were provided with sulforaphane in the drinking water at concentrations of 20 mg/kg and 40 mg/kg, respectively, for 8 weeks. Preliminary stability studies using LC-MS indicated that SF is stable in water at room temperature for at least 5 days (data not shown). Fresh SF solutions were made from stock twice a week. The selection of SF doses was based on preliminary studies in our laboratory and previous studies [24, 26].

2.4. Mean Arterial Pressure (MAP) Measurements. Blood pressure was measured as previously described by Vermehren-Schmaedick et al. [29]. All blood pressure measurements were performed in conscious animals. For each SHR rat, blood pressure was measured once every week using a noninvasive method based on determining the tail blood volume with a volume-pressure recording sensor and an occlusion tail-cuff (CODA system; Kent Scientific, Torrington, CT). Our previous studies indicate that blood pressure values obtained with the noninvasive tail-cuff method are not significantly different from the values obtained in the same animals by direct measurements in the femoral artery [29]. Blood pressure was measured by multiple readings in individual rats, until an average of 15 stable measurements was obtained. Results are shown as the average of MAP values obtained from individual rats.

2.5. Tissue Collection. At the end of the study, rats were anesthetized with isoflurane; the abdominal cavities were opened, and the kidneys were rapidly removed and rinsed with ice-cold saline. Kidney tissues were then flash frozen in liquid nitrogen and stored at −80∘C until use.

2.6. Preparation of Microsomes and Measurement of Protein Content. Kidney microsomes were prepared as described previously [30]. Briefly, kidney samples were homogenized in 2 mL ice-cold homogenization buffer (50 mM Tris buffer, 150 mM KCl, 0.1 mM dithiothreitol, 1 mM ethylenediaminetetraacetate (EDTA), 20% glycerol, and 0.1 mM phenylmethylsulfonlfon fluoride) using a Polytron homogenizer. The homogenate was centrifuged at 10000 x g for 30 min at 4°C in a Sorvall MTX 150 Micro-Ultracentrifuge (Thermo Scientific, Asheville, NC). The supernatant was carefully transferred to clean ultracentrifuge tubes and centrifuged at 100 000 x g for 30 min at 4°C. The supernatant (cytosol) was removed and transferred to labeled tubes and stored at −80°C until use. Microsomal protein concentrations were determined in triplicate by the method of Lowry et al. [31] using bovine serum albumin as a calibration standard. Absorbance was measured at 750 nm on a Synergy 2 microplate reader using Gen5 Software (BioTek, Winooski, VT).

2.7. Microsomal Arachidonic Acid (AA) Hydroxylation and Analysis of 20-HETE Formation. Oxidation of AA to its metabolite 20-HETE was determined in reaction mixtures (500 µL) containing 100 mM phosphate buffer (pH 7.4), 40 µM AA, 2 mM MgCl₂, 1 mM NADPH, and rat kidney microsomes equivalent to 0.4 mg protein. Reactions were initiated with NADPH and were terminated after 15 minutes at 37°C with 1.0 M HCl and vigorous shaking. In vitro experiments using renal cortical microsomes from untreated Sprague-Dawley rats were similarly utilized to study the effect of SF on 20-HETE formation in vitro. In these experiments, microsomes were exposed to different SF concentrations (0.5, 12.5, and 50 µM) in presence and absence of NADPH. 20-HETE formation rate in both in vitro and in vivo studies was measured as described previously [32]. Briefly, the reaction mixtures were extracted with ethyl acetate and the organic extracts were evaporated with nitrogen gas and the residues were reconstituted in the HPLC mobile phase. AA and 20-HETE were resolved on an Agilent Eclipse Plus C₁₈ column (4.6 × 250 mm; Agilent Technologies, Santa Clara, CA) with UV detection at 200 nm. Initial mobile phase composition was 45% acetonitrile in water with 0.1% acetic acid. Linear gradient (0.5%/min) was utilized over 30 min, followed by a sudden increase of 20%/min to 100% acetonitrile. Flow rate was maintained at 0.3 mL/min and column temperature at 40°C.

2.8. Immunoquantification of CYP4A in Rat Kidney Microsomes. Rat kidney microsomes (10 µg per lane) were incubated at 100°C for 5 minutes in Laemmli sample buffer.
Figure 2: Effect of SF on MAP in SHR rats. Male 14-week-old SHR rats (n = 5/group) were exposed to SF in their drinking water at concentrations of 0, 20, and 40 mg/kg for 8 weeks. MAP was measured noninvasively once every week. Change in MAP is expressed as mean ± SD of 5 animals. (a) Changes in MAP in SHR over 8 weeks; (b) difference in MAP between the end and the beginning of the 8-week treatment, that is, (MAP at week 8)−(MAP at week 0). *Significant difference from control with P < 0.05.

3. Results

Exposure of rats to different doses of sulforaphane (SF) had no obvious adverse effect and the gain in body weight was not significantly different in treatment groups compared to control animals (data not shown).

3.1. Effect of Sulforaphane Treatment on Mean Arterial Pressure (MAP). To determine the effect of SF on blood pressure in SHR rats, we measured the MAP of all groups once a week. Figure 2(a) shows the MAP in rats during baseline conditions and after exposure to low dose (20 mg/kg) or high dose (40 mg/kg) of SF. During the study, control rats showed a progressive rise in their MAP from 163 ± 13 to 187 ± 8 mm Hg after 8 weeks, an average increase of 25 ± 5.5 mm Hg. In contrast, exposure of SHR rats to even the low dose of SF prevented the expected progressive rise in MAP with a net decrease of 4.5 ± 15 mm Hg. Moreover, administration of SF in the high dose resulted in a more substantial decrease in MAP from 184 ± 7 at the beginning of the study to 171 ± 7.8 at the end of the 8-week treatment, with a net decrease of 14.9 ± 13 mmHg. The MAP in both low- and high-dose SF treatment groups showed a significant reduction when compared to the control group at the end of the 8-week treatment (Figure 2(b), P < 0.05) without a significant difference between the two SF treatment groups.
3.2. Effect of SF Treatment on Arachidonic Acid (AA) Metabolism. Previous studies have shown that CYP4A is the predominant P450 isoform in the rat kidney responsible for catalyzing the ω-hydroxylation of (AA) to the potent vasoconstrictor metabolite, 20-HETE. To examine whether the observed reduction in blood pressure in SHR rats by SF could be associated with inhibition of CYP4A-mediated formation of 20-HETE, we first measured this reaction in vitro using renal cortical microsomes from untreated Sprague-Dawley rats. Microsomes were preincubated with or without SF (5, 12.5, and 50 μM) in the presence of NADPH before adding AA (100 μM) and other incubation components. An HPLC-UV method was utilized for the quantification of 20-HETE in the incubation mixture and measurement of 20-HETE formation rate as previously described [2, 32]. Under these chromatographic conditions, 20-HETE and AA were eluted with retention times of 35 and 37 min., respectively (Figure 3(a)). Additionally, and as shown in Figure 3(b), 20-HETE formation was NADPH-dependent. This may indicate that inhibition of 20-HETE formation by SF is mechanism-based as NADPH was required in the incubation mixture for inactivation of CYP enzymes. 20-HETE formation was inhibited by in vitro inactivation with SF in renal cortical microsomes in a concentration-dependent manner. At a SF concentration of 50 μM, 20-HETE formation rate was reduced to 57–63% of control (Figure 4, in vitro).
The effect of the in vivo administration of SF on AA metabolism was measured in rat renal microsomes after 8 weeks of exposure to SF in the drinking water at concentrations of 20 mg/kg and 40 mg/kg. Similar to the observed in vitro results, AA ω-hydroxylation was inhibited significantly by both low-dose and high-dose SF compared to control rats, with no significant difference between the two SF treatment groups (Figure 4, in vivo).

3.3. Effect of SF Treatment on CYP4A Protein Levels. To examine the effect of SF on the expression level of CYP4A protein, western blotting of renal microsomes was performed using a polyclonal antibody against rat CYP4A1/2/3. As shown in Figure 5, inhibition of AA ω-hydroxylation was associated with a loss of CYP4A immunoreactive protein. Similar to the observed dose-dependent inhibition of AA hydroxylase activity, SF also caused a significant inhibition of CYP4A protein expression compared to the control group, but no significant difference was observed between the two SF treatment groups (Figure 5).

3.4. Effect of SF Treatment on Soluble Epoxide Hydrolase (sEH) Activity. sEH plays a major role in the metabolic conversion of the cardioprotective eicosanoids to inactive metabolites. To further study the effect of SF treatment on sEH activity, kidney microsomes from control rats or rats exposed to different concentrations of SF were utilized as explained briefly in Section 2 [34]. The results shown in Figure 6 indicate that sEH activity was significantly inhibited by 30% and 51% following SF treatment with doses of 20 mg/kg and 40 mg/kg, respectively. No significant difference was observed between the two treatment groups.

Since establishing a cause-effect relationship between the observed effect of SF on MAP, CYP4A activity, and sEH activity was not possible with our study, we investigated the correlation between these variables. When SF-dependent changes in MAP were compared to changes in 20-HETE formation rate as the measures of CYP4A activity, a good correlation was observed (Pearson r; 0.992 and P; 0.041). While 20 mg/kg and 40 mg/kg SF doses reduced MAP by approximately 8 and 12%, 20-HETE formation rate was reduced by 21 and 41%, respectively (Figure 7). Similarly, changes in MAP reasonably correlate (Pearson r; 0.9954 and P; 0.046) with changes in sEH activity in a SF dose-dependent manner. Namely, compared to 8 and 12% reduction in MAP, sEH activity was reduced by 30 and 51% following administration of 20 and 40 mg/kg SF, respectively (Figure 7).

4. Discussion

This study was undertaken to investigate possible mechanisms that contribute to the beneficial effect of sulforaphane (SF) on blood pressure in hypertensive rats. The results show for the first time that treatment of spontaneously hypertensive rats (SHR) with SF significantly prevents the progressive rise in blood pressure, reduces the expression and activity of renal CYP4A isozymes responsible for 20-HETE formation, and reduces the activity of soluble epoxide hydrolase (sEH) responsible for the degradation of the vasodilator metabolites EETs.

SF is a naturally occurring compound that exhibits cytoprotective, antioxidant, and anti-inflammatory vasoprotective properties [35]. Most studies examining the mechanism of action of sulforaphane glucosinolate (SGS) proposed that preventing and/or inhibiting the development of cardiovascular diseases is related to the ability of SF to reduce oxidative stress and associated inflammation and, thus, ameliorate diseases that become more common with aging, such as hypertension [26, 36]. For example, the consumption of broccoli sprouts rich in SGS decreases oxidative stress in spontaneously hypertensive-stroke prone rats (SHRsp), lowering their blood pressure and decreasing inflammation in all tissues examined, including heart, arteries, kidney, and central nervous system [24, 26]. Furthermore, when pregnant SHRsp were fed with this source of SGS, their adult offspring had lower blood pressure and less inflamed organs than the parental generation [27]. Whether this antihypertensive effect of SGS is due to antioxidant effect is not yet established. Additionally, if the antihypertensive effects of SF result exclusively from its antioxidant properties, then other antioxidants should also show similar antihypertensive properties. However, results of three randomized clinical trials testing the effects of the antioxidant vitamin E on hypertension have proven disappointing [37–39]. Similarly, a nonsignificant reduction in blood pressure was reported in vitamin D-treated patients [40]. Accordingly, it is conceivable that SF reduces blood pressure by more than one mechanism.

This study is the first to investigate the effect of SF on modulation of AA-metabolizing enzymes in the kidney, as a potential mechanism for its antihypertensive property. Even though AA and its metabolites are present in numerous organs, including the liver, lungs, and brain, the kidney plays a central role in arterial blood pressure regulation through both local and hormonal mechanisms. AA metabolites, such as 20-HETE, are known to have a strong vasoconstrictive effect on the renal afferent arteriole that, in turn, is likely to trigger the powerful renin-angiotensin-aldosterone (RAA) cascade, one of primary targets of antihypertensive therapy. The fact that 20-HETE also has a direct inhibitory effect on sodium reabsorption, which is opposite to the RAA-mediated sodium retention, is likely to explain the less pronounced changes in blood pressure during a global downregulation of 20-HETE formation, as in the current study.

To avoid inducing any stress-related effect on blood pressure and also to simulate the commonly used route of SF administration, we introduced SF to rats in their drinking water rather than oral gavage. Our stability studies using LC-MS indicate that SF is stable in water at room temperature for at least 5 days. Also, several studies have indicated that SF is bioavailable in both human and animal models [41, 42]. Selection of SF doses was designed to reflect the normal daily servings of broccoli, that is, clinically relevant doses. Broccoli naturally contains glucoraphanin, not SF. The conversion to the more active SF takes place either by the action of the enzyme myrosinase, also present in fresh broccoli, or in the body during the digestion process. However, this
conversion is highly variable. Assuming 100% conversion and considering the molecular weights of glucoraphanin (436 g/mole) and sulforaphane (177 g/mole), one serving of broccoli (10 oz.), which contains approximately 30 mg of glucoraphanin, will release approximately 12 mg of SF. Administration of 20 mg/kg and 40 mg/kg of SF to SHR rats with a body weight range (400–600 g) is expected to provide 8–24 mg of SF daily.
Notably, the observed attenuation of the expected rise in blood pressure by SF after an 8-week period was comparable to the results achieved with commonly used antihypertensive medication in the same rat model. For example, telmisartan (5 mg for 8 weeks), indapamide (1 mg/kg for 8 weeks), hydrochlorothiazide (20 mg/kg for 8 weeks), and metoprolol (100 mg/kg) with hydralazine (50 mg/kg; for 10 weeks) resulted in an average MAP reduction of 25, 17, 15, and 22 mmHg, respectively [43]. Such reduction in blood pressure was associated with a similar pattern of inhibition in the formation rate of 20-HETE in kidney microsomes. It is well established that 20-HETE induces hypertension through its potent vasoconstriction in the renal and peripheral vasculature and by promoting sodium retention [3]. Considerable evidence has now accumulated suggesting that development of hypertension in the SHR strain is, at least in part, attributed to the elevated production of 20-HETE in the kidney and mesenteric arteries [14]. Our data are consistent with this hypothesis and with several findings that inhibitors of 20-HETE synthesis lower blood pressure by 10–15 mmHg in SHR [14, 44]. In attempts to identify the cytochrome P450 activities of sEH. Those results contribute novel knowledge to the antihypertensive properties of SF.

Future studies will be undertaken to examine whether an earlier exposure to SF can prevent the development of hypertension and whether SF can reverse the established hypertension in older SHR rats. Additionally, we will examine the effect of specific inhibitors or inducers for the 20-HETE formation pathway on the observed antihypertensive effect of SF in SHR rats.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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In 10 ISRN Pharmacology

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